Characterizing Allosteric Regulation and Conformational Dynamics in Staphylococcus Aureus Pyruvate Carboxylase

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CHARACTERIZING ALLOSTERIC REGULATION AND CONFORMATIONAL DYNAMICS IN STAPHYLOCOCCUS AUREUS PYRUVATE CARBOXYLASE

By

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A Dissertation submitted to the Faculty of the Graduate School, Marquette University, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Pyruvate carboxylase (PC) produces oxaloacetate from pyruvate and bicarbonate in an ATP-dependent manner. The catalytic activity of PC places it at a pivotal intersection between catabolism and anabolism. Understanding the essential metabolic role of PC requires a more complete description of how its activity is regulated and how that regulation is manifested through changes in conformational dynamics.

PC activity is regulated by the mutually exclusive binding of the allosteric activator, acetyl-CoA, and the allosteric inhibitor, L-aspartate. The binding site for acetyl-CoA has largely been identified, but the binding location for the acetyl moiety is unknown. Given that the acetyl moiety enhances the binding affinity drastically, a definition of the binding site will uncover new molecular insights regarding the allosteric mechanism. This work defines the binding site of the acetyl moiety at the biotin carboxylase dimer interface in *Staphylococcus aureus* PC and identifies essential residues Arg21, Lys46, and Glu418 to allosteric activation, and inhibition.

Early studies on vertebrate PC reported enzyme-mediated hydrolysis of the acetyl moiety from acetyl-CoA. The complete definition of the acetyl moiety binding site offers new opportunities to understand enzyme-mediated hydrolysis. The current study confirms that microbial PC enzymes catalyze acetyl-CoA hydrolysis. Residues in the dimer interface protect against enzyme catalyzed hydrolysis of the acetyl moiety. This suggests that a secondary binding site in the carboxyltransferase domain is responsible for hydrolyzing acetyl-CoA.

The regulatory mechanism that controls carrier domain translocation remains unknown. Using numerous biophysical tools, the current study demonstrates that the conformational dynamics of PC are altered by acetyl-CoA, increasing the rate of carrier domain translocation and coordinating carrier domain positioning in the tetramer. Acetyl-CoA does this by modulating the flexibility of the carboxyltransferase dimers at the corners of the tetramer.

This work provides a molecular basis for both allosteric activation and inhibition and confirms that microbial PC enzymes catalyze acetyl-CoA hydrolysis. These studies provide insights into how allostery modulates the conformational dynamics of PC in a coordinated manner, lending greater insights into the metabolic role of PC at the crossroads of metabolism.
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CHAPTER 1. INTRODUCTION

Allostery, or action at a distance, is the phenomenon when a binding event or modification of a biological macromolecule occurs and causes a change at a distant functional site on the macromolecule (Wodak et al., 2019). Allostery can alter the conformational dynamics of a macromolecule, impacting the transition between conformational states and the macromolecule’s propensity to adopt certain conformations. Allosteric studies have historically been focused on how effector molecules modulate enzymes through conformational transitions. Both the fields of allosteric and conformational dynamics have evolved greatly since their inception, but they remain inextricably linked. To fully understand the allosteric regulation of an enzyme system, the influence of allosteric effectors on conformational dynamics must be explored.

Allostery

The Significance of Allosteric Regulation in Life Processes

A large subset of proteins are subject to allosteric regulation. Consequently, allostery impacts nearly every cellular function and occurs across all domains of life (Wodak et al., 2019). Allostery is particularly important in cell signaling, transcriptional regulation, and metabolism (Wodak et al., 2019). Additionally, metabolites often serve as these effector molecules which provides a way for the cell to sense and respond to the levels of these small molecules (Lindsley & Rutter, 2006).

Metabolism is highly allosterically regulated, and an example of this regulation is AMP-activated protein kinase (AMPK), which serves as a “fuel sensor” in the cell. The
Carboxylation of acetyl-CoA by acetyl-CoA carboxylase is the first committed step in fatty acid synthesis, and when energy supply in the cell is low the cell needs to turn off growth processes such as fatty acid synthesis. When AMP:ATP ratios are high, and cellular energy is low, AMP binds to and allosterically activates AMPK, which is the kinase that inhibits acetyl-CoA carboxylase, effectively halting fatty acid synthesis. This inhibition of acetyl-CoA carboxylase halts fatty acid synthesis so that the cell can conserve energy. Allosteric regulation of AMPK aids the cell in the metabolic shift from the “fed” to the “starved” metabolic state. AMPK is an evolutionarily conserved energy sensing protein, whose function is entirely dependent on allostery (Kahn et al., 2005).

Metabolism is allosterically regulated through feedback and feedforward mechanisms. A downstream metabolite can act as an allosteric effector for an upstream protein in a metabolic pathway or an upstream metabolite can act as an allosteric effector for a downstream protein in a pathway. Glutamine synthetase is a prominent protein in the nitrogen cycle, and downstream metabolites, can bind to and inhibit the enzyme (Kodama & Nakayama, 2020). Allostery modulates metabolism, and understanding how metabolism is shifted by allosteric regulation can provide insights and therapeutic targets for diseases that have altered nitrogen and carbon metabolisms, such as cancer.

Studying allosteric regulation is also important for drug design because allosteric sites are often excellent targets. Allosteric sites are distinct from the active site and compounds very dissimilar from the substrate/product can be used. Benzodiazepines, a class of drugs used for sedation, bind to an allosteric site in ligand gated ion channels and elicit desired drug effects (Nussinov & Tsai, 2013, 2015; Richter et al., 2012). A
complete understanding of the ways in which allostery can alter a protein/therapeutic
target is essential to develop effective allosteric drugs.

The History and Basic Concepts of Allostery

In the late 1950’s, a common theme of negative feedback loops was emerging in
studies of metabolic pathways (Changeux, 2011). Research conducted on the metabolic
enzymes threonine deaminase and aspartate transcarbamoylase revealed that a metabolite
that was structurally dissimilar from the enzyme substrate blocked the catalytic activity of
the enzyme in a competitive and sigmoidal fashion (Umbarger, 1956). From these
observations it was proposed that there were multiple sites in enzymes that can contribute
to enzyme control. The first mention of the word “allosteric” appeared in Monod’s 1961
publication in reference to allosteric inhibition occurring in aspartate transcarbamoylase
(Monod & Jacob, 1961). Early on, after principles of allostery were established, allostery
was used to interpret structural data of hemoglobin, the oxygen transport protein.
Hemoglobin became the center of allosteric studies in the 1960s.

Hemoglobin is tetrameric and consists of two heterodimers composed of an α and
β subunit (Figure 1-1). Each subunit contains a heme prosthetic group which binds an
oxygen (Figure 1-1A and B). The binding of these four oxygen molecules to hemoglobin
occurs in a sigmoidal fashion, meaning that when you plot the percentage of oxygen
saturation as a function of oxygen partial pressure, a sigmoidal curve is observed. This
sigmoidicity is indicative of cooperative binding, where the binding of one oxygen
molecule increases the binding affinity for the next oxygen. Cooperative oxygen binding
is essential to hemoglobin function, enabling a large amount of oxygen to be
loaded/unloaded under relatively small changes in oxygen partial pressure. The first
crystal structure of hemoglobin allowed this phenomenon to be studied at a structural level and revealed that the binding of oxygen to hemoglobin caused a conformational change (Perutz et al., 1960). In the Perutz model, the tetramer exists in one of two conformations: the T or tensed deoxygenated state and the R or relaxed oxygenated state (Figure 1-1C). Both conformations are suited for ligand binding, but the R state has a higher affinity for oxygen. After these two distinct conformations were discovered, Monod, Wyman and Changeux proposed a model for the conformational change where each subunit in the tetramer switches from the T state to the R state simultaneously, and this model was referred to as the concerted quaternary structural transition model or MWC model (Monod et al., 1965) (Figure 1-1C). Shortly after this model was proposed, Koshland, Némethy and Filmer proposed a sequential model for this conformational change where, when one subunit binds an oxygen and goes from T to R, it promotes a T to R transition in a neighboring subunit to facilitate oxygen binding (Koshland et al., 1966) (Figure 1-1C). This model is referred to as the sequential or KNF model.

Following more structural and NMR studies, it is now generally accepted that cooperativity in hemoglobin results from a combination of the two models. The tetramer undergoes the transition from T to R when at least one subunit of each heterodimer (α or β) is ligand bound. The binding of an oxygen to a subunit sequentially increases the binding affinity for the next oxygen, but the quaternary conformational change from T to R state occurs in a concerted fashion (Yuan et al., 2015).
Allosteric effectors can be either homotropic or heterotropic. A homotropic allosteric effector influences the binding of an identical ligand in a distant site. A heterotrophic effector is a non-substrate molecule that affects the binding of the substrate. The allosteric effector can modulate the enzyme in a K-type or V-type manner. Allosteric effectors that modulate the binding affinity are K-type allosteric effectors. In K-type allostery there is a change in $K_m$, which is the Michaelis-Menten constant that

### Figure 1-1. The T and R states of hemoglobin.

**A.** PDB 2DN2. The deoxygenated or T state of hemoglobin with the $\alpha$ subunits in cyan and the $\beta$ subunits in grey. **B.** PDB 1LFQ. The oxygenated or R state of hemoglobin with the $\alpha$ subunits in cyan and the $\beta$ subunits in grey. **C.** A representation of the concerted MWC model of conformational change is shown on the top and a representation of the sequential KNF model is shown on the bottom.
represents the substrate concentration in which the enzyme is at half maximal velocity and is a metric for binding affinity. Allosteric effectors that change the turnover rate, $k_{cat}$, or the maximal velocity, $V_{max}$, are V-type allosteric effectors. Allosteric effectors can also be a combination of K-type and V-type effectors.

As the field of biochemistry has advanced and structural biology techniques have expanded, our understanding of allostery has changed. Previously, allostery was viewed in a simplistic manner, where the binding of an effector molecule elicits a structural change that either activates or inhibits the protein. More comprehensibly, allostery alters the free energy conformational landscape of a protein, altering the free energy of one or more conformations, so that the population of enzyme favors one or more conformational states (Tsai & Nussinov, 2014). In the most simplistic case, the binding of an allosteric effector thermodynamically alters the protein so that the local energy minimum of the activated conformation is lower than that of the apo protein (Figure 1-2A).

Additionally, it is now well accepted that allosteric effectors can modify the free energy basin of a conformational state of an enzyme. Rather than shifting the population of enzyme to favor a certain conformation, the allosteric effector can broaden or narrow the free energy landscape and the conformational space the enzyme can adopt (Guo & Zhou, 2016) (Figure 1-2B). Recently, it was found that the allosteric activator of a glutamate receptor resulted in a broader conformational space, resulting in a higher probability of the receptor adopting a conformation with a partially open cleft (Dai & Zhou, 2015). Rather than the cleft being in the open or closed conformation, it more commonly occupies an intermediate state in the presence of the allosteric effector (Dai et al., 2015). This intermediate state is indicative of the allosteric regulator modifying the
dynamics of an enzyme system rather than influencing which conformation is preferred (Figure 1-2C).

**Figure 1-2. Allosteric influences on conformational states of an enzyme.** A. The binding of an allosteric effector can influence the enzyme’s conformational free energy landscape to favor a specific conformation. B. The binding of an allosteric effector can broaden the conformational space the enzyme can occupy. C. The binding of an allosteric effector can narrow the conformational space the enzyme can occupy. The black line represents the free energy of an enzyme as a function of conformation in the absence of allosteric effector and the green lines represents the enzyme in the presence of an allosteric activator. Figure adapted from Guo et. al. 2016.

**Conformational Dynamics**

*The Development of the Conceptual Framework of Conformational Dynamics*

Studying conformational change and conformational dynamics has mechanistic implications that are relevant to not only enzyme mechanisms but also to drug design, natural product synthesis, gene expression and many other biological processes. Understanding how an enzyme undergoes conformational change is essential for a complete mechanistic understanding of how the enzyme performs catalysis and can be further harnessed for drug design.
This field of conformational dynamics has evolved through four conceptual frameworks that include, in chronological order, lock and key, induced fit, linked equilibria, and conformational selection (Abdelsattar et al., 2021). As structural biology techniques and biophysical techniques have expanded, the idea of how enzymes undergo conformational change has shifted. While the lock and key model was originally thought to be the model for substrate binding to enzyme, the idea has evolved into other more accurate frameworks such as induced-fit, linked equilibria, and conformational selection.

The lock and key model was first described by Emil Fischer in the 1894 (Lichtenthaler, 1995). Emil Fischer, who was best known for his discovery of the structure of glucose, was studying fermentation when he proposed a “lock and key” model for molecular recognition in which a substrate must fit into an enzyme like a lock in a key. This model has had significant influence, not only in the field of enzymology but also pharmacology and drug design. This model implies complementarity of the interactions between substrate and enzyme, which is still at the forefront of how current models describe ligand binding.

Induced fit was first proposed by Koshland in 1958, where he expanded on the idea of the “key-lock” theory to also include changes induced by the substrate on the enzyme (Koshland, 1958). Koshland proposed three postulates that comprised the induced fit model: a precise orientation between substrate and enzyme is required, the substrate may cause a structural change in the active site amino acids, and these changes induced by substrate binding will bring the catalytic amino acids into proper orientation whereas a non-substrate will not (Koshland, 1958). The induced fit model explains that conformational change is not required for ligand binding, but that conformational change
is required for catalysis. This model implies that ligand binding is a prerequisite for multiple conformational states of the enzyme.

Linked equilibria was proposed by Monod, Wyman and Changeoux in 1965 (Monod et al., 1965). Linked equilibria was the first model to mathematically describe the quantity of the enzyme population that could adopt a certain conformation (Abdelsattar et al., 2021). The Boltzmann distribution describes the probability of sampling a particular conformation, depending on the energy of that specific conformation and the temperature. This distribution is represented by the Boltzmann Distribution Equation (Equation 1-1), where \( p_{ij} \) is the probability of observing the \( i \)th conformation or \( j \)th binding state at equilibrium, \( \Delta G_{ij} \) is the free energy difference between the two conformations/binding states, \( T \) is the temperature and \( R \) is the gas constant. This model does not refute, but rather elaborates on, the lock and key model by combining it with the conceptual framework of the Boltzmann distribution.

Equation 1-1

\[
p_{ij} = e^{-\frac{\Delta G_{ij}}{RT}}
\]

Linked equilibria was based on the foundational study that revealed the structure of hemoglobin (Perutz et al., 1960). In addition to this structure revealing the importance of allosteric effects, this data also contributed to shifting away from the idea that conformational change only occurs in the presence of ligands. The first model to suggest that an enzyme can adopt multiple conformations in the absence of ligand was the linked equilibria model. In this model, the presence of the ligand changes the population distribution of the conformers but does not create new conformations. In other words, the conformational state pre-exists but that pre-existing state is further stabilized by the
ligand binding. This phenomenon can be described by the Linked Equilibria Equation (Equation 1-2), where $\Delta G^{\circ}_{\text{bound}}$ is the standard free energy of the conformer with ligand bound, $\Delta G^{\circ}_{\text{free}}$ is the standard free energy for the conformer with no ligand bound, and $\Delta \Delta G^{\circ}_{\text{binding}}$ is the difference in binding free energy between the conformer and a standard energy reference state (unfolded protein) (Abdelsattar et al., 2021).

$$\Delta G^{\circ}_{\text{bound}} = \Delta G^{\circ}_{\text{free}} + \Delta \Delta G^{\circ}_{\text{binding}}$$

Finally, the conformational selection model was first proposed where the enzyme exists in a dynamic equilibrium of conformations and that the conformational equilibrium is shifted by substrates and effector molecules. Conformational selection was first proposed in the 1990s with the expansion of structural biology techniques bringing greater evidence that enzymes were highly dynamic in the absence of ligand. The improvement in structural techniques allowed the observation of not only global conformational changes but also localized structural changes in enzyme active sites. Structural data from X-ray crystallography was increasingly showing evidence for highly flexible and dynamic active sites in the absence of bound ligand. One of the first studies that observed this phenomenon was an NMR spectroscopy study in the 90s, done in a calcium binding protein, that directly showed a reduction in dynamics of the enzyme active site with the ligand bound versus the apo enzyme (Akke et al., 1993). The conformational selection model is consistent with the linked equilibria concept, but simply adds a population dynamics framework. Conformational selection suggests that ligands can shift the population of enzyme to favor a specific conformation because, as
more ligand is added, the population shifts to the bound conformer which is governed by the binding free energy (Abdelsattar et al., 2021).

Studying conformational dynamics is important because it can influence the way drugs are designed. The lock and key model has had a massive influence on the pharmaceutical industry where drugs were designed based on their complementarity with the target. Over the last 20 years, as the conceptual framework has shifted to favor conformational selection, drug design approaches have changed significantly. For example, fragment based drug design has been developed to fully utilize the chemical space of an enzyme active site (Erlanson et al., 2016). This methodological development was greatly influenced by mechanistic insights into how enzyme conformational change is related to ligand binding.

Understanding the conformational dynamics of an enzyme provides mechanistic information into how an enzyme executes catalysis. A complete explanation for an enzyme’s function must include dynamics information because it defines the changes in flexibility/rigidity in the structure that aids in the conformational transitions that contribute to the catalyzed reaction. Studying conformational dynamics in enzymes that necessitate large conformational changes to function is important due to the mechanistic implications these conformational transitions can have.

*Modern Models that Describe Protein-Ligand Interactions*

Modern day studies that attempt to elucidate how ligand binding influences conformational change are typically guided by two models: induced fit and conformational selection, which evolved from the initial lock and key model and the linked equilibria concept. As previously described, induced fit implies that ligand binding
induces a specific conformational change that does not occur in the absence of ligand. Conformational selection implies that multiple conformations of the enzyme can pre-exist in the absence of ligand, but ligand binding shifts the conformational equilibrium to favor a specific conformation (Figure 1-3A).

From the start of the molecular recognition theories, studies have used kinetics experiments to show that a hyperbolic increase in the observed rate constant, that represents conformational change, as ligand concentration increases is representative of an induced fit model of conformational change (Chakraborty & Di Cera, 2017). Conversely, when there is a hyperbolic decrease in observed rate constant as concentration of ligand increases, the system is assumed to follow conformational selection. A study conducted in trypsin-like proteases showed that a hyperbolic increase in the observed rate constant as ligand concentration increases can occur in a system where conformational change is governed by conformational selection when the rate-limiting step is not the conformational change (Pozzi et al., 2012). Thrombin, a trypsin-like protease, exists in two states: inactive (zymogen) and active (protease). The zymogen form is irreversibly converted to the protease form through proteolysis, but the active protease then exists in a conformational equilibrium where one conformation is active and the other is in an inactive conformation where the active site is “collapsed” (Pineda et al., 2004). These two enzyme states exist in the absence of ligand, but the conformational change between the two states is not the rate-limiting step, and as ligand concentration increases the observed rate also increases. Therefore, the assumption that a hyperbolic increase in observed rate constant as ligand concentration increases follows an induced fit model can only be held when the conformational change is the rate-limiting step.
The field of enzyme dynamics has undergone a shift towards accepting that conformational selection is the most accurate model to describe how ligand binding events occur in most enzymes systems (Chakraborty & Di Cera, 2017). Induced fit is a much more restrictive model that describes a small number of enzymes. Although it is widely accepted that conformational selection is the best description for most enzyme systems, conformational selection and induced fit need not be mutually exclusive and can co-exist in a linear or linked fashion. Linear induced fit and conformational selection implies that multiple conformations pre-exist in the absence of ligand, but the binding of ligand induces a specific conformational change that promotes the ligand-bound state (Figure 1-3C). Linked induced fit and conformational selection implies that the enzyme can exist in two conformations: one suited for ligand binding and one not suited for ligand binding (Figure 1-3B). When the ligand interacts with the conformation suited for ligand binding, it induces a specific conformation that promotes the ligand-enzyme bound conformation. When the ligand binds to the enzyme when it is not suited for ligand binding, it promotes a different conformation, where the ligand interaction is not stabilized (Redhair & Atkins, 2021). Conformational selection can exist in many forms and better represents most enzyme systems.
Swinging Domain Enzymes Require Dynamic Conformational Changes

Swinging domain enzymes, or enzymes that possess a prosthetic group that is covalently tethered to the enzyme that transfers reaction intermediates, are enzymes that undergo large conformational changes with each round of catalysis. These enzymes are increasingly of interest due to their implication in the production of natural products and pharmaceuticals. For example, polyketide synthase (PKS), non-ribosomal peptide
synthetases (NRPS) and fatty acid synthase (FAS) produce a wide range of molecules that have antibacterial, antifungal, and immunosuppressive properties (Castoe et al., 2007). There is great interest in manipulating these systems for designer production of small molecule drugs and various other products, but the complexity of these systems precludes simple re-engineering efforts. Some of these systems, such as PKS, can have up to 60 domains. Pyruvate carboxylase is a swinging domain enzyme that has only four domains. The use of this simpler system to study carrier domain enzymes will provide insights into multi-domain catalysis that can be harnessed towards more commercially appealing enzyme systems.

Pyruvate Carboxylase

Pyruvate carboxylase (PC) is an essential metabolic enzyme that produces oxaloacetate, an important intermediate in the TCA cycle. PC is an anaplerotic enzyme, meaning it replenishes the TCA cycle with carbon, which is important because carbon is constantly being drawn from the TCA cycle for synthesis of macromolecules such as amino acids, nucleotides and glucose.

Tetrameric Structure of PC

PC is composed of four distinct domains: biotin carboxylase (BC, blue) domain, allosteric domain (AD, green), carboxyltransferase (CT, yellow) domain, and the biotin carboxyl carrier protein (BCCP or carrier, red) domain (Figure 1-4, 1-5). Due to the two faces of the tetramer’s antiparallel layout, each BC domain interacts with a BC domain on the other face of the tetramer, and each CT domain sits on top of another CT domain. The tetramer is held together through the BC and CT dimer interfaces. PC catalyzes the carboxylation of pyruvate to oxaloacetate. Each round of catalysis involves two half
reactions. The first half reaction occurs in the BC domain where a bicarbonate dependent ATP cleavage occurs. Here the carrier domain is bound in the BC domain and a biotin cofactor covalently tethered to the carrier domain becomes carboxylated. Then the carrier domain translocates to the second active site which is in the CT domain. Here the carboxyl group is transferred from the biotin cofactor to pyruvate and the final product, oxaloacetate, is formed (Figure 1-4).

PC was first discovered in 1960 in *Aspergillus niger*, and has since been isolated from a variety of different organisms (Woronick, C. L., and M. J. Johnson, 1960). In the 60’s it was purified and characterized from various vertebrate tissues, such as chicken and sheep liver, and from yeast, so there is a wealth of knowledge about PC from many
phylla. Following the development of recombinant protein expression technology, a large number of PC from bacteria and fungi were also characterized. In 2022 a large cryo EM study was conducted on *Homo sapiens* PC, so there is a wealth of information across many phyla regarding this enzyme (Chai et al., 2022). This study exemplified how structurally similar *HsPC* is to microbial PCs that are used in these studies.

I am specifically studying *Staphylococcus aureus* PC (*SaPC*). The PC gene in *Staphylococcus aureus* (*pycA*) has been identified as a gene that is required for full virulence in systemic and abscess infections, thus providing further clinical rationale for studying PC, specifically *SaPC* (Benton et al., 2004). Learning how *SaPC* is allosterically regulated could provide insights into how to inhibit this enzyme to interfere with the virulence mechanism of *Staphylococcus aureus*. *SaPC* has been extensively kinetically and thermodynamically characterized (Lasso et al., 2010; Westerhold et al., 2016, 2017; Yu et al., 2009). There are many full-length crystal and cryo-EM structures of *SaPC* both with and without allosteric effectors and ligands bound, making it an ideal system to study allostery and conformational dynamics in (Lasso et al., 2010; Yu et al., 2009, 2013) (Table 1-1).

**Structural Variations in the PC Tetramer**

There are structural variations that are seen in PC, specifically in the tetrameric arrangement of the domains, that are dependent on the organism that PC is from. The tetramer can have a symmetric configuration, where the ADs on both faces of the tetramer are equidistant. Conversely, the tetramer can be assembled in an asymmetric manner, where the ADs on one face of the tetramer are much closer than on the other face of the tetramer.
The first crystal structure of full length PC was of *Rhizobium etli* PC (RePC) and it revealed the domain layout and demonstrated that catalysis involved multiple subunits (St. Maurice et al., 2007). The BC domain was faced towards the CT domain of the opposing subunit on the same face of the tetramer. Tetrameric RePC was found to be asymmetric (Figure 1-5). The first structure of SaPC was published in 2008, and it revealed a symmetric tetramer, where both faces of the tetramer have subunits that are equidistant, and ADs do not come into contact (Xiang & Tong, 2008). Similarly to SaPC, the *Aspergillus nidulans* PC (AnPC) tetramer was also symmetric (Yumeng Liu, unpublished). Interestingly, in the AnPC crystal structure, the carrier domain was positioned in the BC domain of the subunit across the face of the tetramer, which is a conformation that had not been seen before. This was an indication that carrier domain motion was more flexible than previously believed.
Figure 1-5. Variants of PC that are asymmetric and symmetric. **A.** The crystal structure of *Re*PC tetramer, PDB 2QF7. The top face of the tetramer reveals that the AD of the two subunits come into contact. The AD on the top face of the tetramer are circled. **B.** The crystal structure of *Re*PC tetramer, PDB 2QF7. The bottom face of the tetramer reveals that the AD on this face are much further apart. The AD on the bottom face of the tetramer are circled. **C.** The crystal structure of *Sa*PC tetramer, PDB 3BG5. The AD on the top face of the tetramer are circled. **D.** The crystal structure of *Sa*PC tetramer, PDB 3BG5. The AD on the bottom face of the tetramer are circled. The *Sa*PC tetramer is symmetric.
There are 16 full length structures of *SaPC* and 15 of them reveal a symmetric tetramer. A cryo EM study conducted in 2014 had one class of *SaPC* structures in which the tetramer was asymmetric configuration. This cryo EM structure also had a resolution of only ~11.5 Å, therefore whether *SaPC* can adopt the asymmetric tetramer is still unknown (Lasso et al., 2014). The asymmetric configuration does not appear in *Homo sapiens* PC (*HsPC*) or *Lactococcus lactis* PC (*LlPC*). In the early to mid 2010s the debate about the catalytic importance of the asymmetric vs symmetric tetramer was a major part of PC studies but now it is recognized that it is not a widely seen conformation. The asymmetric tetrameric conformation is only observed in *RePC*, which has low intrinsic activity, therefore the asymmetric configuration is an exception in *RePC* and not relevant to PC catalysis from a range of phyla (López-Alonso et al., 2022).

**BCCP Positioning in the PC Tetramer**

The tetrameric conformation of PC also varies due to the positioning if the carrier domain. As previously mentioned, the carrier domain, or BCCP domain, translocates from the BC domain active site to the CT domain active site to transfer a carboxyl group. This movement is essential to PC’s function, and the movement and regulation of carrier domain translocation is an active area of investigation. The BCCP domain has three known binding locations: the BC domain active site, the CT domain active site, and the CT domain exo site.

Crystal structures of PC usually show the BCCP domain in the CT domain, suggesting that the BCCP-CT interaction is more stable than the BCCP-BC interaction (Lasso et al., 2010; St. Maurice et al., 2007; Xiang & Tong, 2008; Yu et al., 2009) (Table 1-1). Recently, a cryo EM structure with the first catalytically relevant BCCP-BC
interaction was published, but the global conformational analysis of the cryo EM dataset showed that this interaction is much more rare than the BCCP-CT interaction (López-Alonso et al., 2022).
Table 1-1. Full length cryo EM and crystal structures of PC.

<table>
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<tr>
<th>Species</th>
<th>PDB/EMD</th>
<th>Method</th>
<th>Mutation</th>
<th>Ligands</th>
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* Adenosine triphosphate (ATP), PYR (pyruvate), HCO₃⁻ (bicarbonate), cyclic-di-adenosine monophosphate (c-di-AMP), oxaloacetate (OAA), phosphoiminophosphonic acid-adenylate ester (ANP), adenylyl-imidodiphosphohate (AMP-PNP), adenosine-5'-O-(3-thiotriphosphate) (ATP-g-S), acetyl coenzyme A (AcCoA), and coenzyme A (CoA). In the BCCP location column the A, B, C and D before the BCCP location represents the chain that that BCCP is a part of.
**BC and CT Domain Half Reactions**

The first half reaction occurs in the BC domain, where substrates MgATP and bicarbonate bind. In the most generally accepted mechanism, a catalytic triad involving residues Lys\textsubscript{239}, Glu\textsubscript{213}, and Glu\textsubscript{298}, help lower the pK\textsubscript{a} of the active site general base, Glu\textsubscript{298}, which deprotonates bicarbonate. The enables a nucleophilic attack from bicarbonate on the γ-phosphate of MgATP, resulting in formation of the carboxyphosphate intermediate. The electrostatic interaction between Lys\textsubscript{239} and the γ-phosphate is essential for carboxyphosphate formation (Zeczycki et al., 2011). Carboxyphosphate then decomposes to CO\textsubscript{2} and PO\textsubscript{4}\textsuperscript{3-} (inorganic phosphate). The inorganic phosphate then deprotonates the N\textsubscript{1} ureido nitrogen on the biotin cofactor that is tethered to Lys\textsubscript{1112} on the BCCP domain, forming the biotin enolate. The nucleophilic biotin enolate, stabilized by Arg\textsubscript{346}, attacks the CO\textsubscript{2} forming carboxybiotin, which is the final product of the BC domain half reaction (Zeczycki et al., 2011). It has been proposed that Arg\textsubscript{346} subsequently forms a salt bridge with Glu\textsubscript{343} to exclude carboxybiotin from the BC domain active site, preventing decarboxylation, and promoting BCCP disassociation from the BC domain (Zeczycki et al., 2011) (Figure 1-6).
The second half reaction occurs in the CT domain where pyruvate is bound. Pyruvate interacts with Arg<sub>614</sub>, Arg<sub>541</sub>, Gln<sub>545</sub> and the divalent metal that is permanently bound in the CT active site. When pyruvate binds in the CT domain active site it induces a structural change. This structural change is referred to as the substrate-induced biotin binding pocket, where Tyr<sub>621</sub> swings down and forms an interaction with Asp<sub>583</sub>. The $\pi$-sulfur interaction between carboxybiotin and Tyr<sub>621</sub> aids in carboxybiotin binding in the CT domain active site (Lietzan & St. Maurice, 2013a).
First carboxybiotin decarboxylation is facilitated by interactions with Gln838 and Ser879 (Zeczycki et al., 2009). Next, Thr876 acts as a proton shuttle, serving both as a general acid to protonate the biotin enolate at N1 and a general base to deprotonate pyruvate with the resulting formation of the enol-pyruvate intermediate. The nucleophilic enol-pyruvate intermediate then attacks the liberated CO2 forming the product oxaloacetate (Figure 1-7).

**Figure 1-7. The CT domain half reaction in SaPC.** Residues that are in the CT domain are colored in blue. The biotin cofactor that is tethered to the BCCP domain is colored in red. This figure is adapted from the figure in Zeczycki et. al., 2009.
Kinetic Characterization of PC Reaction

To kinetically characterize the BC domain half reaction, the reverse half reaction can be measured. The reverse half reaction is ATP formation, or ADP phosphorylation. Various compounds can be used as the phosphoryl donor, such as carbamoyl phosphate or acetyl phosphate (Figure 1-8B). This assay utilizes hexokinase and glucose-6-phosphate dehydrogenase (G6PDH) as the coupling enzymes and monitors the formation of NADPH at 340 nm (Zeczycki et al., 2011). When characterizing the full forward reaction, pyruvate carboxylation, malate dehydrogenase (MDH) is used as the coupling enzyme and the disappearance of NADH is monitored at 340 nm (Figure 1-8A).

To kinetically isolate the CT domain reaction the reverse CT domain reaction, oxaloacetate decarboxylation can be monitored. This activity can be followed even in constructs of PC that lack the BC domain. The reaction is often performed in the presence of oxamate since oxamate increases the rate of this reaction by accepting the CO₂ group from oxaloacetate (Attwood & Cleland, 1986; Lietzan et al., 2014; Marlier et al., 2013). This assay uses lactate dehydrogenase (LDH) as the coupled enzyme and observes the consumption of NADH at 340 nm (Figure 1-8C).
Structure of BC and CT Domains

The BC domain is comprised of 3 subdomains, the N-terminal A subdomain, the B subdomain lid and the C-terminal C subdomain (Waldrop et al., 1994) (Figure 1-9A). The A subdomain is comprised of 4 α-helices and 5 β-sheets. A helix-turn-helix motif connects the A and B subdomains. The B subdomain lid extends out from the BC domain and undergoes a conformational change upon ATP binding. When ATP is bound the B subdomain lid closes down over the BC active site (Lietzan et al., 2011; Thoden et al., 2000) (Figure 1-9B). The closed lid contributes to the ATP binding site, which is at the interface between the B and C subdomains. The C subdomain is the largest subdomain and contains 8 β-sheets. Structural motifs in the BC domain revealed that enzymes with a
BC domain are part of the ATP grasp enzyme family, which include many carboxylate-amine ligases (Thoden et al., 2000). The connection to the ATP grasp family helped elucidate the BC domain reaction mechanism because other ATP grasp enzymes also proceed through acylphosphate intermediates (Fawaz et al., 2011).

**Figure 1-9. The structure of the BC domain of SaPC.** A. PDB 3BG5. The three subdomains of the BC domain are the N-terminal A subdomain (cyan), the B subdomain lid (magenta) and the C terminal C subdomain (orange). B. The closure of the B subdomain lid. PDB ID 3BG5. The B subdomain lid in the open conformation (grey) and in the closed conformation (cyan). C. The interaction between the BC and BCCP domains. The BCCP domain from PDB 7ZZ3 was superimposed onto PDB 3BG5. ATP is from 3BG5 structure and is shown in sticks. The bicarbonate ion is from PDB 7ZZ3 and is shown sticks. The biotinylated lysine from PDB 7ZZ3 is also shown in sticks.
The CT domain consists of two structural motifs: an N terminal TIM barrel and a C-terminal funnel (St. Maurice et al., 2007). The TIM barrel consists of 8 α-helices and 8 β-sheets and comprises the bulk of the CT domain (Lietzan & St. Maurice, 2013a). The TIM barrel contains the CT domain catalytic residues. The C-terminal funnel leads into the CT domain active site. When the CT domain substrate binds, a subtle conformational change occurs and there is a slight narrowing of the funnel (López-Alonso et al., 2022). A Lewis acid metal (Mn²⁺) is bound in the active site and helps coordinate pyruvate (Figure 1-10). The carrier domain has been observed to bind in two distinct areas of the CT domain: the active site and the exo site (Figure 1-10C and D). When the carrier domain is bound in the active site, the biotinylated lysine is positioned near the substrate pyruvate, so that the carboxyl group can be transferred to form oxaloacetate. When the carrier domain is bound in the exo site, the biotinylated lysine is located at the periphery of the CT domain. No catalysis occurs when the carrier domain is bound in the exo site. The BCCP domain has been observed to primarily be bound in the CT exo site in crystal structures of SaPC (Xiang & Tong, 2008; Yu et al., 2009). The function of the exo binding site and its role in the catalytic cycle, if any, remains an active area of investigation.
Figure 1-10. The structure of the CT domain of SaPC. 

A. PDB 3BG5. The two subdomains of the CT domain are the N-terminal TIM barrel (grey) and the C-terminal funnel (green). The three blue helices are not a part of either subdomain. 

B. The CT domain with no substrate bound is shown in grey (PDB 3HO8) and the CT domain with pyruvate bound shown in cyan (PDB 3BG5). The CT domain active site is shown with Mn$^{2+}$ in purple sphere representation and pyruvate in stick representation from 3BG5 structure. 

C. The binding of the BCCP domain in the CT domain active site. 

D. The binding of the BCCP domain in the CT domain exo site. The BCCP domain is outlined in red surface representation. The active site is shown with pyruvate in stick representation and Mn$^{2+}$ in sphere representation.
Role of PC in Metabolism and Disease

As previously mentioned, PC is an anaplerotic enzyme, replenishing carbon to the TCA cycle. HsPC is expressed and plays a pivotal role in liver, kidney, adipose, pancreatic and astrocyte tissues through many different mechanisms. Briefly, PC is important in liver and kidney tissue because of its role in gluconeogenesis and fatty acid synthesis, in adipose tissue for de novo lipogenesis, in pancreatic tissue for glucose homeostasis and inflammation protection, and in astrocytes for neurotransmitter production from α-ketoglutarate (Jitrapakdee et al., 2008). Due to PC’s vital role in metabolism in these various tissues, PC is implicated in many diseases that have a higher energy requirement.

PC’s role in replenishing carbon to the TCA cycle makes it crucial for redox metabolism. PC’s role in redox metabolism essential to its role in glucose induced insulin secretion in pancreatic islet cells. Insulin exocytosis requires a high ATP/ADP ratio. PC aids in the production of nicotinamide adenine dinucleotide phosphate + hydrogen (NADPH) through pyruvate shuttling. After pyruvate is carboxylated to form oxaloacetate, oxaloacetate is converted to malate and/or citrate. Malate and citrate are then shuttled to the cytoplasm, through either the pyruvate/malate or the pyruvate/citrate shuttles, respectively. In the cytosol, malate is converted to pyruvate by malic enzyme concomitantly producing NADPH. Cytoplasmic citrate is converted to oxaloacetate and acetyl-CoA by ATP citrate lyase, and the oxaloacetate is converted to pyruvate and NADPH through the action of malate dehydrogenase and malic enzyme. NMR studies have revealed that this pool of NADPH is linked to insulin secretion (Lu et al., 2002).
In healthy β-islet cells, the predominant end product of glycolysis is pyruvate, due to the low expression levels of lactate dehydrogenase in this cell type (Sekine et al., 1994). Due to the higher concentration of pyruvate, healthy β-islet cells have higher PC activity. In a fed/overfed metabolic state PC activity is upregulated, which increases pyruvate shuttling, generating more reducing equivalents as explained above. After insulin secretion, induced by glucose, PC activity is reduced in pancreatic β-islet cells, while PC is simultaneously being overexpressed in adipocyte cells to expand adipose tissues as a means to store excess diacylglycerides and fatty acids.

In unhealthy β-islet cells, or in a diabetic scenario, the liver is insulin resistant and hepatic glucose production is increased leading to a hyperglycemic state. Hepatic PC activity is upregulated because of an increased uptake of substrates and overall increase in flux through the liver, which causes the generation of more glucose via gluconeogenesis (Large & Beylot, 1999). Hyperglycemia down regulates PC expression and activity in pancreatic tissue, which lowers insulin production and secretion. This reduced level of insulin in turn contributes to the increase of glucose production in the liver, creating a so called glucotoxicity loop (Figure 1-11). The hyperglycemic state contributes to insulin resistance and lower PC expression in adipose tissue. This overproduction of glucose in the liver, in part from PC, and reduction of PC in pancreatic and adipose tissues, exacerbates inflammation and other pathologies of insulin resistance (Jitrapakdee et al., 2008).
In addition to the role played by PC in redox metabolism, PC activity is also associated with glutathione (GSH) metabolism which serves to protect pancreatic islet cells from oxidative stress. GSH synthesis metabolite profiles revealed that increased glucose metabolism is protective against inflammation (Fu et al., 2021). When glucose levels are increased, glucokinase, the hexokinase isoform in pancreatic tissue, is increased which modulates PC activity. Increased PC activity contributes to the availability of aspartate. Stable levels of aspartate contributes to the production of arginosuccinate, which is the rate limiting urea cycle substrate. Sufficient levels of
arginosuccinate contributes to increased arginase activity, which increases the production of urea as opposed to nitric oxide (NO) which is produced from arginine (Fu et al., 2020). PC-directed partitioning of the aspartate-arginosuccinate shunt decreases NO production and subsequent inflammation. This is an essential protective mechanism for pancreatic islet cells, especially in diabetic scenarios.

PC’s role in redox metabolism has linked it to multiple types of cancers, such as non-small cell lung cancer, breast cancer, and thyroid cancer (Fan et al., 2009; Kumashiro et al., 2013; Lin et al., 2020; Liu et al., 2022). In thyroid cancer cell lines, PC protects against oxidative stress which promotes mitogen-activated protein kinase (MAPK) signaling, which in turn decreases expression of iodine uptake associated proteins and causes malignant progression (Liu et al., 2022). In breast cancer cell lines, PC increases the NADPH/NADP+ ratio which decreases ROS in tumor cells which leads to the increase in tumor proliferation and metastasis (Wilmanski et al., 2017).

Inhibition of PC in vitro, using multiple breast cancer cell lines, showed reduction in invasion and migration of the cancer cells. Inhibition of PC in vivo, in a breast cancer mouse model, reduced metastasis and lung colonization (Lin et al., 2020). Inhibiting PC in multiple thyroid cancer cell lines reduced cell proliferation and migration (Liu et al., 2022). In a diabetic mouse model PC knockdown has been shown to improve insulin resistance (Kumashiro et al., 2013). Small molecule inhibitors have been generated and bind in the CT domain or at unknown locations (Burkett et al., 2019; Lin et al., 2020). All of these studies, and others, demonstrate that PC is a therapeutic target for a multitude of diseases. In order to develop inhibitors/therapeutics against PC, the fundamentals of how PC is regulated and undergoes conformational change must be understood.
Figure 1-12. The role of PC in metabolism. PC serves to replenish carbon to the TCA cycle and is the connection to the urea cycle. NAD represents nicotinamide adenine dinucleotide, FAD represents flavin adenine dinucleotide, GSH represents glutathione, NO represents nitric oxide, AsAT represents aspartate aminotransferase, and MDH represents malate dehydrogenase. The compounds in a box filled with blue are located in the cytoplasm. The compounds in a box filled with yellow are located in the mitochondria. The compounds in a box filled with blue outlined in yellow are located in both the cytoplasm and the mitochondria. The green arrows represent pyruvate shuttling, the black arrows represent the TCA cycle, and the blue arrows represent the urea cycle. Figure made with BioRender.
Allosteric Activation of PC by Acetyl-CoA

Pyruvate carboxylase is allosterically activated by acetyl-CoA, a two-carbon, high-energy metabolite that is extremely important in many cellular processes (Figure 1-13). Acetyl-CoA is at the intersection between many different metabolic processes, and the localization and sub-compartmental levels of this metabolite can serve as a sensor for the cellular metabolic state. High levels of cytosolic and nuclear acetyl-CoA correlate with the fed state and contributes to directing energy to lipid synthesis and promotes histone/protein acetylation. Conversely, high levels of mitochondrial acetyl-CoA correlate with the starved state and leads to the synthesis of ketone bodies (Shi & Tu, 2015). Furthermore, acetyl-CoA directly regulates metabolic enzymes that participate in cellular respiration. When carbon is plentiful in the TCA cycle, acetyl-CoA binds to and allosterically inhibits pyruvate kinase (Fenton & Blair, 2002). This aids in the switch from the starved state to the fed state. When the TCA cycle needs replenishing, acetyl-CoA allosterically activates PC, which increases the production of oxaloacetate, facilitating the condensation of oxaloacetate with acetyl-CoA in the first step of the TCA cycle (Figure 1-12). Acetyl-CoA is an essential metabolite, not just because it provides two carbon building blocks for energy molecules, but also because it plays a critical role in regulation both at a small scale, by modulating individual proteins, and on a cellular scale, by serving as a critical sensor and switch for the metabolic state (Shi & Tu, 2015).
Acetyl-CoA is a non-essential allosteric activator of PC, meaning that PC retains a baseline activity in the absence of the activator. The degree of sensitivity to acetyl-CoA is dependent on the organism from which PC is isolated. RePC and SaPC are acetyl-CoA sensitive. RePC has 35-fold greater activity when acetyl-CoA is present, whereas SaPC has ~15-fold greater activity (Sirithanakorn et al., 2016; Westerhold et al., 2017). AnPC is insensitive to acetyl-CoA, and exhibits no change in activity when it is present (Adina-Zada, Zeczycki, et al., 2012). The locus of activation of acetyl-CoA is the BC domain, where the presence of the activator decreases the $K_m$ of the BC domain substrates bicarbonate and MgATP (Ashman et al., 1972). Acetyl-CoA increases the binding affinity of BC domain substrates and metal ions which increases the overall BC domain

![Figure 1-13. The regulatory role acetyl-CoA plays in the cell. A. A representation of acetyl-CoA localization in the cell when the cell is in a fed state or a starved state. The molecules produced from acetyl-CoA, when the cell is in that particular metabolic state, are depicted below. B. A representation of acetyl-CoA in the TCA cycle. The green arrow represents allosteric activation, and the red blunted arrow represents allosteric inhibition. PC stands for pyruvate carboxylase, PDH stands for pyruvate dehydrogenase, AsAT stands for aspartate aminotransferase.](image)
occupancy, leading to less abortive ATP cleavage, effectively coupling the BC and CT domain half reactions.

To determine what causes a variant of PC to be acetyl-CoA sensitive or insensitive, enzyme chimeras of RePC and AnPC were generated (Yumeng Liu, unpublished). A chimeric protein with the BC domain from RePC and the AD, CT and BCCP domains of AnPC, was sensitive to acetyl-CoA. Conversely, a chimeric protein with the BC domain from AnPC and the AD, CT, and BCCP from RePC was not sensitive to acetyl-CoA. This demonstrates that the BC domain determines the sensitivity to acetyl-CoA. Although AnPC is insensitive to acetyl-CoA activation, the acetyl-CoA binding site is still conserved and there is kinetic evidence that it can still bind. While acetyl-CoA does not influence the overall pyruvate carboxylation reaction in AnPC, it does activate the reverse oxaloacetate decarboxylation reaction in a concentration dependent manner, demonstrating that the activator is binding to AnPC (Yumeng Liu, unpublished). Additionally, acetyl-CoA relieves L-aspartate inhibition, which is known to have an overlapping binding site, demonstrating that acetyl-CoA must be binding to AnPC (Osmani et al., 1984). These studies regarding AnPC emphasize the importance the BC domain has on allosteric activation by acetyl-CoA.

Acetyl-CoA binds in the AD near the BC dimer interface. Arg$_{420}$, Arg$_{422}$ and Arg$_{465}$ bind to the phosphates that are part of the nucleotidyl portion of the activator (Adina-Zada, Sereeruk, et al., 2012). The pantothenic acid arm of acetyl-CoA threads into the BC dimer interface (Figure 1-14). The exact binding site of the acetyl moiety of acetyl-CoA, in the BC dimer interface, is not defined because, until recently, it does not appear in the electron density, likely because it is being hydrolyzed (Scrutton & Utter,
The binding of acetyl-CoA does not result in a large conformational change in the BC domain active site, but acetyl-CoA binding does result in tetrameric changes in the domain angles between one another.

**Figure 1-14. The binding site of CoA in SaPC.** A. PDB 3HO8. CoA binds in the BC dimer interface. The BC dimer interface is outlined in surface representation. CoA is shown in sphere representation. B. A molecular view of the CoA binding site in the AD and BC dimer interface. Residues Arg420, Arg422, and Arg465 are shown in stick representation and interact with the phosphates on the nucleotidyl portion of CoA.

A recent cryo EM study conducted on *Lactococcus lactis* PC (*Ll*PC) revealed a set of global movements in the presence of acetyl-CoA. Using multibody refinements on a large cryo EM data set, a correlation between acetyl-CoA binding and the change of the angle between BC and CT domains on the same subunit was revealed. The presence of acetyl-CoA results in a $1.4^\circ$ shift in angle, with the BC domain shifting inward towards the CT domain. The tetramers that exhibited this shift in angle were classified as BC$_{\text{react}}$ and CT$_{\text{react}}$, therefore this shift in angle is associated with catalysis (López-Alonso et al., 2022).
While the main locus of activation is in the BC domain, acetyl-CoA does influence the CT domain substrate, pyruvate. Acetyl-CoA decreases the $K_m$ for pyruvate, which represents an increase in binding affinity, but it does not activate the carboxyltransferase half reaction (Hudson et al., 1975).

**Allosteric Activation by Acetyl-CoA Modulates Carrier Domain Movement**

A previous study conducted by our group showed that carrier domain translocation pathways are refined by the presence of acetyl-CoA (Liu et al., 2018a). This study utilized systematically inactivated hybrid tetramers. Through deliberate remixing of these hybrid tetramers to target specific carrier domain translocation pathways, it was shown that not only one translocation pathway in PC was catalytically productive, but two others as well (Figure 1-15). The fact that the carrier domain has three translocation pathways is an indication that the carrier domain more flexible than previously described. When acetyl-CoA is present, only one translocation pathway is promoted (Figure 1-15, pathway $a$), while it had no influence on pathways $b$ and $c$. The $k_{cat}^{AcCoA}/k_{cat}^{°}$ for pathway $a$ in the remixed hybrid tetramer was greater than for WT PC, suggesting that the degree of acetyl-CoA activation is increased in the hybrid tetramer, emphasizing the allosteric control acetyl-CoA directs to carrier domain translocation. This study indicates that acetyl-CoA is activating the enzyme, in part, by refining the translocation of the carrier domain, providing an initial indication that the conformational dynamics of PC are influenced by allosteric activation.
As a further indication of acetyl-CoA allosteric activation altering carrier domain movement, it has been shown that acetyl-CoA couples the BC and CT domain half reactions (Legge et al., 1996). In the presence of acetyl-CoA, the rate of ATP formation and carboxybiotin formation in the BC domain is greatly increased, and the rate of abortive ATP cleavage, which results in carboxybiotin decomposition, is greatly reduced. The reduction in abortive ATP cleavage can be interpreted as an enhanced coupling of the BC domain half reaction to the CT domain half reaction. The fact that acetyl-CoA
couples the two half reactions together is consistent with acetyl-CoA influencing carrier domain translocation.

Thermodynamic linkage analysis studies also revealed that the $K_m$ values of BC domain substrate, MgATP, and CT domain substrate, pyruvate, are thermodynamically coupled, in the pyruvate carboxylation reaction, but only in the presence of acetyl-CoA. This indicates that although acetyl-CoA binds closer to the BC domain, there are long range effects to the other catalytic domain. The thermodynamic activation parameters for SaPC show that the transition state entropy was less negative in the presence of acetyl-CoA, which indicates SaPC is a more ordered system in the presence of the allosteric activator (Westerhold et al., 2017). Allosteric activation’s modulation of conformational dynamics in PC could be contributing to the ordering of the system.

*Allosteric Inhibition of PC by L-Aspartate*

Microbial and fungal PCs are allosterically inhibited by L-aspartate. When carbon is plentiful in the TCA cycle, levels of the amino acid L-aspartate are high, and L-aspartate binds to and inhibits PC (Figure 1-13B). Initially it was discovered that vertebrate PC’s were inhibited by a different tricarboxylic acid, L-glutamate (Scrutton, 1978). Following this discovery, it was soon revealed that microbial PC’s were inhibited by L-aspartate (Osmani et al., 1981). Like acetyl-CoA, L-aspartate’s main locus of inhibition is the BC domain, competitively inhibiting the bicarbonate dependent ATP cleavage reaction. L-aspartate has no effect on the CT domain reverse reaction. Since L-aspartate has no effect on the CT domain reaction but is still inhibitory to pyruvate carboxylation at saturating MgATP concentrations, it is believed that L-aspartate either inhibits carboxybiotin formation or carrier domain translocation (Sirithanakorn et al.,
L-Aspartate had little effect on the coupling of MgATP to oxaloacetate formation monitored through a P_i release assay (Legge et al., 1996). L-Aspartate binds to PC cooperatively and competitively inhibits with respect to acetyl-CoA. The fact that the two primary allosteric regulators of PC, L-aspartate and acetyl-CoA, are mutually exclusive has led to the hypothesis that there is an overlap in their binding sites (Osmani et al., 1981; Sirithanakorn et al., 2014; Yu et al., 2009). However, mutagenesis of residues that bind the nucleotidyl portion of acetyl-CoA had no effect on L-aspartate inhibition (Adina-Zada, Sereeruk, et al., 2012). Consequently, it was assumed that the effector molecules have distinct binding sites (Adina-Zada, Sereeruk, et al., 2012; Sirithanakorn et al., 2014). Compared to acetyl-CoA, L-aspartate has a significantly reduced binding affinity. The \( K_i \) of L-aspartate for \( SaPC \) is \( \sim 20 \) mM, whereas the \( K_a \) of acetyl-CoA for \( SaPC \) is \( \sim 1 \) \( \mu \)M. Consequently, much higher concentrations of L-aspartate are required to inhibit the enzyme than acetyl-CoA is required to activate the enzyme. Currently, the binding site of L-aspartate remains undefined, in part because its low binding affinity has made it difficult to obtain a structure of PC in complex with L-aspartate. While some attempts have been reported, the inhibitor has not appeared in the electron density (Lietzan et al., 2011).

The affinity of PC for L-aspartate is dependent on the source organism. For fungal PC enzymes, the \( IC_{50} \) values range from 0.6 to 4 mM (Osmani et al., 1981). For bacterial \( RePC \) the \( IC_{50} \) is 1.3 mM (Sirithanakorn et al., 2014), but for \( Sinorhizobium meliloti \) PC, which is a bacterium also part of rhizobia, 10 mM L-aspartate only inhibits \( \sim 10\% \) of pyruvate carboxylation activity, therefore there is a great range of L-aspartate sensitivity even among closely related organisms (Dunn et al., 2001).
The Significance of the BC Dimer Interface

Acetyl-CoA carboxylase (ACC), like PC, is a biotin dependent carboxylase. ACC is the enzyme involved in the rate limiting step of fatty acid biosynthesis. ACC also has a BC dimer, and the BC dimer interface is important for long range communication. It was shown in *E. coli* BC ACC that, when one subunit contains an active site mutation, the other subunit becomes inactive and the hybrid dimer only retains 2% WT activity, although monomeric *E. coli* BC ACC retains 91% activity *in vitro* (Janiyani et al., 2001; Shen et al., 2006). This indicates that there is long range communication between the two active sites located 50 Å apart. Although dimerization is not a requirement for *E. coli* BC *in vitro*, dimerization is required, in both bacterial and eukaryotic ACC, for activity and ultimately fatty acid biosynthesis *in vivo* (Smith & Cronan, 2012).

BC dimerization is required for *Sa*PC BC domain activity. To create monomeric *Sa*PC BC, obstructive mutations were made in the BC dimer interface. In *Sa*PC, residues Arg21, Glu25, Lys411, and Phe472 were all mutated to residues of the opposite charge. These residues were chosen because they are homologous to residues that are implicated in dimerization in *E. coli* BC from ACC. These BC dimer interface mutants yielded inactive protein, thus proving dimerization is required for activity (Yu et al., 2013). This is unsurprising because, as previously mentioned, PC is only catalytically active as a tetramer (St. Maurice et al., 2007).

Additionally, the CT dimer interface of PC from various bacterial species has an allosteric site. cyclic-di-adenosine monophosphate (ci-di-AMP), a secondary messenger that is important in cell signaling binds to and allosterically activates PC. Ci-di-AMP binds in the CT dimer interface of *Listeria monocytogenes* PC (*Lm*PC), *Lactococcus*
*lactis* PC (*LlPC*), and *Enterococcus faecalis* PC (*EfPC*). The ci-di-AMP binding site is not conserved in *SaPC*, likely because this signaling molecule is not as important in this bacterium. In *Staphylococcus aureus* ci-di-AMP contributes to osmotic regulation, but this secondary metabolite is not required for viability (Zeden et al., 2018). Although ci-di-AMP does not allosterically regulate *SaPC*, it shows that there could be an allosteric site located in the CT domain. It also shows that dimer interfaces are common places for effector molecules to bind. Acetyl-CoA and ci-di-AMP both bind in dimer interfaces, as well as soraphen, which is an allosteric modulator of the BC subunit of ACC (Shen et al., 2004; Wei & Tong, 2015). Dimer interfaces are common places for ligands and effector molecules to bind, and important for long range communication in PC.

The BC dimer of ACC has obligatory half of the sites reactivity, meaning that when one BC subunit is active the other subunit is inactive (Janiyani et al., 2001). Due to the similarity between the BC dimer from ACC and from PC, it was long thought that the BC dimer in PC also exhibited half the sites reactivity. This is still an active area of investigation, but a recent cryo EM study that did a correlative global conformational analysis found a strong positive correlation between active BC domains in the same BC dimer, disproving obligatory half the sites reactivity (López-Alonso et al., 2022). Additionally, sedimentation velocity experiments have confirmed the presence of intra- and inter-organism tetramers and showed that hybrid tetramers with only one catalytically active subunit on each face of the tetramer retained comparable activity to WT tetramers (Adina-Zada et al., 2019; Rattanapornsompongb et al., 2020). It is increasingly acknowledged that, although the BC dimers are highly conserved, PC likely does not display half the sites reactivity.
Overview

PC is an essential metabolic enzyme, and because of its role in replenishing carbon to the TCA cycle, it is implicated in many diseases that have an altered metabolism, such as cancer, making it a proven drug target (Liu et al., 2022). PC is subject to both allosteric activation and inhibition, depending on the metabolic state of the cell. For PC to perform catalysis a large conformational change must occur. The mechanism of allosteric regulation and how this regulation alters the conformational dynamics of PC is poorly understood, although this regulation has large functional implications that is relevant in therapeutic design.

In this dissertation, allosteric regulation and conformational dynamics are being studied in the well characterized SaPC. Here I determined the previously uncharacterized binding site for the acetyl moiety of acetyl-CoA, which contributes significantly to the overall binding affinity, but is susceptible to enzyme-catalyzed hydrolysis. Residues Arg_{21}, Lys_{46} and Glu_{418} all interact with the acetyl moiety and contribute to binding and the allosteric mechanism. The role of the residues that interact with the acetyl moiety was investigated and were determined to be implicated in both allosteric activation and allosteric inhibition.

Swinging domain enzymes, such as PC, undergo large conformational changes to transfer reaction intermediates. Many swinging domain enzymes, such as PKS, can have up to 60 domains (Castoe et al., 2007). PC is much simpler with only 4 domains and 4 subunits. Whether these large conformational changes are regulated, and how ligand binding influences these motions, are major questions in the field. To study the conformational dynamics in PC, multiple biophysical tools were utilized. Here, it is
determined that the carrier domain translocation in PC is governed by conformational selection, and that BC domain substrates influence the rate of carrier domain translocation. It was concluded that the carrier domain exists in a dynamic equilibrium that is altered by the presence of substrates and allosteric effectors, and a model was proposed for carrier domain translocation in the tetramer that is highly coordinated in the presence of the allosteric activator acetyl-CoA, limiting the number of subunits undergoing simultaneous catalysis. This work contributed to a greater understanding of the molecular basis of allosteric regulation in PC and proposed a refined model for the complete catalytic cycle in the context of the PC tetramer.
CHAPTER 2. CHARACTERIZING THE MOLECULAR BASIS OF ALLOSTERIC REGULATION IN THE BIOTIN CARBOXYLASE DIMER INTERFACE

Introduction

Allosteric regulation can change an enzyme in a myriad of ways, altering the binding affinity of substrates, the thermodynamic activation parameters, the conformational dynamics, and the maximal rate of the reaction. Establishing a clear molecular basis for allosteric regulation can elucidate the ways in which the effector molecule is affecting the enzyme at areas distant from its binding site when no clear structural change is observed.

Allosteric activator acetyl-CoA is a non-essential activator that has both V and K-type activation because it alters both the $V_{\text{max}}$ of the overall reaction and the $K_M$ of substrates. The sensitivity to acetyl-CoA is dependent on the organism and can range from having no affect to activating the enzyme ~35-fold (Adina-Zada, Zeczycki, et al., 2012). The BC domain determines whether the enzyme is sensitive to acetyl-CoA (Yumeng Liu, unpublished). Acetyl-CoA binds in the AD near the BC domain. Arg420, Arg422 and Arg465 bind to the phosphates that are part of the nucleotidyl portion of the activator (Adina-Zada, Sereeruk, et al., 2012). The binding site of the acetyl moiety of acetyl-CoA is not defined because it does not appear in the electron density, likely because it is being hydrolyzed. The locus of activation of acetyl-CoA is the BC domain and the presence of the activator decreases the $K_M$ of BC domain substrates bicarbonate and MgATP (Ashman et al., 1972). Acetyl-CoA increases the binding affinity of BC domain substrates and metal ions which increases the overall BC domain occupancy,
which leads to less abortive ATP cleavage, effectively coupling the BC and CT domain half reactions.

While the main locus of activation is in the BC domain, near the acetyl-CoA binding site, acetyl-CoA does influence the CT domain. Acetyl-CoA decreases the $K_M$ for CT domain substrate, pyruvate. Thermodynamic linkage analysis studies also revealed that BC domain substrate, MgATP, and CT domain substrate, pyruvate, are thermodynamically coupled but only in the presence of acetyl-CoA. This indicates that although acetyl-CoA binds closer to the BC domain there are long range effects to the other catalytic domain. The thermodynamic activation parameters for $SaPC$ show that the transition state entropy was less negative in the presence of acetyl-CoA, which indicates $SaPC$ is a more ordered system in the presence of the allosteric activator (Westerhold et al., 2017).

Acetyl-CoA has been shown to affect the conformational dynamics of PC. The most characterized translocation pathway of the BCCP domain is from the BC domain of the same subunit to the CT domain of the subunit the runs antiparallel, on the same face of the tetramer (Lasso et al., 2010). A study that utilized hybrid tetramers with systematically inactivated domains revealed that there were two other translocation pathways that were also catalytically productive: one pathway where the BCCP domain translocates from the BC domain to the CT domain of the same subunit, only interacting intermolecularly, and another where the carrier domain translocates from the BC domain of the opposing subunit to the CT domain of the same subunit (Liu et al., 2018b). Acetyl-CoA only activates the most characterized translocation pathway, not all three pathways,
which indicates that acetyl-CoA narrows the available carrier domain motions, making it a more ordered system.

Microbial PC is allosterically inhibited by L-aspartate. Like acetyl-CoA, L-aspartate’s main locus of inhibition is the BC domain, competitively inhibiting the bicarbonate dependent ATP cleavage reaction. L-aspartate has no effect on the CT domain reverse reaction, the oxamate induced oxaloacetate decarboxylation reaction. Since L-aspartate has no effect on the CT domain reaction but is still inhibitory to pyruvate carboxylation at saturating MgATP concentrations, it is believed that L-aspartate either inhibits carboxybiotin formation or carrier domain translocation. There was found to be little effect on the coupling of MgATP to oxaloacetate formation monitored through a P\textsubscript{i} release assay (Legge et al., 1996). L-aspartate binds to PC cooperatively and competitively inhibits with respect to acetyl-CoA. The fact that allosteric regulators L-aspartate and acetyl-CoA are mutually exclusive has led to the hypothesis that there is an overlap in their binding sites, but when mutagenic studies on residues that bind the nucleotidyl portion of acetyl-CoA had no effect on L-aspartate inhibition, it was assumed that the effector molecules have distinct binding sites (Adina-Zada, Sereeruk, et al., 2012; Sirithanakorn et al., 2014). Here, we mutate residues that possibly bind to the acetyl moiety of the activator and hypothesize that the overlap between L-aspartate and acetyl-CoA binding sites is in the BC dimer interface.

Here, we propose that the acetyl moiety of acetyl-CoA is positioned at the BC dimer interface. We further propose that this binding site represents the site of overlap between L-aspartate and acetyl-CoA. Using acetyl-CoA analogs, site-directed mutagenesis and X-ray crystallography, we probed the structure and function of the
acetyl moiety binding site at the BC dimer interface and demonstrate an important contribution from the acetyl thioester linkage of the allosteric activator, along with three critical residues (Lys\textsubscript{46}, Glu\textsubscript{418}, and Arg\textsubscript{21}) that lie in close proximity to the acetyl moiety. We demonstrate that these residues mediate allosteric activation by acetyl-CoA and allosteric inhibition by L-aspartate. A single mutation at the BC dimer interface, E418A, was sufficient to eliminate both allosteric activation and allosteric inhibition. These findings expand the definition of allostery in PC to include an important allosteric site at the BC dimer interface.

**Materials and Methods**

**Materials**

Acetyl-CoA was purchased from CoALA Biosciences (Austin, TX). Except where noted, all other chemicals and materials were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), or Research Products International Corp. (Mount Prospect, IL). All DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA).

**SaPC Constructs**

Pyruvate carboxylase from *S. aureus* (SaPC; UniProtKB - A0A0E8G8A7) was previously cloned into a modified pET-27b vector and was generously supplied by Dr. Liang Tong, Columbia University (Yu et al., 2009). To reclone SaPC into the pTXB1 vector (New England Biolabs, Ipswich, MA), the gene encoding SaPC in pET-27b was PCR amplified using forward (5'- GCC ATA TGA AAC AAA TAA AAA AG -3') and reverse primers (5'- CGT GAT GCA GTT AGT TGC TTT TTC AAT TTC AAT TTG TAA AAA AG -3'). The
PCR amplicon was subjected to restriction digestion with NdeI and SapI prior to ligation into a gel-purified pTXB1 vector digested with NdeI and SapI. WT SaPC pTXB1 was fully sequenced.

The full sequence of SaPC pTXB1 revealed a PCR-introduced G571S mutation. The mutation was corrected back to a glycine at residue 571 by mutagenesis using the whole-plasmid PCR technique according to the Quikchange II mutagenesis protocol from Agilent Technologies, Inc. (Santa Clara, CA), with forward primer 5’- GCG GAC GTA TTT AAA GAT GGT TTC TCA G -3’ and reverse primer 5’- CTA GTG AGA AAC CAT CTT TAA ATA CGT CCG C -3’. The complete gene sequence of WT SaPC in the pTXB1 vector was confirmed by DNA sequencing.

**SaPC pTXB1 Expression and Purification**

SaPC pTXB1 enzymes were purified with sequential chitin affinity and anion exchange chromatography. Harvested cell paste (20 – 30 g) was re-suspended in 200 mL column binding buffer (20 mM HEPES, pH 7.8; 200 mM NaCl; 0.5 mM ethylenediaminetetraacetic acid (EDTA); 1 mM Tris (2-carboxyethyl) phosphine (TCEP)) with the inclusion of protease inhibitors phenylmethylsulfonyl flouride (PMSF) (1 mM), pepstatin A (1 μM); and L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine (E-64) (5 μM). Cells were lysed by sonication for 8 minutes at a temperature ranging from 4-10 °C and pelleted by centrifugation. The supernatant was loaded on a 10 mL chitin resin column (NEB, Ipswich, MA), with the column flow through re-applied to the column a total of three times. The column was washed with 100 mL of column binding buffer. The resin was subsequently incubated overnight at 4 °C with 30 mL of cleavage buffer (20 mM HEPES, 200 mM NaCl, 1 mM EDTA, and 50 mM dithiothreitol (DTT)).
The following day, the cleaved protein was eluted from the column. Two additional incubations with 30 mL of cleavage buffer were applied for at least 4 hours before eluting protein. The eluant from each of the incubations was pooled and dialyzed overnight at 4 °C against a buffer compatible with anion-exchange chromatography (20 mM triethanolamine, pH 8.0; 50 mM NaCl; 1 mM EGTA; and 2 mM DTT). The dialyzed protein was loaded onto a 10 mL Q-Sepharose Fast Flow resin column (GE Healthcare), washed with 100 mL of dialysis buffer and eluted with a gradient from 50 mM to 1 M NaCl in dialysis buffer. *SaPC* typically elutes between 400 - 800 mM NaCl. The purified protein was pooled and dialyzed against storage buffer (20 mM HEPES, pH 7.2; 15 mM NaCl; 10 mM MgCl₂, and 1 mM TCEP) for two successive changes of 4 hours or more. The protein was concentrated to a final concentration of 10 – 25 mg/mL and was drop frozen in liquid nitrogen prior to storage at -80 °C. All protein concentrations were determined using the predicted extinction coefficient corresponding to absorbance at 280 nm.

**SaPC Mutagenesis**

All *SaPC* genes were expressed and purified in an identical manner. Mutations in *SaPC* were generated according to the Agilent Quikchange II Site-Directed Mutagenesis protocol. Primers were obtained from Integrated DNA Technologies. K46A *SaPC* was mutated using primers 5'- GAT ATG CTG CAG ATG AAT CCT ATT TAG TTG -3' (forward) and 5'- CAA CTA AAT AGG ATT CAT CTG CAG CAT ATC -3' (reverse). E418A *SaPC* was mutated using primers 5'- CGA GCT ATG CGT ATT CGT GGT GTT AAA AC -3' (forward) and 5'- GTT TTA ACA CCA CGA ATA CGC ATA GCT CG -3' (reverse). R21A *SaPC* was mutated using primers 5'- CGT ATA TTC GCA GCG GCG
GCA GAA TTA GAC -3' (forward) and 5'- GTC TAA TTC TGC CGC CGC TGC GAA TAT ACG -3' (reverse). K411A ScaPC was mutated using primers 5'- GCG ATA TCA TTT GCG CAA GCA GAA GAA AAA -3' (forward) and 5'- TTT TTC TTC TGC TTG CGC AAA TGA TAT CGC -3' (reverse). All constructs used in this study were confirmed by complete DNA sequencing of the gene in its entirety.

**Synthesis of Acetyl-CoA Analogs**

The analogs acetyl-oxa(dethia)-CoA and acetyl-aza(dethia)-CoA were synthesized using a previously published chemoenzymatic approach (Boram et al., 2023). A similar chemoenzymatic approach was taken to generate acetyl-carba(dethia)-CoA. Briefly, 5-Amino-2-pentanone ethylene ketal from Sigma Aldrich was condensed with previously prepared pantothenic acid acetonide in dichloromethane with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to yield ethylene ketal and acetonide protected acetyl-carba(dethia)pantetheine. The acetyl-carba(dethia)pantetheine was liberated with the application of TFA at a pH of ~2 for a few hours in a water/methanol solvent then converted to the CoA with appropriate biosynthetic enzymes as previously reported. The acetyl-carba(dethia)-CoA product was purified by reverse-phase HPLC and confirmed with LCMS analysis against an authentic standard.

**Pyruvate Carboxylation Assay**

Pyruvate carboxylation activity was measured by monitoring the change in absorbance at 340 nm resulting from the conversion of oxaloacetate to malate in the presence of the coupled enzyme, malate dehydrogenase (MDH), as previously described (Burkett et al., 2019). Reactions were performed in a kinetics buffer (0.1 M Tris-HCl, pH
7.8; 0.1 M KCl, and 7 mM MgCl₂). All substrates and coupling reagents were prepared as a 10× stock solution that provided final reaction concentrations of 25 mM NaHCO₃, 2.5 mM ATP, 0 – 4 mM acetyl-CoA, 12 mM sodium pyruvate, 0.12 mM NADH, and 10 U/mL MDH. The 10× stock substrate solution, consisting of NaHCO₃, ATP, sodium pyruvate, NADH, and MDH was used to initiate the reaction. The final PC concentration in the assay ranged from 2 - 30 μg/mL per reaction. All assays were performed in triplicate, and reported errors are the standard deviation resulting from the three trials. Data was fit to Equation 2-1, which describes allosteric activation varying the concentration of activating compound. $k_{cat}$ is the catalytic rate constant, $k_{cat}^0$ is the catalytic rate constant in the absence of activator, $k_{cat}^{-app}$ is the apparent catalytic rate constant at each concentration of compound, $K_a$ is the activation constant, $h$ is the Hill coefficient and [A] is the concentration of activator.

$$k_{cat}^{app} = \frac{k_{cat}^0 + k_{cat}[A]^h}{K_a^h + [A]^h}$$

The titration data with 3’5’ diphosphoadenosine was fit to Equation 2-2 which describes allosteric activation and subsequent inhibition at high concentrations where [A] is the concentration of 3’5’ diphosphoadenosine, $K_a$ is the activation constant, and $h$ is the Hill coefficient. The data was best fit when the $K_i$ was fixed at 5 mM and $k_{cat}^0$ was experimentally determined and subsequently fixed at 1 s⁻¹.

$$k_{cat}^{app} = \frac{k_{cat}^0 + k_{cat}([A]^{\frac{1}{K_a}})^h}{1 + ([A]^{\frac{1}{K_a}})^h + ([A]^{\frac{1}{K_i}})^h ([A])^h}$$
The titration data with L-aspartate were fit to Equation 2-3, which describes allosteric inhibition at saturating substrate concentrations where $k_{cat}$ is the catalytic rate constant, $k_{cat}^{0}$ is the catalytic rate constant in the absence of inhibitor, $K_i$ is the inhibition constant, $h$ is the Hill coefficient and $[I]$ is the concentration of L-aspartate.

$$k_{cat}^{app} = \frac{k_{cat}^{0} [I]^h}{K_i^h + [I]^h}$$

**Pyruvate Carboxylation Inhibition Assays**

Pyruvate carboxylation activity was measured in the presence of CT domain inhibitors by monitoring the change in absorbance at 340 nm resulting from the conversion of oxaloacetate to malate in the presence of the coupled enzyme, MDH, as previously described (Burkett et al., 2019). Reactions were performed in kinetics inhibition buffer (0.1 M Tris-HCl, pH 7.8, 0.15 M KCl, 7 mM MgCl$_2$, 0.5% triton X-100, and 1% dimethyl sulfoxide (DMSO)). All substrates and coupling reagents were prepared as a 10× stock solution that provided final reaction concentrations of 25 mM NaHCO$_3$, 2.5 mM ATP, 12 mM sodium pyruvate, 0.12 mM NADH, and 10 U/mL MDH. This 10× stock substrate solution was used to initiate the reaction. Reactions included 0-2.5 mM acetyl-CoA and 5-500 μg/mL of SaPC. All assays were performed in triplicate, and reported errors are the standard deviation resulting from the three trials. The normalized velocity was calculated from Equation 2-4, where $v'$ is the initial velocity, $v_{max}$ is the maximum velocity, and $v_{min}$ is the minimum velocity. The normalized velocities were then plotted against the log of the concentration of inhibitor used and the data was fit to Equation 2-5 where $v_{max}$ represents the normalized maximum velocity, $v_{min}$ represents the
minimum normalized velocity, $[I]$ represents the concentration of inhibitor and $h$
represents the Hill coefficient. $v_{\text{min}}$ was constrained to 0 and $v_{\text{max}}$ was constrained to 1.

$$\text{normalized velocity} = \frac{(v - v_{\text{min}})}{(v_{\text{max}} - v_{\text{min}})}$$

Equation 2-4

$$\text{normalized velocity} = v_{\text{min}} + \frac{(v_{\text{max}} - v_{\text{min}})}{\left(1 + \left(\frac{IC_{50}}{[I]}\right)^h\right)}$$

Equation 2-5

**ADP Phosphorylation Assay**

ADP phosphorylation activity of PC was measured spectrophotometrically at 340 nm by following the conversion of glucose-6-phosphate to 6-phosphogluconate using the coupling enzymes hexokinase and glucose-6-phosphate dehydrogenase, as previously described (Lietzan & St. Maurice, 2013b). Reactions were performed in an ADP phosphorylation assay buffer (100 mM Tris-HCl, pH 7.8, 0.1 M KCl, and 7.5 mM MgCl₂). The final PC concentration in the assay was 30 μg/mL per reaction, and PC was used to initiate the reaction. Final concentrations of 20 mM carbamoyl phosphate, 0.24 mM NADP⁺, 3 mM ADP, and 200 μM glucose were used. 10 U/mL of glucose-6-phosphate and 2 U/mL hexokinase was used. Acetyl-CoA was titrated from 0 to 8mM. All assays were performed in triplicate, and reported errors are the standard deviation resulting from the three trials.

**Size Exclusion Chromatography**

Size exclusion chromatography was performed on a BioRad FPLC system using a Superose 6 10/300 GL size exclusion column from Amersham Pharmacia Biotech
(Piscataway, NJ). 1 mL of 1 mg/mL of WT SaPC and mutants were loaded and eluted at 0.3 mL/min in FPLC buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 2 mM TCEP). Eluted samples were monitored at 280 nm.

**Protein Crystallization for R21A SaPC**

All crystals of R21A SaPC were obtained at room temperature by the batch crystallization method. The drop consisted of 13.3% MEPEG 2K, 133 mM KNO$_3$, 50 mM MOPS pH 7.0, 1.5% v/v 2-propanol, 0.625 mM acetyl-CoA, 1.25 mM adenosine triphosphate (ATP) and 9.65 mg/mL R21A SaPC. The drop volume was 10 µL. Under these conditions, crystals grew spontaneously. Crystals grew to full size of about 0.25mm after 2-4 days, with a cube-like morphology. Crystals were transferred via a quartz capillary to a synthetic stabilizing solution that consisted of 14.63% MEPEG 2K, 146.5 mM KNO$_3$, 50 mM MOPS pH 7.0, 1.5% v/v 2-propanol, 1.375 mM ATP, and 0.6875 mM acetyl-CoA. Crystals were incubated in the stabilizing solution for 5 minutes prior to being incrementally transferred into a cryoprotectant solution that consisted of 14.63% MEPEG 2K, 146.5 mM KNO$_3$, 50 mM MOPS pH 7.0, 1.5% v/v 2-propanol, 1.375 mM ATP, 0.6875 mM acetyl-CoA and 20% v/v ethylene glycol. The crystals were successively transferred 5 times into a solution with increasing amounts of the cryoprotectant solution. The crystals, indexed as a = 94 Å, b = 254 Å, c = 126 Å, and α = 90° β = 110° γ = 90°, belonged to space group P 2$_1$ 2$_1$ 2$_1$ with 4 subunits in the asymmetric unit.
Protein Crystallization for K46A SaPC

All crystals of K46A SaPC were obtained at room temperature by the batch crystallization method. The drop consisted of 11.4% MEPEG 2K, 90 mM KNO$_3$, 50 mM MOPS pH 7.0 1.5% v/v 2-propanol, 0.625 mM acetyl-CoA, 1.25 mM ATP and 8.28 mg/mL K46A SaPC. The drop volume was 10 µL. Under these conditions, crystals did not spontaneously nucleate. After a 24-hour period, the crystallization solution was seeded with a 1:100,000 seed stock solution obtained by manually crushing crystals that grew spontaneously at 13% MEPEG 1.5K, 200 mM KNO$_3$, 50 mM MOPS pH 7.0, and 1.5% v/v 2-propanol, 0.625 mM acetyl-CoA, and 1.25 mM ATP. Seeded crystals grew to full size of 0.25mm after 2-4 days, with a cube-like morphology. Crystals were transferred via a quartz capillary to a synthetic stabilizing solution that consisted of 12.5% MEPEG 2K, 99 mM KNO$_3$, 50 mM MOPS pH 7.0 1.5% v/v 2-propanol, 0.69 mM acetyl-CoA, and 1.38 mM ATP. Crystals were incubated in the stabilizing solution for 5 minutes prior to being incrementally transferred into a cryoprotectant solution that consisted of 12.5% MEPEG 2K, 99 mM KNO$_3$, 50 mM MOPS pH 7.0 1.5% v/v 2-propanol, 0.69 mM acetyl-CoA, 1.38 mM ATP, and 20% ethylene glycol. The crystals were successively transferred 5 times into a solution with increasing amounts of the cryoprotectant solution. The crystals, indexed as $a = 96 \text{ Å}$, $b = 245 \text{ Å}$, $c = 126 \text{ Å}$, and $\alpha = 90^\circ$, $\beta = 99^\circ$, $\gamma = 90^\circ$, belonged to space group $P 2_1 2_1 2_1$ with 4 subunits in the asymmetric unit.
Protein Crystallization for WT SaPC with Acetyl-carba(dethia)

All crystals of WT SaPC were obtained at room temperature by the batch crystallization method. The drop consisted of 11% PEG 1.5K, 137.5 mM NaCl, 50 mM EPPS pH 7.5, 1.5% v/v 2-propanol, 2.5 mM acetyl-carba(dethia)-CoA, 2.5 mM adenosine-5’-O- (3-thiotriphosphate) (ATP-γ-S) and 5.03 mg/mL WT SaPC. The drop volume was 10 µL. Under these conditions, crystals did not spontaneously nucleate. After a 24-hour period, the crystallization solution was seeded with a 1:10,000 seed stock solution obtained by manually crushing crystals that grew spontaneously at 13% PEG 1.5K, 137. mM NaCl, 50 mM EPPS pH 7.5, and 1.5% v/v 2-propanol. Seeded crystals grew to full size of 0.25mm after 2-4 days, with a cube-like morphology. Crystals were transferred via a quartz capillary to a synthetic stabilizing solution that consisted of 12.2% PEG 1.5K, 151.25 mM NaCl, 50 mM EPPS pH 7.5, 1.5% v/v 2-propanol, 2.75 mM ATP-γ-S, and 2.75 mM acetyl-carba(dethia)-CoA. Crystals were incubated in the stabilizing solution for 5 minutes prior to being incrementally transferred into a cryoprotectant solution that consisted of 24.4% PEG 1.5K, 302.5 mM NaCl, 100 mM EPPS pH 7.5, 3% v/v 2-propanol, 2.75 mM ATP-γ-S, 2.75 mM acetyl-carba(dethia)-CoA and 20% v/v ethylene glycol. The crystals were successively transferred 5 times into a solution with increasing amounts of the cryoprotectant solution. The crystals, indexed as \(a = 97 \text{ Å}, b = 165 \text{ Å}, c = 374 \text{ Å},\) and \(\alpha = \beta = \gamma = 90^\circ\), belonged to space group \(P 2_1 2_1 2_1\) with 4 subunits in the asymmetric unit.
Data Collection, Structure Determination, and Refinement for all Crystals

X-ray diffraction data were collected at the Life Sciences Collaborative Access Team beamline 21-ID-F at the Advanced Photon Source, Argonne National Laboratory, on a Rayonix MX-300 s/n 023 detector with an X-ray wavelength of 0.97872 Å. A total of 360 diffraction images were collected at an oscillation angle of 1° (360° total) with an exposure time of 2s, with the detector set to a distance of 300 mm. The images were processed using autoPROC software (Vonrhein et al., 2011), which merged and scaled the anisotropic diffraction data in AIMLESS (Evans & Murshudov, 2013), with the programs TRUNCATE and UNIQUE (French & Wilson, 1978; Winn et al., 2011) to determine the resolution limit. The phases were determined by molecular replacement method using WT SaPC (PDB 3HO8) (Yu et al., 2009) as the search model using the program Phaser (McCoy et al., 2007). For WT SaPC with acetyl-carba(dethia)-CoA, the molecular replacement was done by fitting the whole tetramer at once. For R21A SaPC and K46A SaPC the molecular replacements were done by fitting the AD and CT domains, residues 493-1092, first and then the BC domains, residues 1-492, were fit last. The models then went through many rounds of manual model building using COOT (Emsley et al., 2010), and refinement using Phenix.Refine (Afonine et al., 2012). Refinements were done using translation/liberation/screw (TLS) and non-crystallographic symmetry restraints (NCS). Water molecules were added using COOT and Phenix.Refine with manual verification. No waters were built into the K46A SaPC structure. The refined ligand coordinates for acetyl-CoA, CoA, and acetyl-carba(dietha) CoA were optimized using eLBOW (electronic ligand building and optimization workbench) (Moriarty et al., 2009). Data collection and processing statistic are listed in Table 2-4.
Results

**Non-Hydrolysable Acetyl-CoA Analogs Fully Activate SaPC**

In pyruvate carboxylase, the acetyl moiety is known to contribute several orders of magnitude to the activation constant for acetyl-CoA, but the molecular basis for this phenomenon has not been fully described. Efforts to define the role of the acetyl moiety have been impeded by the lack of electron density corresponding to the acetyl moiety in co-crystal structures of PC with acetyl-CoA. Presumably, this is a consequence of either enzyme-mediated or spontaneous hydrolysis of acetyl-CoA (Frey & Utter, 1976). In an effort to better define the contributions of the acetyl moiety to the allosteric activation of PC by acetyl-CoA, non-hydrolysable/slowly hydrolysable dethia analogs of acetyl-CoA [acetyl-carba(dethia)-CoA, acetyl-oxa(dethia)-CoA, and acetyl-aza(dethia)-CoA (Figure 2-1B) were synthesized and assessed for their ability to activate SaPC. The activation of SaPC by each of these analogs was evaluated for both the overall degree of activation ($k_{cat}^{app}/k_{cat}^r$) and the activation constant ($K_a$). All three analogs had the same degree of activation as acetyl-CoA but displayed elevated $K_a$ values relative to acetyl-CoA (Figure 2-2). Both acetyl-carba(dethia)-CoA and acetyl-oxa(dethia)-CoA had $K_a$ values comparable to CoA, while acetyl-aza(dethia)-CoA had a $K_a$ value that was two orders of magnitude higher than CoA. This indicates that the thioester linkage plays a key role in positioning the acetyl moiety of acetyl-CoA in the allosteric site. In addition, 3’5’-diphosphoadenosine, which lacks both the acetyl moiety and the pantothenic acid arm of acetyl-CoA, was assessed for activation of SaPC. At concentrations up to 7 mM, 3’5’-diphosphoadenosine activated SaPC, but it was inhibitory at concentrations greater than 7 mM (Figure 2-3). The inhibitory effect at high concentrations of 3’5’-
diphosphoadenosine can be attributed to competitive inhibition with ATP in the BC domain active site, which has been observed in PC with other nucleotide analogues (Adina-Zada et al., 2011; Rattanapornsompong et al., 2021). A fit of the 3’5’-diphosphoadenosine titration data to Equation 2-2, which is derived from a kinetic scheme for non-essential activator inhibition, yielded an estimated $K_a$ value of ~5.9 mM, which is three orders of magnitude greater than the $K_a$ values of the acetyl-CoA analogs (Table 2-1). Taken together, our data confirm that the acetyl thioester makes a critical contribution to the overall binding affinity of acetyl-CoA, but that this moiety is not required to achieve full activation of the enzyme.

Figure 2-1. Acetyl-CoA analogs. A. Chemical structure of allosteric activator acetyl-CoA. B. Non-hydrolysable or slowly hydrolyzing acetyl-CoA analogs. From left to right acetyl-carba(dethia)-CoA, acetyl-oxa(dethia), and acetyl-aza(dethia)-CoA.
Figure 2-2. Acetyl-CoA analogs activate SaPC. A. Pyruvate carboxylation assay for WT SaPC titrated with acetyl-CoA (black), CoA (blue), acetyl-oxa(dethia) (green), acetyl-carba(dethia)-CoA (purple), and acetyl-aza(dethia)-CoA (red) titration. B. Pyruvate carboxylation assay for WT SaPC in the presence of 20mM L-aspartate titrated with acetyl-CoA (black), CoA (blue) and right acetyl-carba(dethia)-CoA (purple), acetyl-oxa(dethia)-CoA (green), and acetyl-aza(dethia)-CoA (red). Data was fit to Equation 2-1 and error bars represent standard deviations from three independent measurements.

Figure 2-3. 3’5’diphosphoadenosine titration with WT SaPC. A. 3’5’diphosphoadenosine activated SaPC up to 7 mM but then become inhibitory. The data was fit to Equation 2-2. The $K_a$ was $5.9 \pm 3.1$ mM and the hill coefficient was $1.6 \pm 0.7$. B. Structure of 3’5’ diphosphoadenosine.
The dethia acetyl-CoA analogs were also assessed for their ability to overcome allosteric inhibition by L-aspartate in the pyruvate carboxylation assay. L-aspartate, an allosteric inhibitor of PC, is competitive with respect to acetyl-CoA in various bacterial and fungal PC enzymes (Osmani et al., 1981; Sirithanakorn et al., 2014). To assess whether the dethia analogs could displace L-aspartate, they were titrated in the presence of a fixed concentration of 20 mM L-aspartate. In the presence of L-aspartate, the acetyl-carba(dethia)-CoA and acetyl-oxa(dethia)-CoA analogs behaved much like acetyl-CoA and CoA: all of these activators had the same degree of activation in the presence of L-aspartate and the apparent $K_a$ values increased ~4-fold in the presence of L-aspartate. Notably, the Hill coefficients for acetyl-CoA, acetyl-carba(dethia)-CoA and acetyl-oxa(dethia) all increased in the presence of L-aspartate (Table 2-1), which is consistent with displacement of L-aspartate (Sirithanakorn et al., 2014). Acetyl-aza(dethia)-CoA, which exhibits a much higher $K_a$ value compared to the other analogs, was unable to overcome L-aspartate inhibition, suggesting that it was unable to displace L-aspartate (Figure 2-2B). Interestingly, unlike the other activators, CoA did not exhibit a substantially increased Hill coefficient in the presence of L-aspartate (Table 2-1). This suggests that, if both L-aspartate and CoA are bound to the enzyme, allosteric activation dominates over allosteric inhibition.
**Table 2-1. Activation of WT SaPC pyruvate carboxylation reaction by acyl CoAs.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_a$ (μM)</th>
<th>$h$</th>
<th>$k_{cat}^{app}/k_{cat}^*$</th>
<th>$K_a$ (μM)</th>
<th>$h$</th>
<th>$k_{cat}^{app}/k_{cat}^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>0.94 ± 0.09</td>
<td>1.5 ± 0.1</td>
<td>13.0 ± 0.2</td>
<td>3.3 ± 0.6</td>
<td>14 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoA</td>
<td>40.0 ± 3.4</td>
<td>1.4 ± 0.2</td>
<td>14.0 ± 0.5</td>
<td>1.8 ± 0.2</td>
<td>16 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-carba(dethia)-CoA</td>
<td>53.0 ± 3.8</td>
<td>1.5 ± 0.2</td>
<td>13.0 ± 0.4</td>
<td>3.1 ± 0.6</td>
<td>16 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-oxa(dethia)-CoA</td>
<td>73.0 ± 11</td>
<td>1.5 ± 0.3</td>
<td>13.0 ± 0.6</td>
<td>3.3 ± 0.7</td>
<td>16 ± 0.2</td>
<td></td>
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</tr>
<tr>
<td>Acetyl-aza(dethia)-CoA</td>
<td>2500 ± 1000</td>
<td>1.3 ± 0.4</td>
<td>15.0 ± 2.4</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>3'5' diphosphodenosine</td>
<td>5900 ± 3100</td>
<td>1.6 ± 0.7</td>
<td>5.8 ± 0.8</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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</table>

**BC Dimer Interface Mutations at the Acetyl Moiety Binding Site Disrupt Allostery**

To identify and characterize residues that interact with the acetyl moiety of acetyl-CoA, a series of residues at the BC dimer interface were mutated to alanine. In crystal structures, residues Lys46, Glu418, and Arg21 are located at the BC dimer interface, and in close proximity to the thiol of CoA (Figure 2-4A). These residues are conserved among PC enzymes but are not conserved in the larger family of biotin carboxylases (Figure 2-4B). Mutation of Lys46, Glu418, Arg21 and Lys411 to alanine was designed to specifically probe the potential for the BC dimer interface to mediate the allosteric effect of acetyl-CoA (Figure 2-4C).

Since the mutated residues were located at the BC domain interface, the impact of the mutations on the oligomerization state of the mutant enzymes was assessed.

Analytical size exclusion chromatography was performed for WT SaPC, ΔBCΔBCCP
SaPC and each of the BC interface mutants (Figure 2-4D). WT SaPC ran as a tetramer while the ∆BC∆BCCP SaPC construct, which lacks both the BC and BCCP domains, ran as a dimer as previously demonstrated for the identical construct in *Rhizobium etli* PC (Lietzan et al., 2011). The R21A SaPC mutant had the same elution profile as WT SaPC, while the other mutations demonstrated a slight increase in the percentage of dimer. Nevertheless, in each case, the mutated construct populations greatly favored the tetrameric species and did not differ significantly from the profile of the WT enzyme (Figure 2-4D).
**Figure 2-4. Probing the acetyl moiety binding site through mutagenesis.** A. Residues within a 10 Å radius of the thiol of CoA in *Sa*PC (PDB ID 3HO8) were candidates for mutagenesis. The distance from the thiol of CoA in chain A is denoted for each residue. The light blue shading denotes residues originating from one subunit (chain A) while the darker blue shading denotes residues originating from the second subunit (chain C). B. Multiple sequence alignment for PC from various organisms and other biotin dependent carboxylases: biotin carboxylase (BC), acetyl coenzyme A carboxylase (ACC), urea carboxylase (UC), 3-methylcrotonyl coenzyme A carboxylase (MCC), and propionyl coenzyme A carboxylase (PCC). The asterisk represents conserved residues in PC that are within 10 Å of the thiol of CoA, highlighted in panel A. C. The BC dimer interface highlighting the residues targeted for mutagenesis. Inter-subunit hydrogen bonding and ionic interactions are represented by a dotted line and the bond distances are denoted next to each bond. The dimer interface is represented by a thick blue line. D. Elution profiles for K46A *Sa*PC (blue), E418A *Sa*PC (red), R21A *Sa*PC (green), and K411A *Sa*PC (purple). The elution profile for each mutant is compared to the elution profile for tetrameric WT *Sa*PC (black), and dimeric ∆BC∆BCCP *Sa*PC (brown).
The mutations K46A, E418A, R21A and K411A were kinetically characterized (Figure 2-5 and Table 2-2 and Table 2-3). The side chain of Lys411 resides at the BC dimer interface but is distant (~12 Å) from the putative acetyl moiety binding site. It, therefore, was employed as a control to account for perturbations at the BC dimer interface that are independent of acetyl-CoA. All the mutants retained pyruvate carboxylation activity in the presence of both acetyl-CoA and CoA but the response of each mutant to activation by acetyl-CoA and CoA differed compared to WT SaPC. Relative to WT SaPC, the K46A SaPC mutant exhibited a reduced degree of activation and a substantially elevated $K_a$ value for both acetyl-CoA and CoA. The R21A SaPC mutant also exhibited a substantially elevated $K_a$ value for acetyl-CoA and CoA, but both allosteric effectors were able to activate the R21A mutant to the same degree as WT SaPC (~14-fold). Of all the mutants surveyed, the K411A SaPC mutation, located distant from the acetyl-CoA binding site, displayed kinetic constants most similar to WT. This mutant exhibited only a slightly elevated $K_a$ value relative to WT SaPC and exhibited no change in the degree of activation relative to WT SaPC. The most dramatic effect was observed in the E418A SaPC mutant. This mutant was unresponsive to activation by both acetyl-CoA and CoA, even at extremely high concentrations of the activators (4 mM acetyl-CoA and 30 mM CoA) (Figure 2-5A).
**Table 2-2.** Activation of the pyruvate carboxylation reaction by acetyl-CoA and CoA for *SaPC* mutations in the BC dimer interface.

<table>
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<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>% WT Activity</th>
<th>Acetyl-CoA</th>
<th>CoA</th>
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<tr>
<td>WT</td>
<td>1.5 ± 0.067</td>
<td>N/A</td>
<td>1.5 ±</td>
<td>15 ± 5.5</td>
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<tr>
<td><em>SaPC</em></td>
<td>0.45 ± 0.017</td>
<td>31%</td>
<td>12 ± 0.27</td>
<td>49 ± 6.5</td>
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<tr>
<td>K46A</td>
<td>0.039 ± 0.0013</td>
<td>3%</td>
<td>0.14 ±</td>
<td>0.30 ±</td>
</tr>
<tr>
<td>E418A</td>
<td>0.089 ± 0.0039</td>
<td>6%</td>
<td>14 ± 0.91</td>
<td>53</td>
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<tr>
<td>R21A</td>
<td>0.23 ± 0.0062</td>
<td>16%</td>
<td>14 ± 0.19</td>
<td>14 ± 0.27</td>
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</table>

In order to determine the impact of these mutations on the catalytic activity of the BC domain, the mutants were assessed for the acetyl-CoA dependence on the phosphorylation of ADP by carbamoyl phosphate, a well-established assay of the BC
domain half reaction (Figure 2-5B and Table 2-3). It was determined that 50 mM carbamoyl phosphate was saturating for all mutants (Figure 2-6) and this concentration of carbamoyl phosphate was held constant for all titrations with acetyl-CoA. The rate of ADP phosphorylation was reduced for all the mutants, with E418A SaPC having the lowest relative rate. Surprisingly, K46A SaPC, E418A SaPC and R21A SaPC were all unable to be activated by acetyl-CoA in the BC domain reaction, up to 1 mM acetyl-CoA. K411A SaPC responded to acetyl-CoA but was not activated to the same degree as WT SaPC and had an elevated $K_a$ value. In all cases, including WT, there was slight inhibition of activity at concentrations of acetyl-CoA greater than 4 mM (Figure 2-7).

The precise binding site for the allosteric inhibitor, L-aspartate, is unknown, but it has been shown to be mutually exclusive with acetyl-CoA, strongly suggesting that the allosteric sites may overlap (Cazzulo & Stoppani, 1968). To assess whether BC interface mutations near the acetyl moiety binding site could also impact L-aspartate inhibition, we titrated L-aspartate in the pyruvate carboxylation reaction. For WT SaPC we observed allosteric inhibition as previously described (Sirithanakorn et al., 2014). WT SaPC and K411A SaPC were fully inhibited with $K_i$ values of 18 ± 1 and 24 ± 3 mM, respectively. However, K46A SaPC, E418A SaPC and R21A SaPC were not inhibited by L-aspartate up to 100 mM (Figure 2-5C). Inhibition was observed in all mutants at concentrations exceeding 100 mM L-aspartate (Figure 2-8). However, L-glutamate, which is not an inhibitor of bacterial PC, also inhibited WT SaPC at concentrations greater than 100 mM, indicating that inhibition at these elevated concentrations is a nonspecific effect (Figure 2-8).
**Figure 2-5. Carbamoyl phosphate titration with WT SaPC and mutants.**
Michaelis-Menten plot for phosphoryl donor, carbamoyl phosphate, in the ADP phosphorylation reaction. WT SaPC (black), K411A SaPC (purple), R21A SaPC (green), K46A SaPC (blue), and E418A SaPC (red). Data was fit to the Michaelis-Menten equation.

**Figure 2-6. Kinetic characterization of BC dimer interface mutants** A. The degree of activation ($k_{cat\text{app}}/k_{cat}$) for the carboxylation of pyruvate as a function of acetyl-CoA (solid line, filled circles) and CoA (dashed line, open circles) concentrations for WT SaPC (black), K411A SaPC (purple), R21A SaPC (green), K46A SaPC (blue), and E418A SaPC (red). Data were fit to Equation 2-1. B. The degree of activation ($k_{cat\text{app}}/k_{cat}$) for the phosphorylation of ADP as a function of acetyl-CoA concentration for WT SaPC (black), K411A SaPC (purple), R21A SaPC (green), K46A SaPC (blue), and E418A SaPC (red). K411A SaPC and WT SaPC data were fit to Equation 2-1. R21A SaPC, K46A SaPC and E418A SaPC were fit to a linear regression. C. Pyruvate carboxylation activity, plotted as $k_{cat\text{app}}/k_{cat}$, as a function of L-aspartate for WT SaPC (black), K411A SaPC (purple), R21A SaPC (green), K46A SaPC (blue), and E418A SaPC (red). K411A SaPC and WT SaPC data were fit to Equation 2-3. R21A SaPC, K46A SaPC and E418A SaPC were fit to a linear regression. Error bars are representative of the standard deviation from 3 independent measurements.
Figure 2-7. Nonspecific L-aspartate inhibition. L-aspartate titrated in the pyruvate carboxylation reaction with WT SaPC and mutants. WT SaPC (black), K411A SaPC (purple), R21A SaPC (green), K46A SaPC (blue), and E418A SaPC (red). WT SaPC was also titrated with L-glutamate (pink, thick line) as a control because it is not an inhibitor of PC. Data was fit to Equation 2-3.

Figure 2-8. Acetyl-CoA titration with BC dimer interface mutants. Acetyl-CoA titrated in the ADP phosphorylation reaction. Acetyl-CoA is slightly inhibitory in all cases above 4 mM. WT SaPC (black), K411A SaPC (purple), R21A SaPC (green), K46A SaPC (blue), and E418A SaPC (red). Data was fit to Equation 2-1.
Structure of R21A SaPC with Acetyl-CoA Reveals the Acetyl Moiety Binding Site

To further characterize the role of the BC dimer interface on allostery in PC, we sought to structurally characterize the BC dimer interface mutants in the presence of acetyl-CoA. A crystal of R21A SaPC, grown in the presence of 0.69 mM acetyl-CoA and 1.4 mM ATP, diffracted to 2.7 Å resolution and revealed strong difference density for acetyl-CoA in chain A (Figure 2-10). Chain C also had strong difference density for coenzyme A, but this was best modeled by the hydrolyzed CoA thiol rather than the intact acetyl-CoA thioester. The omit maps for acetyl-CoA and CoA are best contoured at 2 σ, but polder maps generated for both acetyl-CoA in chain A, and CoA in chain C, strongly support the modeled position and orientation of these ligands (Figure 2-9). All four chains had BCCP-biotin located in the exo binding site. In all of these cases, however, much of the BCCP domain was disordered and could not be fully modeled.

Crystal contacts ordered the B-subdomain lid in the BC domains of Chains A and B, allowing the B-subdomain lid to be modeled into the electron density in an open conformation. In chains C and D, however, the B subdomain lid was fully disordered and could not be modeled.

The overall structure of R21A SaPC is similar to the structure of WT SaPC in complex with CoA (PDB ID 3HO8, Figure 2-11). The BC domains of chain A for 3HO8 and the R21A SaPC structure aligned very well, with an rmsd of 0.6 Å. When these subunits were aligned, the other subunit of the dimer also remained well aligned, with an rmsd of 1.4 Å (Figure 2-11). This indicates that the BC dimer interface is very similar in both structures, reinforcing that the BC dimer interface in R21A SaPC is intact and that neither the mutation nor the presence of unhydrolyzed acetyl-CoA significantly alters the
dimer interface in *Sa*PC. In the larger context of the tetramer, there are differences in the relative positioning of the individual subunits. Such differences are commonly observed in published PC structures and have been explored in detail in other reports (Lasso et al., 2010, 2014; López-Alonso et al., 2022; Wei & Tong, 2015; Xiang & Tong, 2008; Yu et al., 2009).

**Figure 2-9.** Polder map difference density for acetyl-CoA and CoA. **A.** Polder map difference density for acetyl-CoA contoured at 3σ, with CC_{peak}^{1,2} 0.53, CC_{peak}^{1,3} 0.92, and CC_{peak}^{2,3} 0.45. **B.** Polder map difference density for CoA contoured at 3σ with CC_{peak}^{1,2} 0.57, CC_{peak}^{1,3} 0.89, and CC_{peak}^{2,3} 0.50.
Figure 2-10. R21A SaPC in complex with acetyl-CoA. A. Omit F₀-Fc electron density contoured at 2 σ (green mesh) corresponding to acetyl-CoA in chain A. CoA from chain C overlaid (white) with acetyl-CoA (grey) from chain A. B. Omit F₀-Fc electron density contoured at 2 σ (green mesh) corresponding to CoA in chain C. Acetyl-CoA from chain A overlaid (grey) with CoA (white) from chain C. C. Acetyl-CoA from chain A with F₀-Fc electron density contoured at 3 σ (green mesh) with 2F₀-Fc electron density contoured at 1 σ (blue mesh) surrounding residues K46, from chain C, and E418, from chain A. The distance from the acetyl moiety to K46 and E418 are displayed. The water that occupies the space that Arg21 typically is in, in WT SaPC, is shown as a red sphere.
Unlike many other crystal structures, where the difference density for acetyl-CoA is strong for the nucleotidyl portion but diminishes for the acetyl phosphopantetheine arm, the structure of R21A SaPC revealed relatively strong difference density in chain A for the entire acetyl-CoA molecule, including the acetyl moiety (St. Maurice et al., 2007; Yu et al., 2009). Acetyl-CoA in chain A, and CoA in chain B, have their nucleotidyl portions in the same orientation, but the density in chain A extends further to encompass the acetyl moiety (Figure 2-10A and B). The position and orientation of acetyl-CoA, in chain A, is very similar to the acetyl-CoA molecules modeled in recent cryo EM structures of Lactococcus lactis PC and Homo Sapiens PC (Chai et al., 2022; López-Alonso et al., 2022) (Figure 2-12) and to previously published structures of SaPC in complex with CoA. Acetyl-CoA is stabilized by a well-characterized set of interactions between Arg420, Arg422 and Arg465 and the phosphates of acetyl-CoA (Adina-Zada, Sereeruk, et al., 2012). In addition to their inter-subunit interactions at the dimer interface, residues Lys46, Glu418 and, likely, Arg21, interact with the acetyl moiety of acetyl-CoA (Figure 2-10C). The density for the carbonyl oxygen of the acetyl moiety was modeled to position the oxygen just 3.4 Å from Glu418, the residue at the BC dimer interface that is most critical to mediating the allosteric effect of acetyl-CoA (Figure 2-10C and Figure 2-5A). Residue Lys46, which also contributes significantly to mediating the allosteric effect, is located 4.5 Å from the modeled position of the carbonyl oxygen. It must be noted that both the K46A and R21A SaPC mutants were much less discriminating in their relative activation by acetyl-CoA compared to CoA, with only ~4-5 fold reduction in $K_a$ (compared to ~30-fold for WT SaPC) on going from CoA to acetyl-CoA (Table 2-2). This reduced discrimination for acetyl-CoA may have
contributed to the success in capturing acetyl-CoA in the structure, but it also introduces a caveat that the structure of R21A SaPC may not accurately reflect the true orientation of acetyl-CoA in the wild-type enzyme.

**Figure 2-11. 3HO8 and R21A SaPC overlay.** BC domain from SaPC with CoA, PDB ID 3HO8 (grey), aligned with the BC domain from chain A of R21A SaPC with acetyl-CoA (cyan). BC domains that were aligned are outlined in surface representation. The BC domains of chain C, which forms the dimer with chain A BC domain, have an rmsd of 1.4 Å.
Structure of K46A SaPC with Acetyl-CoA has Intact BC Homodimer Interface

A crystal of K46A SaPC, grown in the presence of 0.69 mM acetyl-CoA and 1.4 mM ATP, diffracted to 3.0 Å resolution, but had weak density for acetyl-CoA in all 4 chains. Weak acetyl-CoA density is likely due to the reduced binding affinity in K46A SaPC compared to WT SaPC as shown in the kinetic data (Figure 2-5 and Table 2-2). The overall structure of K46A SaPC is similar to a previously published structure of WT SaPC with CoA (PDB ID 3HO8). The BC domains of chain A for 3HO8 and the K46A SaPC with acetyl-CoA structure aligned decently, with an rmsd of 0.82 Å. When these subunits were aligned, the other subunit of the dimer also remained well aligned, with an
rmsd of 1.98 Å (Figure 2-13). This is further proof that the BC dimer interface is intact, and the changes seen in the binding affinity for acetyl-CoA and the reduction of the degree of activation seen in the kinetic data are not due to large structural perturbations but due to the absence of the Lys$_{46}$ interaction with the acetyl moiety.

In chain D of this structure there is a lot of scattered difference density, likely caused from translational non-crystallographic symmetry. The crystallographic quality metrics R-work and R-free values were 0.26 and 0.32 respectively. The density for CoA was relatively weak for all chains and the binding site for the acetyl moiety could not be determined from this structure. The worst density for the ligand was in chain A and the best density was in chain B but was much worse than the omit density in the R21A SaPC structure (Figure 2-14). Ultimately, we decided to not deposit this structure to the Protein Data Bank, but just used it to help conclude that the BC dimer interfaces of the mutants are still intact.

Figure 2-13. 3HO8 and K46A SaPC with acetyl-CoA overlay. BC domain from SaPC with CoA, PDB ID 3HO8 (grey), aligned with the BC domain from chain A of K46A SaPC with acetyl-CoA (green). BC domains that were aligned are outlined in surface representation. The BC domains of chain C, which forms the dimer with chain A BC domain, have an rmsd of 1.98 Å.
Structure of WT SaPC with Acetyl-carba(dethia)-CoA Reveals Similar Binding Site

Of the analogs we examined, acetyl-carba(dethia)-CoA had the highest binding affinity and was able to overcome L-aspartate inhibition, indicating that it was a good candidate for structural characterization with SaPC (Figure 2-2). Additionally, contrary to acetyl-oxa(dethia)-CoA which hydrolyses slowly, acetyl-carba(dethia)-CoA is a completely non-hydrolysable analog. A crystal of full-length SaPC, grown in the presence of 1.5 mM acetyl-carba(dethia) CoA, diffracted to 2.5Å resolution and revealed relatively strong difference density for acetyl-carba(dethia)-CoA in all four binding sites (Figure 2-15). The density surrounding the analog is best defined in chains B and C. Only one biotin appeared in chain C, located in the CT domain exo site. Poor density is seen in the CT domains of chains A and D, but is better defined in chains B and C.

Figure 2-14. Ligand density in K46A SaPC structure. A. Omit Fo-Fc electron density contoured at 3σ (green) in chain A of K46A SaPC structure. B. Omit Fo-Fc electron density contoured at 3σ (green) in chain B of K46A SaPC structure.
The overall structure is very similar to *SaPC* in complex with CoA (PDB ID 3HO8, Figure 2-16). The BC domains of chain A for 3HO8 and the WT *SaPC* with acetyl-carba(dethia)-CoA structure aligned very well, with an rmsd of 0.48 Å. When these subunits were aligned, the other subunit of the dimer also remained well aligned, with an rmsd of 1.0 Å (Figure 2-16). The electron density is strongest for the nucleotidyl portion of the analog but diminishes for the acetyl phosphopantotheine arm, consistent with previous X-ray crystal structures of PC with bound coenzyme A analogues (St. Maurice et al., 2007; Yu et al., 2009). The position and orientation of acetyl-carba(dethia)-CoA is very similar to previously published structures of *SaPC* in complex with CoA and is stabilized by a well-characterized set of interactions between Arg420, Arg422 and Arg465 and the phosphates of the dethia analogue. Compared to previous structures, this structure reveals difference density for the acetyl moiety of the analog that is in closer proximity to Glu418 (Yu et al., 2009). Unfortunately, the difference density

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**Figure 2-15. Structure of SaPC with the non-hydrolysable acetyl-CoA analog acetyl-carba(dethia) CoA.** A. Acetyl-carba(dethia)-CoA in the *SaPC* BC dimer interface with key residues highlighted. The arginines that bind the phosphate groups are colored in blue CPK coloring and the residues in the BC dimer interface are in grey CPK coloring. B. Omit Fo-Fc electron density contoured at 3σ (green) and 2σ (grey) corresponding to acetyl-carba(dethia)-CoA.
corresponding to the acetyl moiety of acetyl-carba(dethia)-CoA was very weak, precluding a precise definition of the acetyl moiety binding site in this structure. Nevertheless, when the acetyl moiety of acetyl-carba(dethia)-CoA was modeled into the weak electron density, the modeled position agreed well with the position of the acetyl moiety of acetyl-CoA from a recent cryo EM structures of *Lactococcus lactis* PC and *Homo Sapiens* PC (Chai et al., 2022; López-Alonso et al., 2022) (Figure 2-17).
Figure 2-16. 3HO8 and WT SaPC with acetyl-carba(dethia)-CoA overlay. BC domain from SaPC with CoA, PDB ID 3HO8 (grey), aligned with the BC domain from chain A of WT SaPC with acetyl-carba(dethia)-CoA (magenta). BC domains that were aligned are outlined in surface representation. The BC domains of chain C, which forms the dimer with chain A BC domain, have an rmsd of 1.0 Å.

Figure 2-17. Acetyl-carba(dethia)-CoA overlay with acetyl-CoA. A. Cryo EM structure from *Lactococcus lactis* PC with resolution of 2.41Å (PDB 7ZZ3) with the acetyl moiety of acetyl-CoA (grey) modeled in the same location as the acetyl moiety from acetyl-carba(dethia) (magenta) CoA analog structure. BC domains of chain A were aligned from both structures B. Cryo EM structure from *Homo sapiens* PC with a resolution of 3.3Å (PDB 7WTE) with the acetyl moiety of acetyl-CoA (grey) modeled in the same location as the acetyl moiety from acetyl-carba(dethia) (magenta) CoA analog structure. BC domains of chain C were aligned from both structures.
As previously mentioned, the density in the CT domains of chains A and D is extremely weak and this is likely due to translational non-crystallographic symmetry present in the data. The crystallographic quality metrics R-work and R-free values were 0.26 and 0.31 respectively. The poor data quality and the lack of clear density for the acetyl moiety binding site was ultimately why we decided not to deposit this structure to the Protein Data Bank, and why we chose to publish the R21A SaPC structure instead.
Table 2-4. Data collection and refinement statistics

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<th>K46A SaPC with acetyl-CoA Xtal 447</th>
<th>WT SaPC with acetyl-carba(dethia)-CoA Xtal 455</th>
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<td>P 1 21 1</td>
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**Acetyl-CoA Alters CT Domain Inhibition**

To investigate the impact of acetyl-CoA and the BC dimer interface on long range communication between the BC and CT domains, we assessed the influence of acetyl-CoA on the inhibition of PC by a well-characterized set of CT domain inhibitors. Oxalate is known to bind in the CT domain with a similar binding orientation to pyruvate and is a potent inhibitor of PC (Lietzan & St. Maurice, 2013b; Mildvan et al., 1966; Ruiz-Amil et al., 1965) (Figure 2-18). Interestingly, the IC$_{50}$ value for oxalate inhibition of WT SaPC increased as a function of acetyl-CoA concentration, resulting in more than a 20-fold increase in the IC$_{50}$ at saturating concentrations of acetyl-CoA (Figure 2-19A, Table 2-5). A similar phenomenon was observed for two other competitive inhibitors with respect to pyruvate, 2-hydroxy-3-(quinoline-2-yl)propenoic acid and phenylpyruvate (Burkett et al., 2019) (Figure 2-20, Table 2-6). CoA also increased the IC$_{50}$ value of oxalate ~13-fold, indicating that this phenomenon is a general feature of allosteric activation and is not specific to the acetyl moiety (Figure 2-19B, Table 2-5). Moreover, the allosteric inhibitor, L-aspartate, did not alter the IC$_{50}$ value of oxalate (Figure 2-19B, Table 2-5). The ability of CoA, and the inability of L-aspartate, to alter the IC$_{50}$ value of oxalate suggests that the BC dimer interface does not contribute to reduced CT domain inhibition in the presence of acetyl-CoA. To test this, we examined the oxalate inhibition of both R21A and K46A in the presence and absence of 2.5 mM acetyl-CoA, which is equivalent to a practical upper limit of ~7 times the $K_a$ value for both mutants. At 2.5 mM acetyl-CoA, both the R21A and K46A mutations displayed an 8-fold increase in the IC$_{50}$ value for oxalate, equivalent to the ~6-fold increase in the IC$_{50}$ value for oxalate inhibition of WT SaPC at ~7 times the $K_a$ value (Figure 2-19C, Table 2-7). Although
E418A SaPC retained only 3% of WT SaPC activity, it was inhibited by all three inhibitors (oxalate, 2-hydroxy-3-(quinoline-2-yl)propenoic acid, and phenylpyruvate). This inhibition was unchanged in the presence of 500 µM acetyl-CoA, but this is consistent with the inability of E418A to respond to activation by acetyl-CoA. K411A SaPC also exhibited a substantially elevated IC$_{50}$ value for CT domain inhibitors in the presence of acetyl-CoA (Figure 2-21, Table 2-6). Taken together, these results indicate that, while acetyl-CoA alters inhibition in the CT domain, the acetyl moiety binding site does not contribute to this phenomenon.

**Figure 2-18. CT domain active site with substrate and inhibitor bound.** Inhibitor oxalate (cyan) overlaid with substrate pyruvate (green) PDB ID 4MFD. CT domain active site residues R541, Q545 and R614 shown as sticks and Mn$^{2+}$ and a water as spheres.
**Figure 2-19. The effect of effector molecules and mutations on oxalate inhibition.**

A. Normalized velocities for the pyruvate carboxylation reaction catalyzed by WT SaPC at increasing concentrations of oxalate in the absence of acetyl-CoA (open circles, dashed line), in the presence of 2.5 µM acetyl-CoA (squares), 5 µM acetyl-CoA (triangles), 10 µM acetyl-CoA (inverted triangles), and 250 µM acetyl-CoA (diamonds). B. Normalized velocities for the pyruvate carboxylation reaction catalyzed by WT SaPC at increasing concentrations of oxalate in the absence of acetyl-CoA (open circles, dashed line), in the presence of 5 mM L-aspartate (triangles), and in the presence of 500 µM CoA (squares). C. Normalized velocities for the pyruvate carboxylation reaction at increasing concentrations of oxalate. WT SaPC is shown in black in the absence of acetyl-CoA (open circles, dashed line) and in the presence of 10 µM acetyl-CoA (closed circles, solid line). R21A SaPC (green) in the absence of acetyl-CoA (open circles, dashed line) and in the presence of 2.5 mM acetyl-CoA (closed circles). K46A SaPC (blue) in the absence of acetyl-CoA (open circles, dashed line) and in the presence of 2.5 mM acetyl-CoA (closed circles). Normalized velocities were calculated using Equation 2-4 and the data was fit to Equation 2-5. Error bars, shown in red, represent the propagation of error from three separate measurements.

**Table 2-5. Absolute IC_{50} values for WT SaPC inhibited by oxalate in the presence of various effector molecules.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>No effectors</th>
<th>Relative IC_{50} (µM)</th>
<th>AcCoA^a</th>
<th>CoA^b</th>
<th>L-aspartate^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SaPC</td>
<td>3.4 ± 0.1</td>
<td>74 ± 8</td>
<td>43 ± 2</td>
<td>2.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

^a250 µM acetyl-CoA; ^b500 µM CoA; ^c5 mM L-aspartate
Figure 2-20. 2-hydroxy-3-(quinoline-2-yl)propenoic acid (HQPA) and phenylpyruvate inhibition of WT SaPC in the presence of acetyl-CoA. A. Normalized velocities for the pyruvate carboxylation reaction at increasing concentrations of HQPA in the presence (solid line, filled circles) and absence (dashed line, open circles) of 250 µM acetyl-CoA. B. Normalized velocities for the pyruvate carboxylation reaction at increasing concentrations of phenylpyruvate in the presence (solid line, filled circles) and absence (dashed line, open circles) of 250 µM acetyl-CoA. Normalized velocities were determined by Equation 2-4 and the data were fit to Equation 2-5. Error bars, shown in red, represent the propagation of error from three separate measurements.

Table 2-6. Absolute IC\(_{50}\) values for CT domain inhibitors for WT SaPC and K411A SaPC.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>oxalate - AcCoA</th>
<th>oxalate + AcCoA</th>
<th>HQPA(^a) - AcCoA</th>
<th>HQPA(^a) + AcCoA</th>
<th>phenylpyruvate - AcCoA</th>
<th>phenylpyruvate + AcCoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SaPC(^b)</td>
<td>3.4 ± 0.1</td>
<td>74 ± 8</td>
<td>4.5 ± 0.3</td>
<td>110 ± 30</td>
<td>420 ± 70</td>
<td>8000 ± 1000</td>
</tr>
<tr>
<td>K411A SaPC(^c)</td>
<td>4.3 ± 0.2</td>
<td>86 ± 11</td>
<td>5.2 ± 0.2</td>
<td>160 ± 30</td>
<td>190 ± 30</td>
<td>10000 ± 2000</td>
</tr>
</tbody>
</table>

\(^a\)HQPA = 2-hydroxy-3-(quinoline-2-yl)propenoic acid; \(^b\)± 250 µM acetyl-CoA (~170× \(K_a\) for WT SaPC); \(^c\)± 500 µM acetyl-CoA (~90× \(K_a\) for K411A SaPC).
Table 2-7. Absolute IC50 values for WT SaPC, R21A SaPC and K46A SaPC inhibited by oxalate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Relative IC50 (µM)</th>
<th>oxalate - AcCoA</th>
<th>oxalate + AcCoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SaPC</td>
<td>3.4 ± 0.1</td>
<td>18 ± 3 a</td>
<td></td>
</tr>
<tr>
<td>R21A</td>
<td>1.6 ± 0.1</td>
<td>6.6 ± 0.7 b</td>
<td></td>
</tr>
<tr>
<td>K46A</td>
<td>6.0 ± 0.5</td>
<td>33.7 ± 11 b</td>
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a 10 µM acetyl-CoA (~7× Ks for WT SaPC); b 2.5 mM acetyl-CoA (~7× Ks for R21A and K46A SaPC).

Figure 2-21. Oxalate, 2-hydroxy-3-(quinoline-2-yl)propenoic acid (HQPA) and phenylpyruvate inhibition of K411A SaPC in the presence of acetyl-CoA. A. Normalized velocities for the pyruvate carboxylation reaction at increasing concentrations of oxalate in the presence (solid line, filled circles) and absence (dashed line, open circles) of 500 µM acetyl-CoA. B. Normalized velocities for the pyruvate carboxylation reaction at increasing concentrations of HQPA in the presence (solid line, filled circles) and absence (dashed line, open circles) of 500 µM acetyl-CoA. B. Normalized velocities for the pyruvate carboxylation reaction at increasing concentrations of phenylpyruvate in the presence (solid line, filled circles) and absence (dashed line, open circles) of 500 µM acetyl-CoA. Normalized velocities were determined by Equation 2-4 and the data were fit to Equation 2-5. Error bars, shown in red, represent the propagation of error from three separate measurements.
Discussion

**Acetyl Thioester makes a Critical Contribution to Activation**

While the allosteric regulation of PC by acetyl-CoA and L-aspartate has been studied for over 60 years (Cazzulo & Stoppani, 1968)(Utter & Keech, 1963), the complete binding site and mechanism of acetyl-CoA activation and L-aspartate inhibition remains an active area of investigation. Acetyl-CoA is known to bind at the allosteric domain and extend into the BC dimer interface, but the exact binding site for the acetyl moiety has not been defined (Adina-Zada, Sereeruk, et al., 2012). The precise binding site for the allosteric inhibitor, L-aspartate, is also unknown; it is postulated to bind near the acetyl-CoA binding site because acetyl-CoA and L-aspartate are mutually exclusive, and because allosteric inhibition affects only the BC domain half reaction (Sirithanakorn et al., 2014). Here, we used structural and kinetic data to precisely define the binding site for the acetyl moiety of acetyl-CoA at the BC dimer interface and argue that this also represents the overlapping binding site for the allosteric inhibitor, L-aspartate.

Many prior studies probing the acetyl-CoA binding site have focused on residues that bind near the nucleotidyl portion of acetyl-CoA (Adina-Zada et al., 2019; Adina-Zada, Sereeruk, et al., 2012; Sirithanakorn et al., 2016). The nucleotidyl portion of the activator binds primarily in the allosteric domain near the BC domain dimer interface and is both necessary and sufficient for activation: 3’5’diphosphoadenosine, a truncated compound that contains only the nucleotidyl portion of acetyl-CoA, activates the enzyme (Figure 2-3), while acetylpantetheine, which lacks the nucleotidyl portion, binds at the acetyl-CoA site but does not activate PC (Scrutton & Utter, 1967). While the nucleotidyl portion of acetyl-CoA is the only essential component for activation, the acetyl moiety
nevertheless plays a significant role in modulating the binding affinity. Many acyl CoAs
activate PC, and the pantetheine groups and β-mercaptoethylamine groups also contribute
to binding affinity. The acetyl moiety contributes most greatly to the binding affinity,
increasing it ~80 fold (Xiang & Tong, 2008). The present study demonstrates that dethia
acyetyl-CoA analogs [acetyl-carba(dethia)-CoA, acetyl-oxa(dethia)-CoA, and acetyl-
aza(dethia)-CoA] were able to fully activate WT SaPC (Figure 2-2A). Additionally,
acetyl-CoA, acetyl-carba(dethia)-CoA, acetyl-oxa(dethia)-CoA, and CoA were able to
fully activate WT SaPC in the presence of 20 mM L-aspartate (Figure 2-2B). Acetyl-
aza(dethia)-CoA did not activate WT SaPC in the presence of the allosteric inhibitor.
These results are consistent with acetyl-aza(dethia)-CoA binding to SaPC in an
orientation that is different from that of acetyl-CoA, CoA or the other dethia analogs.
This contributes to the low binding affinity (as measured by the elevated $K_a$) of acetyl-
aza(dethia)-CoA and makes it unable to displace L-aspartate (Figure 2-2B and Table 2-1).

**Residues in the BC Dimer Interface are Essential for Allosteric Activation**

In the present study, we demonstrate that protein-ligand interactions at the BC
dimer interface and near to the acetyl moiety binding site are essential to communicating
the allosteric mechanism. Residues Arg$_{21}$, Lys$_{46}$, and Glu$_{418}$ were mutated to alanine due
to their conservation in PC from many different organisms and other biotin dependent
carboxylases (Figure 2-4B). These residues were also chosen due to their location in the
BC dimer interface and their proximity to the acetyl moiety binding site of allosteric
activator acetyl-CoA (Figure 2-4A and C). Lys$_{46}$ of SaPC was of particular interest
because it is conserved in nearly all PC enzymes. One notable exception is PC from
*Corynebacterium glutamicum* PC, which encodes a phenylalanine at this position and is
insensitive to allosteric activation by acetyl-CoA (Figure 2-4B). Lys_{411} was also mutated to an alanine because it is in the BC dimer interface but further from the acetyl moiety binding site and therefore can serve as a control to ensure that the change in allosteric activation is not merely due to a disruption of the BC dimer interface. Only one of these residues has previously been subjected to the same mutagenic analysis: the equivalent residue to Glu_{418} was mutated in RePC to alanine but this yielded insoluble protein that prevented a clear determination of the impact of this residue on acetyl-CoA activation (Sirithanakorn et al., 2016). We have employed a revised purification strategy for SaPC that uses a C-terminal, intein-cleavable chitin binding affinity tag in place of the traditional N-terminal poly-histidine tag which lends to substantially higher soluble protein yields for the WT SaPC, and which likely enabled the purification of these mutated constructs where prior attempts have been unsuccessful. In the present study, E418A SaPC was purified as soluble protein that retained 3% of WT activity (Table 2-2). While this mutant retained a low level of catalytic activity, it was not allosterically activated by acetyl-CoA, nor by CoA (Figure 2-5A and B). Although not responsive to either allosteric effectors, E418A SaPC was inhibited by CT domain inhibitors oxalate, HQPA and phenylpyruvate (data not shown).

Several other residues at the BC dimer interface have previously been mutated in SaPC but these mutations were designed to fully disrupt the BC dimer interface through charge noncomplementarity and, in all cases, also resulted in inactive protein (Yu et al., 2013). Arg_{21} was previously mutated to a glutamate in SaPC and yielded tetrameric inactive protein. R21E SaPC, like R21A SaPC had an identical gel filtration profile as WT, suggesting this residue does not play a large role in tetramerization (Figure 2-4D).
(Yu et al., 2013). Unlike R21E, which was inactive, R21A SaPC retained 6% WT activity (Table 2-2). R21A SaPC is more similar to WT SaPC than it is to R21E SaPC, likely because the arginine to glutamate mutation is so disruptive (Figure 2-23). When the BC domains from chain A of WT SaPC and R21A SaPC were aligned they had an rmsd of 0.55 Å. When the BC dimer interfaces, on the opposite corner of the tetramer from the aligned BC domains, were compared they had an rmsd of 15.03 Å (Figure 2-23A). When the BC domains from chain A of R21E SaPC and R21A SaPC were aligned they had an rmsd of 0.73 Å. When the BC dimer interfaces, on the opposite corner of the tetramer from the aligned BC domains, were compared they had an rmsd of 26.10 Å (Figure 2-23B). The BC dimer interfaces were compared this way because this is the most drastic difference that can be seen, and this was how the comparisons were made in the study Yu et al. study on the BC dimer interface’s importance in 2013.
Lys\textsubscript{411}, the residue that was used as a control, was most similar to WT, when mutated to an alanine, in pyruvate carboxylation, ADP phosphorylation and inhibition kinetics studies. Lys\textsubscript{411} is a conserved, positively charged residue, and has been shown to be important for dimerization in another biotin dependent carboxylase, \textit{E. coli} BC (Figure 2-4B). In \textit{E. Coli} BC this residue is an arginine and when mutated disrupts dimerization (Shen et al., 2006). Lys\textsubscript{411} was previously mutated to a glutamate in \textit{SaPC} and was dimeric in solution and catalytically inactive (Yu et al., 2013). Although dimeric in solution, K411E \textit{SaPC} was tetrameric in crystal structures, due to its high protein concentration. Shown here, K411A \textit{SaPC} was predominantly tetrameric, although had
some dimeric species, and retained 16% of WT activity (Table 2-2, Table 2-3 and Figure 2-5). This data combined with our kinetic data shows that Lys411 is important for tetramerization but does not play a role in conveying the allosteric mechanism from the activator acetyl-CoA, likely because it is located too far from the approximate acetyl moiety binding site.

The goal of our mutagenic study was to disrupt the protein-ligand interactions while keeping the BC dimer interface intact. Although K46A, K411A and E418A SaPC had some dimeric species, shown in the analytical gel filtration data, all of the mutants retained catalytic activity, which can only occur when PC is in the tetrameric form, indicating that the BC dimer is intact (Figure 2-4D). K411A SaPC, the control mutant, had some dimeric species, and its behavior was most similar to WT in both of our kinetic assays. Our structure of R21A SaPC does not have any large structural difference, when compared to WT SaPC (Figure 2-11). Additionally, K46A SaPC does not have any large structural perturbations and this mutants BC dimer interface is also intact (Figure 2-13). Taken together, we are confident that the observed kinetic differences in the mutant constructs are not simply due to structural perturbations at the dimer interface but, rather, stem from the mutation of essential residues that are directly responsible for conveying the allosteric mechanism. In the kinetic characterization the $k_{cat}^{app}/k_{cat}$ was plotted to control for the loss of activity compared to WT, effectively discarding the inactive dimeric species from the analysis.

We used two kinetic assays, monitoring pyruvate carboxylation, the forward reaction, and ADP phosphorylation using carbamoyl phosphate as the phosphoryl donor, the reverse BC domain reaction. Shown through the kinetic data, all three residues, Arg21,
Lys\textsubscript{46}, and Glu\textsubscript{418} are implicated in the allosteric mechanism. R21A SaPC and K46A SaPC were both activated by acetyl-CoA and CoA in the pyruvate carboxylation reaction, although K46A SaPC had a significant decrease in degree of activation and both mutants had a reduced binding affinity. Neither R21A SaPC or K46A SaPC responded to acetyl-CoA in the ADP phosphorylation reaction. E418A SaPC did not respond to acetyl-CoA or CoA in both the pyruvate carboxylation reaction or the ADP phosphorylation reaction (Figure 2-5). Glu\textsubscript{418} is \textasciitilde 3.5Å from the carbonyl of the acetyl moiety of acetyl-CoA in chain A of the R21A SaPC structure. Additionally, Glu\textsubscript{418} forms ionic interactions with Arg\textsubscript{21} and Lys\textsubscript{46} across the BC dimer interface and contributes to tetramerization (Figure 2-4C and D). Lys\textsubscript{46} is important for both tetramerization and allosteric activation. The N\text{e} of Lys\textsubscript{46} projects deep into the BC dimer interface near the acetyl moiety of acetyl-CoA, while residing at the furthest extension of loop 44 – 50 that wraps around the adenosyl moiety of acetyl-CoA. As a consequence, Lys\textsubscript{46} is positioned to contribute multiple molecular interactions with the nucleotide base and to translate this binding interaction directly into the BC dimer interface. Lys\textsubscript{46} is just 4 residues from Ser\textsubscript{50} which is located \textasciitilde 3.2Å from the N1 position of the adenosine group of acetyl-CoA, linking the acetyl moiety to the nucleotidyl portion of acetyl-CoA (Figure 2-24). Arg\textsubscript{21} is not important for tetramerization, because the gel filtration data is identical to WT SaPC, but is implicated in acetyl-CoA binding and allosteric activation. The control mutant, K411A SaPC is not important for the allosteric mechanism but is important for tetramerization (Figure 2-4D). The acetyl moiety does not contribute to degree of activation but does increase the binding affinity and interacts with residues that are required for full activation of the enzyme.
WT SaPC, in addition to all of the BC dimer interface mutants, were co-crystallized with acetyl-CoA, but R21A SaPC yielded the quality best structure, and it reveals the binding site of the acetyl moiety of acetyl-CoA. Previous crystal structures of acetyl-CoA and acetyl-CoA analogs were unable to model in the acetyl moiety due to weak density, but chain A of the R21A SaPC structure has density corresponding to the entire compound. The R21A SaPC mutant likely yielded the best results because it had an unperturbed oligomerization state compared to WT, therefore this mutant had the smallest dimeric species and the homogeneity of the sample contributed to the quality of the crystals (Figure 2-4D). The R21A mutation allowed additional space for the acetyl moiety, while maintaining the interactions with Lys46 and Glu418 that stabilize the acetyl moiety. The additional space where Arg21 typically is, is occupied by a water (Figure 2-10C). This additional water only appears in chain A, whereas in chain C, that has CoA,
does not have this water. Acetyl-CoA is bound partially in the AD and BC dimer interface. The acetyl moiety interacts with Lys46 from chain C and Glu418 from its own chain. The acetyl moiety also likely interacts with Arg21, but this could not be concluded from this structure due to the R21A mutation. While chain A had acetyl-CoA bound, chain C had difference density that better corresponded to CoA (Figure 2-10A and B). The BC domains of chains B and D form a dimer and had little difference density corresponding to acetyl-CoA. The BC dimer composed of chains B and D had weak density compared to the BC dimer composed of chains A and C, which had activating compounds bound.

Although the crystal structure of K46A SaPC did not have strong density for the allostERIC activator, it did reveal that the BC dimer interface of the mutant was intact. The binding affinity of acetyl-CoA for K46A SaPC is much weaker than for WT SaPC, as shown by an increase in $K_a$ value. This is likely why there is poor density for the ligand in the crystal structure (Figure 2-14).

The structure of WT SaPC in complex with acetyl-carba(dethia)-CoA revealed relatively strong density for the analog in all four chains, unlike the R21A SaPC in complex with acetyl-CoA/CoA, which only had strong density for the activators in two chains of the tetramer. In RePC crystal structures, two molecules of an activator are always bound, while typically in structures of SaPC four molecules of an activator are always bound (Adina-Zada et al., 2019; St. Maurice et al., 2007; Westerhold et al., 2017; Yu et al., 2009). This is attributed to the symmetrical layout of SaPC and the asymmetrical layout of RePC (Westerhold et al., 2017). In the recent cryo EM structures of L/PC, four acetyl-CoA molecules were bound in the tetramer (López-Alonso et al.,
In the recent *HsPC* cryo EM study only two molecules of acetyl-CoA were bound to the tetramer, and they ordered the BC domains they were bound to while the other BC dimer on the opposite corner of the tetramer were not ordered and could not be modeled into the structure (Chai et al., 2022). A model was proposed in *HsPC* where the two acetyl-CoA molecules disassociate from the tetramer after each round of catalysis and must re-bind to the tetramer to stabilize the BC dimer to undergo another round of catalysis. The R21A *SaPC* is unique in that it only has two molecules bound, acetyl-CoA in chain A and CoA in chain B. The BC dimer with the two activator molecules bound have better density than the opposite BC dimer, where no activator is bound. This is in agreeance with the *HsPC* study that claims one of the main roles of acetyl-CoA is to order the BC dimer but can neither support nor refute the model of unbinding after each round of catalysis. The enzyme catalyzed hydrolysis of the acetyl moiety of acetyl-CoA could aid in the dissociation of the activator between each round of catalysis. In the WT *SaPC* structure in complex with nonhydrolyzable analog, acetyl-carba(dethia)-CoA, four molecules are occupying the binding sites, and this could be because the acetyl moiety cannot be hydrolyzed. The R21A *SaPC* structure where we’ve captured the full acetyl-CoA molecule bound could be a snapshot of the tetramer where one BC dimer has hydrolyzed and released the activating compound and the other BC dimer is in the process of hydrolyzing and their activating compounds, where chain B has already hydrolyzed the acetyl moiety and only chain A is left with the full molecule.

Allosteric inhibitor, L-aspartate, binds cooperatively to PC and inhibits competitively with respect to acetyl-CoA, but the binding site of L-aspartate is unknown. Previous mutagenic studies conducted on residues surrounding the nucleotidyl portion of
acetyl-CoA had no effect on L-aspartate inhibition (Adina-Zada, Sereeruk, et al., 2012; Sirithanakorn et al., 2014). R21A, K46A and E418A SaPC all lost their ability to be inhibited by L-aspartate (Figure 2-5C and Figure 2-8). Control residue, K411A SaPC had a similar $K_i$ to WT. This kinetic data suggests that the overlap between acetyl-CoA and L-aspartate binding sites may be in the BC dimer interface near the acetyl moiety binding site.

CT domain inhibitors oxalate, HQPA, and phenylpyruvate have a decrease in potency when acetyl-CoA is present. This indicates that acetyl-CoA binding in the AD/BC domain has a long range affect to the CT domain active site. This long range affect acetyl-CoA has on the CT domain has previously been shown through acetyl-CoA decreasing the $K_M$ of pyruvate (Westerhold et al., 2017). The increase in IC$_{50}$ value of the inhibitors suggests that acetyl-CoA alters the binding of the inhibitors and decreases their affinity. Additionally, CoA also shifted the IC$_{50}$ value for oxalate, therefore it is not specifically the acetyl moiety that is responsible for the long range effect to the CT domain. The BC dimer interface mutants had no effect on the potency of CT domain inhibitors, which implies that the acetyl moiety and the residues that interact with the acetyl moiety do not contribute to long range communication and that the nucleotidyl portion is responsible for the effect on the CT domain active site. Allosteric inhibitor L-aspartate had no effect on the potency of CT domain inhibitors, further confirming that allosteric inhibition is localized to the BC domain half reaction and does not affect the coupling efficiency between BC and CT domain reactions (Figure 2-22B) (Sirithanakorn et al., 2014).
Molecular insight into the interactions of the acetyl moiety and allosteric mechanism of acetyl-CoA provides insight for therapeutic intervention for various diseases. PC’s role in replenishing the Krebs cycle leads it to be implicated in diseases with higher energy requirements. PC has been implicated non-small cell lung cancer, breast cancer, thyroid cancer, and type II diabetes (Fan et al., 2009; Kumashiro et al., 2013; Lin et al., 2020; Liu et al., 2022).

Inhibition of PC in vitro, using multiple breast cancer cell lines, showed reduction in invasion and migration of the cancer cells (Lin et al., 2020). Inhibiting PC in multiple thyroid cancer cell lines reduced cell proliferation and migration (Liu et al., 2022). In a diabetic mouse model PC knockdown has been shown to improve insulin resistance (Kumashiro et al., 2013). Small molecule inhibitors have been generated and bind in the CT domain or at unknown locations (Burkett et al., 2019; Lin et al., 2020). The allosteric site provides another possible site to utilize in the design of inhibitors. In this work, residues that are essential for allosteric activation have been identified and this clearer molecular understanding of the allosteric site can be harnessed for the design of better inhibitors.

In this study we assessed the role the acetyl moiety plays in the allosteric activation of SaPC by acetyl-CoA. We compared acetyl-CoA to CoA and three non-hydrolysable/slowly hydrolysable analogs of acetyl-CoA and found that the analogs and CoA could activate SaPC to the same degree as acetyl-CoA but had higher $K_a$ values. We showed that acetyl-CoA, CoA, acetyl-carba(dethia)-CoA, and acetyl-oxa(dethia)-CoA could fully activate SaPC in the presence of 20 mM l-aspartate, whereas acetyl-aza(dethia)-CoA could not. Additionally, four residues in the BC dimer interface were
mutated and assessed for oligomerization state, pyruvate carboxylation rate, ADP phosphorylation rate and L-aspartate inhibition. Lastly, we determined that residues Arg\textsubscript{21}, Lys\textsubscript{46} and Glu\textsubscript{418} are essential for communicating the allosteric effect to the CT domain, through CT domain inhibition studies in the presence of acetyl-CoA. This work further clarifies the molecular basis for allosteric activation by acetyl-CoA. Acetyl-CoA primarily activates the BC domain half reaction but influences the distantly located CT domain through subtle structural changes in the CT domain that effect ligand binding. We believe that the BC dimer interface is an allosteric regulation hotspot for the enzyme and that residues Arg\textsubscript{21}, Lys\textsubscript{46} and Glu\textsubscript{418} play a critical role in allosteric activation and inhibition.
CHAPTER 3. INVESTIGATING THE ENZYME CATALYZED HYDROLYSIS OF ACETYL-COA

Introduction

The allosteric activation of pyruvate carboxylase by acetyl-CoA has been studied since the late 1960’s but the molecular basis for this allosteric activation is just now being defined. The structural, thermodynamic and kinetic basis for allosteric activation by acetyl-CoA has been extensively investigated in recent years (Adina-Zada, Zeczycki, et al., 2012; Chai et al., 2022; Westerhold et al., 2017). However, the question of how/whether the enzyme alters acetyl-CoA by hydrolysis has not been considered outside of the initial observation reported in the 1970’s (Frey & Utter, 1976).

In studies conducted on vertebrate PC, purified from chicken liver, it was shown that PC catalyzes the hydrolysis of its allosteric activator, acetyl-CoA. The rate of hydrolysis was determined using two independent methods. One assay monitored the reduction in absorbance at 232 nm. The thioester bond in acetyl-CoA absorbs at 232 nm. Therefore, only acetyl-CoA and not CoA will absorb at that wavelength. The other assay utilized 5’5’-dithiobis-(2-nitrobenzoic acid) (DTNB) which reacts with nonprotein sulphydryl groups and absorbs at 412 nm (Scrutton & Utter, 1967). Using these methods, the rate of acetyl-CoA hydrolysis was determined to be 4 nM/min/mg, which is drastically slower than the rate of pyruvate carboxylation at 15 µM/min/mg (Frey & Utter, 1976).

In the Scrutton and Utter study, maximal rate of acetyl-CoA hydrolysis was achieved when all reaction substrates were present: pyruvate, bicarbonate, ATP and
Mg$^{2+}$. Omitting the BC domain substrates, ATP and Mg$^{2+}$, resulted in a significant reduction in the rate of acetyl-CoA hydrolysis. Omitting the CT domain substrate, pyruvate, had substantially less impact on the rate of hydrolysis. Since BC domain substrates had the greatest impact on the rate of hydrolysis, a relationship was proposed between the BC domain half reaction and acetyl-CoA hydrolysis. This finding preceded the discovery that acetyl-CoA binds partially at the BC dimer interface.

Other activating acyl-CoAs were also hydrolyzed by PC, including propionyl-CoA and crotonyl-CoA. Thioesters that inhibit acetyl-CoA allosteric activation, such as acetylpantetheine and acetylmercaptosuccinate were also hydrolyzed, indicating that the hydrolysis is not specific to acetyl-CoA. Finally, through protein fractionation studies, it was determined that enzyme catalyzed acetyl-CoA hydrolysis could occur in both tetrameric and monomeric PC (Scrutton & Utter, 1967).

An acetylated form of PC was captured after incubation with acetyl-CoA. Therefore, during the enzyme catalyzed hydrolysis of acetyl-CoA the acetyl moiety is transferred onto the enzyme. The acyl-enzyme was proposed to be the activated form of PC (Scrutton & Utter, 1967). However, when PC was incubated with acetylating agents, the rate of pyruvate carboxylation remained unchanged. Additionally, dilution studies demonstrated that the acyl-enzyme had a 30 second half-life, whereas the activated enzyme complex had a half-life of only 1 second (Scrutton & Utter, 1967). This experiment confirmed that the acetylated enzyme is not the activated form. Consistent with these findings, my experiments with non-hydrolysable acetyl-CoA analogs demonstrated that these analogs could fully activate the enzyme, confirming that hydrolysis is not essential to activation (Chapter 2).
The experiments that investigated the enzyme catalyzed hydrolysis of acetyl-CoA were conducted in vertebrate PC. It is not known whether this phenomenon is broadly distributed to include bacterial PC. It seems likely that bacterial PC enzymes can also catalyze the hydrolysis of acetyl-CoA because, until recently, every attempt to co-crystallize PC with acetyl-CoA resulted in an absence of electron density for the acetyl moiety, consistent with hydrolysis of the acetyl moiety. It is not clear whether the acetyl moiety is being hydrolyzed enzymatically or non-enzymatically: most recombinantly expressed bacterial proteins, including PC, are purified in the presence of reducing agents for stability, and reducing agents are also known to hydrolyze acetyl-CoA.

Acetyl-CoA has a 100-fold greater binding affinity for SaPC than CoA. Therefore, hydrolysis could serve to aid in the disassociation of the activator from the enzyme (Yu et al., 2009). In a recently proposed model of catalysis, each round of pyruvate carboxylation results in the dissociation of acetyl-CoA from the tetramer. This dissociation was attributed to a conformational change in the AD that also helps release ADP from the BC active site to enable another round of ATP binding. To facilitate the next round of catalysis, two new molecules of acetyl-CoA must bind to stabilize the BC dimer (Chai et al., 2022). While this model did not mention or consider acetyl-CoA hydrolysis, a hydrolysis mechanism would certainly contribute to activator dissociation after each round of catalysis, just as they proposed. Here, I investigate whether the bacterial SaPC catalyzes the hydrolysis of its allosteric activator, acetyl-CoA, and seek to better characterize the site of hydrolysis using mutated and engineered constructs of SaPC.
Materials and Methods

Materials

Acetyl-CoA was purchased from CoALA Biosciences (Austin, TX). Except where noted, all other chemicals and materials were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), or Research Products International Corp. (Mount Prospect, IL). All DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA).

SaPC Constructs

Pyruvate carboxylase from S. aureus (SaPC; UniProtKB - A0A0E8G8A7) was previously cloned into a modified pET-27b vector and was generously supplied by Dr. Liang Tong, Columbia University (Yu et al., 2009). To reclone SaPC into the pTXB1 vector (New England Biolabs, Ipswich, MA), the gene encoding SaPC in pET-27b was PCR amplified using forward (5’- GCC ATA TGA AAC AAA TAA AAA AG -3’) and reverse primers (5’-CGT GAT GCA GTT AGT TGC TTT TTC AAT TTC G -3’). The PCR amplicon was subjected to restriction digestion with NdeI and SapI prior to ligation into a gel-purified pTXB1 vector digested with NdeI and SapI. WT SaPC pTXB1 was fully sequenced.

The full sequence of SaPC pTXB1 revealed a PCR-introduced G571S mutation. The mutation was corrected back to a glycine at residue 571 by mutagenesis using the whole-plasmid PCR technique according to the Quikchange II mutagenesis protocol from Agilent Technologies, Inc. (Santa Clara, CA), with forward primer 5’- GCG GAC GTA TTT AAA GAT GGT TTC TCA CTA G -3’ and reverse primer 5’- CTA GTG AGA
AAC CAT CTT TAA ATA CGT CCG C -3’. The complete gene sequence of WT SaPC in the pTXB1 vector was confirmed by DNA sequencing.

**SaPC pET-27b Expression and Purification**

SaPC pET-27b clones were co-transformed and co-expressed with the pCY216 vector encoding *Escherichia coli* biotin protein ligase A (BirA) to ensure complete biotinylation of PC. Transformed *E. coli* BL21 (DE3) cells were cultured in M9 minimal media with 25 μg/mL kanamycin and 30 μg/mL chloramphenicol at 37 °C to an optical density (OD$_{600}$) of 0.8 - 1.0, after which protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and L-arabinose to a final concentration of 0.5 mM and 25 mM, respectively. The culture was also supplemented with D-(-)-biotin at a final concentration of 3 mg/L. Induced cells were incubated at 16 °C for 16 - 24 hours before harvesting by centrifugation.

SaPC pET27-b enzymes were purified using sequential Ni$^{2+}$-affinity and anion exchange chromatography. Harvested cell paste (20 – 30 g) was re-suspended in 200 mL lysis buffer (20 mM Tris-HCl pH 7.8; 200 mM NaCl; 0.5 mM EGTA; 5 mM imidazole; 6 mM β-mercaptoethanol; 1 mM PMSF; 1 μM pepstatin A; and 5 μM E-64). Cells were lysed by sonication for 10 minutes at a temperature between 4-10 °C and pelleted by centrifugation. The supernatant was loaded on a 5 mL Ni$^{2+}$-nitrilotriacetic acid proximity resin column (Bio-Rad, Hercules, CA). The column was washed with 12× column volume of wash buffer (20 mM Tris-HCl pH 7.8; 200 mM NaCl; 0.5 mM EGTA; 20 mM imidazole; 6 mM β-mercaptoethanol) and the protein was eluted with a gradient from 20 mM to 250 mM imidazole using wash buffer and elution buffer (20 mM Tris-HCl pH 7.8; 200 mM NaCl; 0.5 mM EGTA; 250 mM imidazole; 6 mM β-mercaptoethanol). Purified
protein was pooled and dialyzed against a buffer compatible with anion-exchange chromatography (20 mM triethanolamine, pH 8.0; 50 mM NaCl; 1 mM EGTA; and 2 mM dithiothreotol (DTT)) at 4 °C overnight. The dialyzed protein was loaded onto a 10 mL Q-Sepharose Fast Flow resin column (GE Healthcare), washed with 10× column volumes of dialysis buffer and eluted from the column in dialysis buffer with a gradient from 50 mM to 1 M NaCl. SaPC typically elutes between 400 - 800 mM NaCl. The purified protein was pooled and dialyzed against storage buffer (20 mM Bis-Tris Propane, pH 7.2; 15 mM NaCl; 10 mM MgCl₂, and 1 mM TCEP) for three successive changes of 4 hours or more. The protein was concentrated to a final concentration of 4 mg/mL − 10 mg/mL and was drop frozen in liquid nitrogen prior to storage at -80 °C. All protein concentrations were determined using the predicted extinction coefficient corresponding to absorbance at 280 nm.

**SaPC pTXB1 Expression and Purification**

SaPC pTXB1 enzymes were purified with sequential chitin affinity and anion exchange chromatography. Harvested cell paste (20 – 30 g) was re-suspended in 200 mL column binding buffer (20 mM HEPES, pH 7.8; 200 mM NaCl; 0.5 mM ethylenediaminetetraacetic acid (EDTA); 1 mM Tris (2-carboxyethyl) phosphine (TCEP)) with the inclusion of protease inhibitors phenylmethylsulfonyl flouride (PMSF) (1 mM), pepstatin A (1 μM); and L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine (E-64) (5 μM). Cells were lysed by sonication for 8 minutes at a temperature ranging from 4-10 °C and pelleted by centrifugation. The supernatant was loaded on a 10 mL chitin resin column (NEB, Ipswich, MA), with the column flow through re-applied to the column a total of three times. The column was washed with 100 mL of column binding
buffer. The resin was subsequently incubated overnight at 4 °C with 30 mL of cleavage buffer (20 mM HEPES, 200 mM NaCl, 1 mM EDTA, and 50 mM dithiothreitol (DTT)). The following day, the cleaved protein was eluted from the column. Two additional incubations with 30 mL of cleavage buffer were applied for at least 4 hours before eluting protein. The eluant from each of the incubations was pooled and dialyzed overnight at 4 °C against a buffer compatible with anion-exchange chromatography (20 mM triethanolamine, pH 8.0; 50 mM NaCl; 1 mM EGTA; and 2 mM DTT). The dialyzed protein was loaded onto a 10 mL Q-Sepharose Fast Flow resin column (GE Healthcare), washed with 100 mL of dialysis buffer and eluted with a gradient from 50 mM to 1 M NaCl in dialysis buffer. SsPC typically elutes between 400 - 800 mM NaCl. The purified protein was pooled and dialyzed against storage buffer (20 mM HEPES, pH 7.2; 15 mM NaCl; 10 mM MgCl\(_2\), and 1 mM TCEP) for two successive changes of 4 hours or more. The protein was concentrated to a final concentration of 10 – 25 mg/mL and was drop frozen in liquid nitrogen prior to storage at -80 °C. All protein concentrations were determined using the predicted extinction coefficient corresponding to absorbance at 280 nm.

**Acetyl-CoA Hydrolysis Assay**

Prior to the hydrolysis reaction, protein that was purified in the presence of 1 mM TCEP was dialyzed 2 times for at least 4 hours in a 1 L dialysis buffer that consisted of 20 mM HEPES, 15 mM NaCl, and 10 mM MgCl\(_2\) to remove the reducing agent so that it could not hydrolyze acetyl-CoA. The absence of reducing agent in the protein sample was confirmed by running a dialysis buffer control for the maximum incubation period, 6 hours, and confirming that the final dialysis buffer did not hydrolyze acetyl-CoA. After
dialysis, 10 µM SaPC was incubated at room temperature with 250 µM acetyl-CoA in HPLC buffer that consisted of 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$ at pH 7.8, 10 mM MgCl$_2$ and 20 mM KCl for a specific amount of time (0-6 hours). Hydrolysis reactions were quenched by adding 20 µL of 7.625% trifluoracetic acid (TFA) to a 200 µL reaction for a final concentration was 0.7625% TFA. This concentration of TFA is equivalent to ~100 mM, which is twice the concentration of buffer (50 mM) and sufficient to overwhelm the buffering capacity. The addition of TFA precipitated the protein. All samples were centrifuged for 10 min at 20,913 xg, and the supernatant was collected. This process was repeated twice more with the collected supernatant to ensure that all the precipitated protein was completely removed from the sample. Analytical high performance liquid chromatography (HPLC) was carried out at Purdue University by Dr. Jeremy Lohman on an Agilent 1100 HPLC with diode array UV/Vis detection over a Luna 5 µm C18(2) 100 Å 250 x 4.6 mm column (Phenomenex). Linear gradients were developed from 0.1% TFA in water (A) to acetonitrile (B) from 2% to 25% over 20 min. The flow rate was kept constant at 1.0 mL/min, and elution was monitored at 254 nm (Lohman & Shen, 2020). A standard curve was generated by running a series of known CoA concentrations (1, 5, 10, 15, 20, 50 and 100 µM). The linear regression equation from the CoA standard curve was Y=51.99X–155.8 with an R$^2$ value of 0.99. The percentage of the peak area for CoA was plotted as a function of time, to control for slight variations of injection volumes. This data was fit to a one phase decay (Equation 3-1), where $a$ represents the y-intercept, or the percentage of the peak area of CoA at time 0, $b$ represents the plateau or the percentage of the peak area of CoA at infinite time, $k_{obs}$ is the observed rate constant of acetyl-CoA hydrolysis and $t$ is time in minutes.
Equation 3-1

\[ y = (a - b) \cdot e^{-k_{\text{obs}}t} + b \]

**rTEV Expression and Purification**

Recombinant tobacco etch virus protease (rTEV) was expressed in pMHTdelta238 plasmid (Blommel & Fox, 2007). A glycerol stock of rTEV pMHTdelta238 was streaked on a Luria-Bertani (LB) plate containing 30 µg/mL chloramphenicol (CAM) and 50 µg/mL kanamycin (KAN). The plate was incubated overnight at 37 °C and individual colonies were used to inoculate 2 × 200 mL terrific broth (TB) cultures containing 30 µg/mL CAM and 50 µg/mL KAN, which were incubated overnight, with shaking, at 37 °C. A 30 mL aliquot of the 200 mL cultures was then used to inoculate 8 × 1 L media, which grew to an optical density at 600 nm of ~1. The cultures were induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM prior to incubation, with shaking, for 20 hours at 16 °C. The cells were harvested by centrifugation. Cells were thawed and sonicated for a total of 8 minutes in a lysis buffer that consisted of 50 mM HEPES pH 7.6, 10 mM imidazole, 300 mM NaCl, 0.5 mM TCEP, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM pepstatin A and 5 µM N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E-64). The cells were then centrifuged for 30 mins at 48,384 xg at 4 °C and the supernatant was loaded onto a Ni\(^{2+}\)-nitrilotriacetic acid (NTA) profinity resin column (Bio-Rad, Hercules, CA). The column was washed with 100 mL lysis buffer and the protein was eluted off the column with an elution buffer that consisted of 50 mM HEPES pH 7.6, 250 mM imidazole, 10 mM NaCl, 0.5 mM TCEP, and 10% glycerol. The
protein was dialyzed overnight at 4 °C in a cation exchange buffer that consisted of 20 mM HEPES pH 7.6, 10 mM NaCl, 0.5 mM TCEP, and 10% glycerol. The protein was then loaded onto a BT Bio-Rex 70 cation exchange resin column (Bio-Rad, Hercules, CA) and washed with 100 mL of cation exchange buffer. The protein was eluted off the cation exchange column via gradient elution from 150 mM NaCl to 1 M NaCl in elution buffers that also consisted of 20 mM HEPES pH 7.6, 0.5 mM TCEP and 10% glycerol. The protein eluted from the column at a concentration of ~400 mM NaCl. The protein was then flash frozen in liquid nitrogen in 250 µL aliquots.

**TEV Cleavage**

rTEV was thawed and incubated with PC at a 40:1 molar ratio, for ~4 hours at room temperature and then overnight at 4 °C while dialyzing against a loading buffer that consisted of 20 mM Tris-HCl pH 7.8, 200 mM NaCl, and 0.5 mM EGTA. The rTEV digestion of PC was then loaded onto a Ni\(^{2+}\)-NTA profinity resin column (Bio-Rad, Hercules, CA) with a 1 mL resin volume and the flow through was collected. The flow through contained the TEV cleaved PC, leaving the poly-His tag, rTEV protein and un-cleaved PC protein bound to the column. The column was then washed with 5 mL loading buffer and the flow through was collected. Then un-cleaved protein was eluted off the column using an elution buffer that consisted of 20 mM Tris-HCl pH 7.8, 200 mM NaCl, 0.5 mM EGTA, and 250 mM imidazole. The TEV cleaved PC was then dialyzed overnight into a storage buffer that consisted of 20 mM HEPES, 15 mM NaCl, and 10 mM MgCl\(_2\) to remove all remaining imidazole.
**Enzyme Assay for CT Domain Activity**

The PC-catalyzed rate of oxaloacetate decarboxylation was determined by measuring the reduction of pyruvate to lactate, using lactate dehydrogenase and monitoring a decrease in absorbance at 340 nm (Lietzan & St. Maurice, 2013b). Reactions were performed in 0.1 M Tris–HCl (pH 7.8), 0.24 mM NADH, lactate dehydrogenase (10 U/mL), 5 mM biocytin, and 1 mM oxaloacetate with a reaction volume of 200 µL. The half-life for the spontaneous decarboxylation of oxaloacetate is 2 – 6 h at room temperature. Therefore, oxaloacetate solutions were prepared fresh, kept on ice for the duration of the kinetic experiments and added immediately prior to the initiation of the reaction (Wolfenden et al., 2011). Reactions were initiated with 200 µg of PC, and all reactions were performed in triplicate.

**Results**

*Staphylococcus aureus PC Catalyzes the Hydrolysis of Acetyl-CoA*

The hydrolysis of acetyl-CoA was monitored using an HPLC assay modified from Lohman and coworkers (Lohman & Shen, 2020). A standard curve was developed from 1 to 100 µM CoA to ensure that the peak area was directly proportional to the CoA concentration (Figure 3-1A). The standard curve also confirmed that CoA concentrations could be accurately measured as low as ~10 µM. The rate of hydrolysis of acetyl-CoA catalyzed by WT SaPC was investigated by incubating 250 µM acetyl-CoA with 10 µM WT SaPC for a series of timepoints from 0 to 6 hours. The concentration of the hydrolysis product, CoA, was determined by measuring the peak area following HPLC separation. The rate of hydrolysis was determined to be 0.005 min⁻¹ (Figure 3-2). The rate
is about ~20 times greater than the rate of chicken liver PC acetyl-CoA hydrolysis. This confirms that the enzyme catalyzed hydrolysis of acetyl-CoA is a phenomenon that extends to bacterial PC. A non-enzymatic buffer control was performed to ensure that the assay buffer components were not contributing to the hydrolysis (Figure 3-1B). Given the slow rate of hydrolysis, bovine serum albumin (BSA) was used as an additional control to ensure that acetyl-CoA hydrolysis was not simply a result of nonspecific protein-mediated hydrolysis. When BSA was incubated with acetyl-CoA over 6 hours, no CoA appeared (Figure 3-1C).

Figure 3-1 Acetyl-CoA hydrolysis at various starting concentrations of acetyl-CoA. A. The standard curve that was performed with known concentrations of CoA (1, 5, 10, 15, 20, 50 and 100 µM). The data was fit to a linear regression and the equation was Y=51.99X+-155.8 with an R² of 0.99. B. The hydrolysis of acetyl-CoA in the presence of the assay buffer. The disappearance of acetyl-CoA (red squares) and appearance of CoA (black circles) over 6 hours. The data was fit to a linear regression. C. The hydrolysis of acetyl-CoA in the presence of BSA. The disappearance of acetyl-CoA (red squares) and appearance of CoA (black circles) over 6 hours. The data was fit to a linear regression.

To estimate the first order rate constants for WT SaPC hydrolysis of acetyl-CoA, the experiment was repeated with various starting concentrations of acetyl-CoA (50, 100, and 200 µM). The $K_c$ of acetyl-CoA for SaPC is ~1 µM in the pyruvate carboxylation reaction. Therefore, we expected 200 µM acetyl-CoA to greatly exceed the concentration
required for saturation. However, a replot of initial velocities as a function of acetyl-CoA concentration revealed that 200 µM was well below saturation (Figure 3-2B). The estimated $V_{\text{max}}$ was 1.2 µM/min and the $K_m$ was 160 µM. The substantial mismatch between the $K_a$ and the $K_m$ values suggests that acetyl-CoA hydrolysis may take place at a site other than the allosteric binding site for acetyl-CoA. Due to the high $K_m$ and low apparent binding affinity, I altered my approach to begin measuring the rate of the approach to equilibrium at a single acetyl-CoA concentration. This was largely a pragmatic decision: full initial velocity experiments with a $K_m$ value this high and a $k_{\text{cat}}$ value this slow would require prohibitively high concentrations of acetyl-CoA and protein. Acetyl-CoA concentrations would potentially need to be as high as 1 mM, which would diminish the accuracy of the HPLC assay due to the overlap of acetyl-CoA and CoA peaks at these concentrations.
Truncated Constructs of PC Catalyze the Hydrolysis of Acetyl-CoA

As previously described, the allosteric binding site for acetyl-CoA resides at the intersection of the allosteric domain and the BC domain, with the acetyl moiety projecting into the BC dimer interface (Chapter 2). Additionally, acetyl-CoA hydrolysis studies conducted in vertebrate PC connected acetyl-CoA hydrolysis to the BC domain half reaction (Scrutton & Utter, 1967). To investigate whether the BC domain is responsible for hydrolysis, a construct of SaPC that lacks both the BC and BCCP domains, ΔBCΔBCCP SaPC, was incubated with acetyl-CoA over various timepoints up to 6 hours, and the rate of hydrolysis was determined (Figure 3-3). Prior analytical gel filtration studies confirmed that ΔBCΔBCCP *Rhizobium etli* PC formed intact dimers,
offering evidence that the CT dimer interface remains intact when the BC and BCCP domains are removed (Lietzan et al., 2011). I predicted that no enzyme catalyzed hydrolysis would be observed with ΔBCΔBCCP SaPC because the binding site for the acetyl moiety of acetyl-CoA would not be intact. Surprisingly, a slow rate of hydrolysis was still observed, at a rate of 0.003 min⁻¹. The rate of acetyl-CoA hydrolysis catalyzed by ΔBCΔBCCP SaPC was about a half of the rate of WT SaPC, leading to the conclusion that acetyl-CoA hydrolysis is likely occurring both in the BC dimer interface, where it is known to bind, and also in another location in the CT domain. To further investigate which domain(s) are responsible for catalyzing the hydrolysis additional constructs were tested: ΔBCΔBCCP RePC and ΔBC SaPC also catalyzed the hydrolysis of acetyl-CoA (Figure 3-4). Both of these additional constructs could catalyze the hydrolysis of acetyl-CoA. These results strongly suggest that multiple binding sites for acetyl-CoA are present across a range of bacterial PC enzymes and that hydrolysis can occur at more than one location on the enzyme.
Figure 3-3. The primary and quaternary structures of PC constructs. A. The polypeptide chain layout of WT PC, ΔBC PC and ΔBCΔBCCP PC. B. The structure of tetrameric WT PC. C. The structure of dimeric ΔBC PC. D. The structure of ΔBCΔBCCP PC. PDB ID 3BG5 was used to generate panels B, C, and D. One subunit is outlined in grey surface representation in panels B, C and D.
The truncated constructs were all purified via affinity chromatography utilizing an N-terminal poly-histidine tag. Poly-histidine tags have the ability to catalyze ester hydrolysis, even when attached to a protein (Schoonen et al., 2017). Therefore, I sought to evaluate whether the N-terminal poly-histidine tag was contributing to acetyl-CoA hydrolysis. To test whether the observed slow rate of hydrolysis observed in all PC constructs was attributable to their N-terminal poly-histidine tags, the tag was removed using recombinant Tobacco Etch Virus (rTEV) protease. The rTEV protease was incubated with ΔBCΔBCCP SaPC and WT SaPC for 20 hours and the cleaved tags, along with un-cleaved enzyme, were removed by running the samples over a Ni²⁺-NTA column and collecting the unbound flow through. The column flow through samples were run on SDS-PAGE gel to assess the cleavage reaction (Figure 3-5). Unfortunately, the TEV
cleavage was unsuccessful for WT SaPC, evidenced by the entirety of the sample being contained in the elution fraction. This is likely because the TEV cleavage site was not accessible to the protease in WT SaPC. WT SaPC incubated with rTEV was loaded on to a Ni²⁺-NTA column and the flow through contained little to no protein, similar to the control WT SaPC incubated in the absence of rTEV. The protein was all bound to the column as a consequence of the intact poly-histidine tag (Figure 3-5). The TEV cleavage was successful for ΔBCΔBCCP SaPC. The ΔBCΔBCCP SaPC protein was incubated with rTEV and loaded onto a Ni²⁺-NTA column. The flow through contained nearly all of the protein, consistent with the removal of the poly-histidine tag (Figure 3-5).
The TEV cleaved ΔBCΔBCCP SaPC was assessed for its ability to catalyze acetyl-CoA hydrolysis. To ensure that the 20 hour rTEV incubation and subsequent dialysis did not affect the integrity of the protein, the activity of ΔBCΔBCCP SaPC was tested using an enzyme assay for CT domain activity. TEV cleaved ΔBCΔBCCP SaPC retained 76% oxaloacetate decarboxylation activity when compared to ΔBCΔBCCP SaPC (Table 3-1). The rate of acetyl-CoA hydrolysis of TEV cleaved ΔBCΔBCCP SaPC was very similar to the rates of hydrolysis for ΔBCΔBCCP SaPC with the poly-histidine tag intact (Figure 3-6). Therefore, the poly-histidine tag is not primarily responsible for
hydrolyzing acetyl-CoA and, instead, some component of PC outside of the BC domain catalyzes the hydrolysis of acetyl-CoA (Table 3-2).

**Table 3-1.** The rates of the reverse CT domain reaction, oxaloacetate decarboxylation, for various PC constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Rate of Oxaloacetate Decarboxylation (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔBCΔBCCP RePC</td>
<td>0.22 ± 0.002</td>
</tr>
<tr>
<td>ΔBCΔBCCP SaPC</td>
<td>0.17 ± 0.006</td>
</tr>
<tr>
<td>ΔBC SaPC</td>
<td>0.16 ± 0.005</td>
</tr>
<tr>
<td>TEV cleaved ΔBCΔBCCP SaPC</td>
<td>0.13 ± 0.003</td>
</tr>
</tbody>
</table>

![Figure 3-6.](image) **Figure 3-6.** TEV cleaved ΔBCΔBCCP SaPC catalyzes the hydrolysis of acetyl-CoA. The disappearance of acetyl-CoA (red squares) and appearance of CoA (black circles) over 6 hours. The data was fit to Equation . TEV cleaved ΔBCΔBCCP SaPC is shown with dashed lines and ΔBCΔBCCP SaPC is shown with solid lines.
**Residues in BC Dimer Interface Protect Acetyl Moiety from Hydrolysis**

Finally, I investigated the impact of residues near the acetyl moiety binding site on the hydrolysis of acetyl-CoA. The BC dimer interface SaPC mutants characterized in Chapter 2 were investigated for their ability hydrolyze acetyl-CoA. Arg\textsubscript{21}, Lys\textsubscript{46}, Glu\textsubscript{418} and Lys\textsubscript{411} are all residues that are conserved and located in the BC dimer interface. These residues form ionic interactions with residues on the opposite face of the tetramer and contribute to tetramerization. Arg\textsubscript{21}, Lys\textsubscript{46}, and Glu\textsubscript{418} interact with the acetyl moiety and contribute to the allosteric mechanism, whereas Lys\textsubscript{411} is \(\sim11\text{Å}\) from the acetyl moiety and does not participate in the allosteric mechanism. Surprisingly, in every case, the mutants exhibited much faster rates of acetyl-CoA hydrolysis than the WT enzyme (Figure 3-7). Mutation of any of the residues that are located in the BC dimer interface to alanine increased the rate of hydrolysis \(\sim4\) fold. This strongly implies that the residues that hold the two faces of the tetramer together have the added role of protecting acetyl-CoA against enzyme-catalyzed hydrolysis. K411A SaPC had an increase in rate of acetyl-CoA hydrolysis, although Lys\textsubscript{411} does not directly interact with the acetyl moiety but does contribute to the tetramerization. This indicates that the tight junction between the two BC domains is imperative to protect the acetyl moiety from enzyme catalyzed hydrolysis.
Table 3-2. The rates of acetyl-CoA hydrolysis for various constructs and mutants of PC.

<table>
<thead>
<tr>
<th>Construct</th>
<th>$k_{obs}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SaPC</td>
<td>0.0048 ± 0.0005</td>
</tr>
<tr>
<td>ΔBC SaPC</td>
<td>0.0068 ± 0.0010</td>
</tr>
<tr>
<td>ΔBCΔBCCP SaPC</td>
<td>0.0030 ± 0.0023</td>
</tr>
<tr>
<td>ΔBCΔBCCP RePC</td>
<td>0.0037 ± 0.0011</td>
</tr>
<tr>
<td>ΔBCΔBCCP SaPC TEV cleaved</td>
<td>0.0017 ± 0.0004</td>
</tr>
<tr>
<td>R21A SaPC</td>
<td>0.036 ± 0.004</td>
</tr>
<tr>
<td>K46A SaPC</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>E418A SaPC</td>
<td>0.025 ± 0.008</td>
</tr>
<tr>
<td>K411A SaPC</td>
<td>0.021 ± 0.002</td>
</tr>
</tbody>
</table>

Figure 3-7. BC dimer interface mutants hydrolyze acetyl-CoA at an increased rate compared to WT. A. Acetyl-CoA hydrolysis with WT SaPC (black), ΔBCΔBCCP SaPC (brown), R21A SaPC (green), K46A SaPC (blue), E418A SaPC (red), and K411A SaPC (purple). The disappearance of acetyl-CoA (squares) and appearance of CoA (circles) over 6 hours. The data was fit to Equation . B. A bar graph of the $k_{obs}$ values calculated from the fitted data from panel A. The error bars represent the standard errors calculated from iterative non-linear curve fitting in Graphpad Prism.
Discussion

Enzyme Catalyzed Hydrolysis of Acetyl-CoA is Conserved in Microbial PC

PC catalyzed acetyl-CoA hydrolysis has not been investigated since it was first reported in chicken liver PC in the 1970’s. I have demonstrated that this property is conserved in bacterial PC enzymes. In principle, acetyl-CoA hydrolysis provides the enzyme with a mechanism to directly alter the binding affinity of its own allosteric activator. The acetyl moiety of acetyl-CoA is easily hydrolyzed and, since the early 2010’s, this hydrolysis has long been thought to be the reason that electron density for the acetyl moiety is not observed in the crystal structures (Yu et al., 2009). Studies that investigated the acetyl-CoA binding site and allosteric mechanism have been focused on residues that bind acetyl-CoA near the nucleotidyl portion because the 3’5’-diphosphoadenosine portion of the molecule is sufficient for full activation (Adina-Zada, Sereeruk, et al., 2012; Sirithanakorn et al., 2016). The studies described in Chapter 2 have further established that residues that interact with the acetyl moiety are essential for allosteric activation. Here, I further investigated the role of the acetyl moiety binding site on the potential to catalyze the hydrolysis of acetyl-CoA.

The BC Domain is not Solely Responsible for Hydrolysis

The only known binding site for acetyl-CoA is in the AD/BC dimer interface (Adina-Zada, Sereeruk, et al., 2012; Yu et al., 2009). This makes it particularly surprising that the truncated PC constructs, which lack the BC dimer interface that binds the acetyl moiety, maintained hydrolytic activity with acetyl-CoA. The rates of hydrolysis are reduced in the absence of the BC domain, but hydrolysis still occurs. The most likely
explanation for this observation is that there is a binding site for acetyl-CoA elsewhere, likely in the CT domain. Other bacterial PCs, such as *Listeria monocytogenes* PC (*Lm*PC), *Lactococcus lactis* PC (*Ll*PC), and *Enterococcus faecalis* PC (*Ef*PC) have allosteric sites in their CT domains (López-Alonso et al., 2022; Sureka et al., 2014). *Lm*PC, *Ll*PC and *Ef*PC are all allosterically inhibited by cyclic-di-adenosine monophosphate (ci-di-AMP), a second messenger signaling molecule. Ci-di-AMP binds in the CT dimer interface of PC ~25 Å from the CT domain active site, and allosterically inhibits the enzyme (Sureka et al., 2014). Although this binding site is not conserved in *Sa*PC, this raises the potential for other allosteric site(s) to exist in the CT domain. It is also possible that the hydrolysis of acetyl-CoA is facilitated by the metal ion in the CT domain active site. If the CT domain metal was responsible for the hydrolysis, then we would expect pyruvate to decrease the rate of hydrolysis, because pyruvate binds to the metal in the active site and would compete out acetyl-CoA. In chicken liver PC, pyruvate did not alter the rate of hydrolysis. Pyruvate’s effect on the rate of hydrolysis in *Sa*PC was not investigated, but if it is similar to chicken liver PC in this regard, then there is likely an alternative site where acetyl-CoA is being hydrolyzed.

**Acetyl-CoA Hydrolysis Does Not Reach Equilibrium**

In all cases, acetyl-CoA hydrolysis stops well before reaching equilibrium. Enzyme catalyzed hydrolysis typically proceeds until ~60% of acetyl-CoA is converted to CoA. The equilibrium constant for acetyl-CoA hydrolysis is ~1.6 × 10^6 [CoA]/[AcCoA] (Guynn et al., 1974). CoA should be in great excess over acetyl-CoA when the reaction reaches equilibrium so the premature termination of the reaction must be a consequence of a slow kinetic step. The hydrolysis reaction not terminating
prematurely as a consequence of enzyme instability because, after 6 hours at room temperature, PC exhibits only a very small reduction in pyruvate carboxylation activity. The attenuation of hydrolysis could result from enzyme acetylation. Many residues can become acetylated, most commonly lysine, serine and methionine (Polevoda & Sherman, 2002). A buildup of acetylated enzyme could impede acetyl-CoA hydrolysis, preventing the reaction from reaching equilibrium. If the spontaneous deacetylation of the enzyme is much slower than the rate of acetylation, the hydrolysis would stop after a certain amount of acetylation occurred. Dilution studies conducted in the 1960’s did show that PC is acetylated in the presence of acetyl-CoA, but this was not investigated further because the acyl-enzyme was not the activated form of the enzyme (Scrutton & Utter, 1967).

**Conserved Residues in the BC Dimer Interface Protect Against Hydrolysis**

The importance of residues Arg21, Lys46, and Glu418 in the allosteric mechanism of acetyl-CoA was described in Chapter 2. I hypothesized that these residues also participate in the enzyme-catalyzed hydrolysis of the acetyl moiety. Interestingly, the $k_{obs}$ of acetyl-CoA hydrolysis for the BC dimer interface mutants were ~5 times greater than for WT SaPC. Since these residues in the dimer interface form interactions with residues in the neighboring subunit, the loss of these interactions is likely to create more space in the dimer interface, where the acetyl moiety binds. This might enable increased access of water molecules to the BC dimer interface and allow them to come into close proximity to acetyl-CoA. For example, in Chapter 2, I present structural evidence that, when Arg21 is mutated, there is additional space near the acetyl moiety that is occupied by a water molecule (Chapter 2). The increased rate of hydrolysis in the BC dimer interface mutants suggests that, in addition to their role in binding the allosteric activator and
communicating the allosteric effect, these conserved residues in the acetyl-CoA binding site are also important for protecting the acetyl moiety from enzyme-catalyzed hydrolysis.

The allosteric activation of PC by acetyl-CoA has been extensively researched, but PC catalyzed hydrolysis of acetyl-CoA has not been investigated for nearly 40 years. My studies reveal that this phenomenon is conserved from vertebrate to bacterial PC. Most notably, acetyl-CoA hydrolysis catalyzed by ΔBCΔBCCP PC indicates that there could be more than one acetyl-CoA binding site in PC. Both ΔBCΔBCCP SaPC and ΔBCΔBCCP RePC hydrolyzed acetyl-CoA, suggesting that an allosteric site(s) in the CT domain could be a common feature in bacterial PCs.

The $K_m$ determined for acetyl-CoA in the hydrolysis is ~160 μM, indicating that this is not likely to be a physiologically relevant phenomenon because the concentration of acetyl-CoA in vivo is ~1-25 μM (Chen et al., 2016). Moreover, the rate of hydrolysis is drastically slower than the rate of pyruvate carboxylation, minimizing any role for acetyl-CoA hydrolysis as part of the catalytic cycle. Instead, the enzyme-catalyzed hydrolysis of acetyl-CoA is likely to be an in vitro artifact. The disassociation of acetyl-CoA proposed to take place after each round of hydrolysis by the Chai et. al. 2022 HsPC cryo-EM study, is not likely to be impacted by this hydrolysis reaction. Nevertheless, while it is not a physiologically relevant reaction, this work has confirmed that PC can catalyze the reaction. This reveals the possibility for an additional allosteric site in the CT domain and suggests that the BC dimer interface’s tight ionic interactions serve to minimize the potential for acetyl-CoA hydrolysis.
**CHAPTER 4. PROBING THE POSITIONAL EQUILIBRIUM OF THE CARRIER DOMAIN OF PYRUVATE CARBOXYLASE USING SITE SPECIFIC CROSSLINKING**

**Introduction**

The catalytic function of PC requires a large, dynamic conformational change involving the translocation of the carrier domain from the BC domain to the CT domain. This conformational change is necessary to transfer the reaction intermediate, a carboxyl group, from where bicarbonate is bound in the BC domain active site to where pyruvate is bound in the CT domain active site. A major question in enzymology centers on how dynamic conformational changes occur and how they are regulated. Two models have traditionally been used to describe the relationship between conformational change and ligand binding: induced fit and conformational selection. These models are not exclusive and can exist in a linear or linked fashion, where multiple conformations exist in the absence of ligand, but ligand induces a specific conformational change, as explained in the Introduction (Redhair & Atkins, 2021). Swinging domain enzymes can be incredibly complex with up to 60 subunits, like type I polyketide synthases, so it is practical to investigate this question in a simpler swinging domain enzyme, such as PC, that has only 4 subunits (Herbst et al., 2018). Kinetics data does not directly report on conformational changes, because the rate limiting step is often not the conformational change. The only way to directly observe conformational changes is to use biophysical tools.

Two models that are typically used describe the relationship between conformational change and ligand binding are induced fit and conformational selection. Previously the conformational change of the carrier domain translocating across the face
of the tetramer, was described as following an induced fit model of conformational change (Westerhold et al., 2016). For example, detailed studies of carboxybiotin decarboxylation in sheep liver PC and chicken liver PC were performed in the presence of various CT domain ligands and were interpreted based on an induced fit model. Kinetic studies that focused on pyruvate binding in the CT domain concluded that substrates and cofactors mediate communication between subunits by inducing conformational changes through the enzyme (Attwood & Wallace, 1986; Easterbrook-Smith et al., 1979; Goodall et al., 1981; Wallace et al., 1985; Zeczycki et al., 2009). While these interpretations are reasonable, kinetic studies cannot directly observe carrier domain positioning and are limited to observations that conflate ligand binding, catalytic turnover, and carrier domain translocation. Thus, while many studies on PC have been interpreted using an induced fit model, these studies have not directly assessed which models.

To clearly address this question, biophysical tools must be used. In the context of PC induced fit would imply that pyruvate would need to be bound in the CT in order for the carrier domain to translocate to the CT domain (Figure 4-1, top panel). Conformational selection would imply that in the absence of ligand the carrier domain can sample multiple conformations, but the presence of ligands and effector molecules can shift the conformational equilibrium to favor a certain conformation (Figure 4-1, bottom panel).
A previous study from our research group demonstrated that carrier domain translocation pathways are modified in the presence of allosteric activator, acetyl-CoA (Liu et al., 2018a). Using systematically inactivated tetramers it was shown that acetyl-CoA only activates one carrier domain pathway, which led to the conclusion that one of the ways in which acetyl-CoA allosterically activates PC is by regulating carrier domain movement. Another indication that acetyl-CoA allosteric activation alters carrier domain movement is the fact that acetyl-CoA couples the BC and CT domain half reactions. Acetyl-CoA reduces abortive ATP cleavage which can be interpreted as coupling the BC domain half reaction to the CT domain half reaction because the reaction intermediate is being transferred and the ATP cleavage results in pyruvate carboxylation (Legge et al., 1996). The fact that acetyl-CoA couples the two half reactions together can indicate that

Figure 4-1. Induced fit and conformational selection in the context of PC. The top panel, outlined in red, represents induced fit. $L_1$ represents BC domain substrates, and $L_2$ represents CT domain substrates. The bottom panel, outlined in green, represents conformational selection. The main difference in the two models is that in conformational selection the conformational change precedes $L_2$ binding.
acetyl-CoA is influencing the translocation of the carrier domain because the carboxyl group is being transferred via carrier domain movement.

The aromatic residue, tryptophan, emits an intrinsic fluorescence signal that is sensitive to changes in the microenvironment. Changes in the fluorescence intensity or peak fluorescence of tryptophan can, therefore, be used to probe protein conformational change. Fortuitously, SaPC only has 5 tryptophan residues in the entire enzyme, and they are all located in the CT domain. This enables the use of intrinsic tryptophan fluorescence (ITF) to observe conformational changes in PC. In the studies outlined below, ITF was used in combination with site specific crosslinking to probe the rate of carrier domain translocation. Gln<sub>891</sub>, located the CT domain, and Asn<sub>1102</sub>, located on the carrier domain, were both mutated to cysteines (Figure 4-2). A homobifunctional crosslinker, bismaleimidoethane (BMOE), was used to crosslink these two cysteines when they are in close proximity (8 Å) (Figure 4-3A). The resulting Q891C/N1102C SaPC crosslinking trap captures the carrier domain when it is bound in the CT domain exo site. When the carrier domain is crosslinked, the microenvironment of the tryptophan residues in the CT domain becomes more hydrophobic, which causes an increase in fluorescence (Figure 4-3C). The fluorescence signal was observed using stopped-flow spectroscopy, with tryptophan excitation at 295 nm and emission observed at 320-350 nm. The fluorescence signal only occurred when crosslinker was present and was dependent on the presence of the biotin cofactor that is attached to the carrier domain (Hakala and Laseke et al., 2022). The single cysteine mutants, Q891C SaPC and N1102C SaPC, did not form crosslinks or have a fluorescence signal when mixed with BMOE (Figure 4-3D).
In preliminary studies, it was demonstrated that the carrier domain was crosslinked in the CT domain, even in the absence of CT domain substrates, indicating that the carrier domain samples multiple conformations in the absence of substrates. This is consistent with the conformational selection model. To follow on these initial studies, I sought to investigate the relationship between substrate concentration and rate of carrier domain translocation. In order to accomplish this, we needed substrate concentration dependent crosslinking experiments so that the change in observed rate constant could be correlated to the change in ligand concentration. Correlating carrier domain movement to ligand concentration can help definitively determine the mechanism of conformational change.

Figure 4-2. The Q891C/N1102C SaPC exo crosslinking trap. The complete tetramer is shown on the left with a zoomed in molecular view of the crosslinked residues on the right. The dashed line represents the crosslinked residues after they have been mutated to cysteines. The CT domain active site and exo site are indicated by dashed circles. Tryptophan residues are shown as dark yellow spheres.
Figure 4-3. ITF and site specific crosslinking were used to probe carrier domain translocation. A. A structure of homobifunctional crosslinker BMOE. B. A schematic representation of the Q891C/N1102C SaPC crosslinking system. The black bar represents the crosslinker and the yellow glow represents the fluorescence signal. This figure was initially generated by Anya Koza. C. The normalized fluorescence data was plotted on a linear scale of 0.175 mg/mL Q891C/N1102C SaPC crosslinked with 200 µM BMOE (red) and 0 µM BMOE (black). D. An 8% acrylamide SDS-PAGE gel of SaPC mutants (1 mg/mL) reacted with increasing amounts of BMOE (0, 10, 300, and 1000 µM) for 15 min. The single mutants did not crosslink and double cysteine mutants did crosslink.
Materials and Methods

Materials

Acetyl-CoA was purchased from CoALA Biosciences (Austin, TX). Except where noted, all other chemicals and materials were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific Inc. (Pittsburgh, PA), or Research Products International Corp. (Mount Prospect, IL). All DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA).

SaPC Constructs

Pyruvate carboxylase from S. aureus (SaPC; UniProtKB - A0A0E8G8A7) was previously cloned into a modified pET-27b vector and was generously supplied by Dr. Liang Tong, Columbia University (Yu et al., 2009). To reclone SaPC into the pTXB1 vector (New England Biolabs, Ipswich, MA), the gene encoding SaPC in pET-27b was PCR amplified using forward (5' - GCC ATA TGA AAC AAA TAA AAA AG -3') and reverse primers (5' - CGT GAT GCA GTT AGT TGC TTT TTC AAT TTC G -3'). The PCR amplicon was subjected to restriction digestion with NdeI and SapI prior to ligation into a gel-purified pTXB1 vector digested with NdeI and SapI. WT SaPC pTXB1 was fully sequenced.

The full sequence of SaPC pTXB1 revealed a PCR-introduced G571S mutation. The mutation was corrected back to a glycine at residue 571 by mutagenesis using the whole-plasmid PCR technique according to the Quikchange II mutagenesis protocol from Agilent Technologies, Inc. (Santa Clara, CA), with forward primer 5’ - GCG GAC GTA TTT AAA GAT GGT TTC TCA CTA G -3’ and reverse primer 5’ - CTA GTG AGA
AAC CAT CTT TAA ATA CGT CCG C -3’. The complete gene sequence of WT SaPC in the pTXB1 vector was confirmed by DNA sequencing.

**SaPC pET-27b Expression and Purification**

SaPC pET-27b clones were co-transformed and co-expressed with the pCY216 vector encoding *Escherichia coli* biotin protein ligase A (BirA) to ensure complete biotinylation of PC. Transformed *E. coli* BL21 (DE3) cells were cultured in M9 minimal media with 25 µg/mL kanamycin and 30 µg/mL chloramphenicol at 37 °C to an optical density (OD₆₀₀) of 0.8 - 1.0, after which protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and L-arabinose to a final concentration of 0.5 mM and 25 mM, respectively. The culture was also supplemented with D-(+)-biotin at a final concentration of 3 mg/L. Induced cells were incubated at 16 °C for 16 - 24 hours before harvesting by centrifugation.

SaPC pET27-b enzymes were purified using sequential Ni²⁺-affinity and anion exchange chromatography. Harvested cell paste (20 – 30 g) was re-suspended in 200 mL lysis buffer (20 mM Tris-HCl pH 7.8; 200 mM NaCl; 0.5 mM EGTA; 5 mM imidazole; 6 mM β-mercaptoethanol; 1 mM PMSF; 1 μM pepstatin A; and 5 μM E-64). Cells were lysed by sonication for 10 minutes at a temperature between 4-10 °C and pelleted by centrifugation. The supernatant was loaded on a 5 mL Ni²⁺-nitrilotriacetic acid proximity resin column (Bio-Rad, Hercules, CA). The column was washed with 12× column volume of wash buffer (20 mM Tris-HCl pH 7.8; 200 mM NaCl; 0.5 mM EGTA; 20 mM imidazole; 6 mM β-mercaptoethanol) and the protein was eluted with a gradient from 20 mM to 250 mM imidazole using wash buffer and elution buffer (20 mM Tris-HCl pH 7.8; 200 mM NaCl; 0.5 mM EGTA; 250 mM imidazole; 6 mM β-mercaptoethanol). Purified
protein was pooled and dialyzed against a buffer compatible with anion-exchange chromatography (20 mM triethanolamine, pH 8.0; 50 mM NaCl; 1 mM EGTA; and 2 mM dithiothreitol (DTT)) at 4 °C overnight. The dialyzed protein was loaded onto a 10 mL Q-Sepharose Fast Flow resin column (GE Healthcare), washed with 10× column volumes of dialysis buffer and eluted from the column in dialysis buffer with a gradient from 50 mM to 1 M NaCl. SaPC typically elutes between 400-800 mM NaCl. The purified protein was pooled and dialyzed against storage buffer (20 mM Bis-Tris Propane, pH 7.2; 15 mM NaCl; 10 mM MgCl₂; and 1 mM TCEP) for three successive changes of 4 hours or more. The protein was concentrated to a final concentration of 4 mg/mL − 10 mg/mL and was drop frozen in liquid nitrogen prior to storage at -80 °C. All protein concentrations were determined using the predicted extinction coefficient corresponding to absorbance at 280 nm.

**SaPC Crosslinking SDS-PAGE**

BMOE was prepared in 100% dimethyl sulfoxide (DMSO) at 6 mM and then diluted to 1 mM in buffer containing 0.1 M Tris-HCl (pH 7.8), 7 mM MgCl₂, and 0.1 M KCl. BMOE was then diluted to a final concentration of 200 μM in the crosslinking reaction. SaPC, in a storage buffer that consisted of 20 mM bis-tris propane pH 7.2, 15 mM NaCl, 10 mM MgCl₂, and 1 mM TCEP, was reacted at room temperature at a final concentration of 1 mg/mL (premixed with substrates for a minimum of 10 minutes when applicable) in the same buffer with the desired concentration of substrate, when applicable. After a defined incubation period, dithiothreitol (DTT) was used to quench the crosslinking reaction at a final concentration of 13 mM for 15 minutes at room temperature. Samples were then mixed with 4× Laemmli Buffer in reducing conditions
and heated to 100 °C for 5 minutes for SDS-PAGE analysis using an 8% polyacrylamide gel. Protein molecular weights were estimated using the Spectra Multicolor High Range Protein Ladder (Thermo Scientific, Waltham, MA). Coloration was achieved with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA).

**Stopped-Flow Spectroscopy**

Stopped-flow spectroscopy was performed using an Applied Photophysics SX20 instrument. Intrinsic tryptophan fluorescence was observed at an excitation wavelength of 295 nm using a 350 nm or 320 nm emission filter. SaPC (0.35 mg/mL) was premixed with substrates for a minimum of 10 minutes when applicable and was loaded in one drive syringe and mixed 1:1 with the substrate/BMOE mixture in the second drive syringe. All solutions were prepared in a buffer containing 0.1 M Tris-HCl (pH 7.8), 0.1 M KCl, and 7 mM MgCl₂. All data are the average of 3-5 replicates. Reported rates are the average value of three independent acquisitions of these replicates. The data was normalized to enable comparative visualization, the normalization was performed to plot the fractional change in intensity: the measured intensity at every time point was divided by the initial measured intensity at $t = 0$. Data were fit to a double exponential burst (Equation 4-1) where $a_1$, and $a_2$ represent the amplitudes, $k_{obs1}$ and $k_{obs2}$ represent the observed rates, $t$ represents time in seconds, $c$ represents the slope of the linear phase, and $a_0$ represents the initial value at $t = 0$. The values for $k_{obs1}$ and $k_{obs2}$ were replotted against the concentration of ADP+PPA and fit to Equation 4-2, which describes an inverse rectangular hyperbola where $k_{max}$ is the observed rate constant in the absence of ligand, $k_{min}$ is the limiting value for the observed rate constant at saturating ligand, and $K_{0.5}$ is the concentration of ligand at which $k_{obs} = (k_{min} + k_{max})/2$. 
When performing stopped-flow spectroscopy experiments with fluorescent crosslinker bBBr, the excitation was 350 nm and emission was observed with the cutoff filter GG435 so that only fluorescence from the crosslinker is observed and ITF (emission at ~320 nm) is excluded.

**Kintek Explorer Simulation and Curve Fitting**

Microscopic rate constants were estimated using Kintek Explorer v10.2.0. Raw data were imported as a concentration series with an 8-step kinetic scheme, with the observable indicated in Equation 4-3, where $a$ and $b$ are scaling factors and $F = \text{BCCP-CT}$ and $FX = \text{BCCP-CT-X}$.

$$k_{obs} = \frac{(k_{\text{min}} - k_{\text{max}})[L]}{K_{0.5} + [L]} + k_{\text{max}}$$

**Enzymatic Activity Assay**

Pyruvate carboxylation activity was measured spectrophotometrically at 340 nm by following the conversion of oxaloacetate to malate using the coupled enzyme, malate dehydrogenase (MDH). This was done in a 96-well plate with 200 µl reaction volumes, using a multi-well plate reader with the reactions initiated by adding the reaction substrates. Reactions were performed in a buffer containing 0.1 M Tris-HCl (pH 7.8), 0.1
M KCl, and 7 mM MgCl₂. All substrates and coupling reagents were prepared as a 10× stock solution that provided final reaction concentrations of 25 mM NaHCO₃, 2.5 mM ATP, 0.25 mM acetyl-CoA, 12 mM sodium pyruvate, 0.12 mM NADH, and 10 U/mL MDH. All measurements were performed in triplicate and the errors are reported as the standard deviation. The final PC concentration in the assay ranged from 2.5 - 10 μg/mL per reaction. Data were fit to Equation 4-4, which describes allosteric activation varying the concentration of activating compound. $k_{\text{cat}}$ is the catalytic rate constant, $k_{\text{cat}}^o$ is the catalytic rate constant in the absence of activator, $k_{\text{cat}}^{\text{app}}$ is the apparent catalytic rate constant at each concentration of compound, $K_a$ is the activation constant, $h$ is the Hill coefficient and $[A]$ is the concentration of activator.

Equation 4-4

$$k_{\text{cat}}^{\text{app}} = \frac{k_{\text{cat}}^o + k_{\text{cat}} [A]^h}{K_a^h + [A]^h}$$

The G938C/N1102C SaPC acetyl-CoA titration data that exhibited inhibition at high acetyl-CoA concentrations were fit to Equation 4-5, which describes allosteric activation and subsequent inhibition at high concentrations where $[A]$ is the concentration of acetyl-CoA, $K_a$ is the activation constant, and $h$ is the Hill coefficient.

Equation 4-5

$$k_{\text{cat}}^{\text{app}} = \frac{k_{\text{cat}}^o + k_{\text{cat}} \left( \frac{[A]}{K_a} \right)^h}{1 + \left( \frac{[A]}{K_a} \right)^h + \left( \frac{[A]}{K_i} \right)^h}$$
**SaPC Kinetics of Inactivation Crosslinking Assay**

1 mg/mL SaPC dilute in a buffer containing 0.1 M Tris-HCl (pH 7.8), 7 mM MgCl$_2$, and 0.1 M KCl. BMOE was prepared in 100% dimethyl sulfoxide (DMSO) at 6 mM and then diluted to 1mM in the same buffer to 1 mM and added to the crosslinking reaction at a final concentration of 200 μM. After a defined incubation period, dithiothreitol (DTT) was used to quench the crosslinking reaction at a final concentration of 13 mM for 15 minutes at room temperature. Samples were then assessed via MDH coupled assay, monitoring pyruvate carboxylation activity, as described in the Materials and Methods. Kinetics of inactivation assays have the $k_{cat}$, the turnover rate, divided by the $k_{cat}$, the turnover rate when crosslinked for 0 seconds, $k_{cat}/k_{cat}$ plotted on the y-axis, as a function of time crosslinked in seconds on the x-axis. Kinetics of inactivation data that does not exhibit recovery of inactivation is fit to Equation 4-6, where $a$ represents the $k_{cat}/k_{cat}$, or the turnover rate when crosslinked for 0 seconds divided by itself, $b$ represents the plateau or $k_{cat}/k_{cat}$ at infinite time, $k_{obs}$ is the observed rate constant of inactivation, and $t$ is the time in seconds.

Equation 4-6

$$y = (a - b) \cdot e^{-k_{obs}t} + b$$

Kinetics of inactivation data that exhibited recovery of inactivation was fit to Equation 4-7, without the 0 second data point, which describes a reciprocal biphasic exponential equation.

Equation 4-7

$$Y = \frac{1}{((a \cdot log(x + 1)^c) \cdot (exp(b \cdot log(x + 1))))}$$
The slope of recovery was calculated in the G938C/N1102C SaPC kinetics of inactivation data in the presence of acetyl-CoA, by calculating the slope using Equation 4-8 with the data point at the minimum $k_{i\text{cat}}/k_{c\text{at}}$ and the maximum $k_{i\text{cat}}/k_{c\text{at}}$ at 1200 seconds, therefore $x_2$ is always 1200.

\[
\text{slope of recovery} = \frac{y_2 - y_1}{x_2 - x_1}
\]

The rate of the approach to minimum $k_{i\text{cat}}/k_{c\text{at}}$ was determined by solving the first derivative of Equation 4-7, to give Equation 4-9 and setting the derivative equal to zero and solving for $x$, to give Equation 4-10. Constants $b$ and $c$ were then used to calculate the time, $x$, to achieve a minimum value (where the slope is equal to zero). The observed rate constant was estimated from the reciprocal of this value.

\[
\frac{dy}{dt} = \frac{(x + 1)^{-b} \log^{-c}(x + 1)}{a}
\]

\[
x = \exp^{-c/b} - 1
\]

**Results**

*Carrier Domain Translocation is Dependent on BC Domain Substrate Concentration*

A substrate titration experiment was performed to determine whether carrier domain translocation correlates with substrate concentration. ITF signals are often very sensitive, which is beneficial when observing conformational changes of an enzyme. A drawback of this technique is that compounds present in the sample can quench and
interfere with the signal, confounding the data interpretation (Ghisaidoobe & Chung, 2014). The allosteric activator, acetyl-CoA, and CT domain substrate, pyruvate, both substantially diminish the ITF signal (Figure 4-4 green and red traces). As a consequence, the direct influence of pyruvate and acetyl-CoA on the carrier domain equilibration was not further investigated using this technique.

Figure 4-4. The ITF signal with various substrates and effector molecules of PC. Q891C/N1102C SaPC was crosslinked in the presence of no substrates (yellow), 5 mM ADP+PPA (blue), 0.25 mM acetyl-CoA (green), and 12 mM pyruvate (red). The data was fit to Equation 4-1.
The combination of adenosine diphosphate (ADP) and phosphonoacetate (PPA) serves to mimic the carboxyphosphate intermediate that is formed in the BC domain reaction. The combination of ADP+PPA have been crystallized with RePC and they were observed to bind in the exact same location as the BC domain substrate, ATP (Lietzan et al., 2011) (Figure 4-5A). Notably, the combination of ADP+PPA did not diminish the

Figure 4-5. ADP+PPA titration in Q891C/N1102C SaPC. A. ADP+PPA (cyan) and ATP (white) bound in the BC domain active site. An overlay of ADP+PPA from RePC (PDB ID 3TW6) and ATP from SaPC (PDB ID 3BG5). B. The raw fluorescence data of Q891C/N1102C SaPC in the presence of 0 mM (red), 0.5 mM (lime green), 1 mM (blue), 1.5 mM (yellow), 2 mM (cyan), 2.5 mM (pink), 3 mM (dark green), and 3.5 mM (purple) ADP+PPA. The data was fit to Equation 4-1. C. The $k_{obs1}$ values were replotted as a function of ADP+PPA concentration. D. The $k_{obs2}$ values were replotted as a function of ADP+PPA concentration. The error bars in panels C and D represent the standard errors calculated from iterative non-linear curve fitting in Graphpad Prism. The data from panels C and D were fit to Equation 4-2.
ITF signal (Figure 4-4). Therefore, ADP+PPA was used to investigate the impact of ligand concentration on carrier domain equilibration.

ADP+PPA were preincubated with Q891C/N1102C SaPC prior to crosslinking to equilibrate the enzyme population. The crosslinker was also preincubated with an identical concentration of ADP+PPA. This ensured that, when the enzyme and crosslinker were mixed, there was no dilution of the ligand. After a 10 minute preincubation period, the evolution of ITF signal intensity was observed by mixing the enzyme with crosslinker in a stopped-flow instrument. The raw fluorescence data was fit to Equation 4-1 to determine the $k_{obs}$ values. Both $k_{obs1}$ and $k_{obs2}$ are dependent on the concentration of ADP+PPA (Figure 4-6C and D). $k_{obs1}$ represents a faster phase while $k_{obs2}$ represents a slower phase. Unfortunately, the rate is too fast to accurately determine $k_{obs1}$ rate constants at ADP and PPA concentrations less than 0.5 mM. Nevertheless, the trends are clear: both $k_{obs1}$ and $k_{obs2}$, when plotted against ADP+PPA concentration, are described by an inverse rectangular hyperbola (Equation 4-2). Replotting the $k_{obs}$ values as a function of ADP+PPA concentration allows the global rate constants $k_{min}$, $k_{max}$ and $K_{0.5}$ to be extracted. The $k_{min}$ is representative of the minimum rate of re-equilibration of the carrier domain or the residual crosslinking of the carrier domain in the presence of saturating BC domain substrates. The $k_{max}$ is representative of the fastest rate of re-equilibration of the carrier domain. The $K_{0.5}$ is a measure of the binding affinity of the combined substrates, ADP+PPA. A replot of $k_{obs1}$ resulted in a $K_{0.5} = 0.52 \pm 0.16$ mM with a $k_{min} = -0.039 \pm 0.091$ s$^{-1}$. Since a $k_{min}$ value less than zero is nonsensical, this reflects the difficulty in accurately determining the values of $k_{obs1}$, particularly at low concentrations of ADP+PPA. The replot of the slower $k_{obs2}$ resulted in a $K_{0.5} = 0.11 \pm$
0.01 mM and a $k_{\text{min}} = 0.013 \pm 0.001 \text{ s}^{-1}$ (Table 4-1). This titration experiment demonstrated that both $k_{\text{obs}1}$ and $k_{\text{obs}2}$ are saturable and that $k_{\text{obs}2}$ approaches a non-zero minimum value. Since $k_{\text{obs}2}$ is a function of carrier domain translocation and the $k_{\text{min}}$ value is greater than zero, this indicates that the carrier domain can translocate to the CT domain, even when the BC domain is saturated with ligands. This is conclusive evidence in favor of the carrier domain following a conformational selection model in SaPC.

$k_{\text{obs}1}$ and $k_{\text{obs}2}$ are both Functions of Crosslinking

The BC domain substrates, ADP+PPA, are pre-incubated with both the enzyme and the crosslinker prior to mixing and observing the ITF signal in the stopped-flow instrument. Consequently, $k_{\text{obs}1}$ nor $k_{\text{obs}2}$ are expected to represent kinetic steps in crosslinking rather than ligand binding. To further attribute the $k_{\text{obs}}$ values to crosslinking, control experiments were performed using WT SaPC, which lacks any cross-linkable cysteine residues.
The fast phase, $k_{obs1}$, occurs on a short timescale and appears as a small decrease in fluorescence (Figure 4-6A). The fast $k_{obs1}$ value likely represents the initial intersubunit capture of the carrier domain. The slower phase, $k_{obs2}$, appears as an increase in fluorescence intensity, and likely represents a slower transition to a lower energy cross-linked state (Figure 4-6A). To confirm that $k_{obs1}$ and $k_{obs2}$ are not a function of the dilution of substrates, protein or crosslinker upon mixing, WT SaPC was mixed with buffer and the fluorescence signal was observed in the stopped-flow instrument (Figure 4-6B and C). WT SaPC was pre-incubated with 0.5, 1.5 and 3 mM ADP+PPA and then mixed with equal concentrations of ADP+PPA. The fluorescence intensity at 350 nm was unchanged as a function of time (Figure 4-6B), confirming that the fluorescence intensity was not simply increasing as a function of increasing ADP+PPA concentration, and that fluorescence intensity signal observed in the Q891C/N1102C SaPC titrations were a true

Figure 4-6. WT SaPC controls for $k_{obs1}$ and $k_{obs2}$. A. A representative trace for Q891C/N1102C SaPC crosslinked ITF signal, shown on a logarithmic scale. $k_{obs1}$ and $k_{obs2}$ components indicated by brackets. B. Fluorescence signal for WT SaPC vs buffer (black), WT SaPC incubated with 0.5 mM ADP+PPA vs 0.5 mM ADP+PPA (blue), WT SaPC incubated with 1.5 mM ADP+PPA vs 1.5 mM ADP+PPA (red), and WT SaPC incubated with 3 mM ADP+PPA vs 3 mM ADP+PPA (green). C. WT SaPC vs buffer (black), WT SaPC incubated with 200 µM BMOE and 3 mM ADP+PPA vs 3 mM ADP+PPA (blue), and WT SaPC incubated with 3 mM ADP+PPA vs WT SaPC incubated with 3 mM ADP+PPA (red).
function of crosslinking. Additionally, WT \textit{Sa}PC was pre-incubated with 200µM BMOE and 3 mM ADP+PPA and mixed with 3 mM ADP+PPA. No change in the fluorescence intensity at 350 nm was observed (Figure 4-6B). Therefore, neither $k_{\text{obs}1}$ nor $k_{\text{obs}2}$ result from the dilution of crosslinker upon mixing. WT \textit{Sa}PC was pre-incubated with 3 mM ADP+PPA and mixed with WT \textit{Sa}PC pre-incubated with 3 mM ADP+PPA. No change in the fluorescence intensity at 350 nm was observed, therefore $k_{\text{obs}1}$ and $k_{\text{obs}2}$ are not due to a dilution in protein (Figure 4-6C). Together these crosslinking experiments using WT \textit{Sa}PC indicate that neither $k_{\text{obs}1}$ and $k_{\text{obs}2}$ are due to dilution of the ligands, protein or crosslinker, and that both $k_{\text{obs}1}$ and $k_{\text{obs}2}$ are a function of crosslinking.

**R346M Mutation Perturbs Carrier Domain Equilibrium**

The $k_{\text{min}}$ value determined from the replot of $k_{\text{obs}2}$ versus ADP+PPA concentration represents the residual crosslinking of the carrier domain in the presence of saturating BC domain ligands. We predicted that a mutation with altered carrier domain equilibrium would lower the $k_{\text{min}}$ value even further and analyzed the impact of a mutation in the BC domain residue, Arg\textsubscript{346}, had on the rate of carrier domain equilibration. This arginine is proposed to serve as a “gate-keeper” for the BC domain active site (Zeczycki et al., 2011). After the biotin carboxylation in the BC domain active site, Arg\textsubscript{346} is repositioned to form a salt bridge with Glu\textsubscript{243}. This rearrangement serves to close off the active site and prevent the re-entry and abortive decarboxylation of carboxybiotin (Figure 4-7A). This ensures the efficient coupling of the reactions in the BC and CT domains by promoting the disassociation of the carrier domain from the BC domain, with a resulting shift in the carrier domain equilibrium position to favor the CT domain. When Arg\textsubscript{346} is mutated to methionine, this “gate” no longer closes, permitting the biotin cofactor to remain in the
BC domain. Compared to the WT enzyme, the R346M mutation is predicted to shift the equilibrium position away from the CT domain as a consequence of the enhanced BC-BCCP domain interaction (Zeczycki et al., 2011). Consistent with these predictions, the R346M mutation was shown to have an increased rate of ADP phosphorylation relative to the WT enzyme (Zeczycki et al., 2011). Similarly, when the R346M mutation was added to the Q891C/N1102C SaPC background, the ADP phosphorylation rate also increased relative to the WT enzyme (Table 4-1).

**Table 4-1.** Macroscopic rate constants determined for Q891C/N1102C SaPC and R346M/Q891C/N1102C SaPC.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pyruvate carboxylation</th>
<th>ATP formation</th>
<th>Replot of $k_{obs}$ vs. [ADP + PPA]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{max}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>WT RePC</td>
<td></td>
<td>2.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>R353M RePC</td>
<td></td>
<td>24.9 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Q891C/N1102C SaPC</td>
<td>8.4 ± 0.1</td>
<td>0.46 ± 0.01</td>
<td>0.081 ± 0.02</td>
</tr>
<tr>
<td>R346M/Q891C/N1102C SaPC</td>
<td>0.18 ± 0.01</td>
<td>0.69 ± 0.01</td>
<td>0.056 ± 0.004</td>
</tr>
</tbody>
</table>

*WT RePC and R535M RePC values from Zeczycki et. al. 2011. Residue 353 in RePC is equivalent to residue 346 in SaPC.
The $k_{\text{obs}2}$ values plotted as a function of ADP+PPA in the R346M/Q891C/N1102C SaPC construct resulted in a smaller value for $k_{\text{min}}$ compared to Q891C/N1102C SaPC (Figure 4-7B). This decrease in $k_{\text{min}}$ is consistent with a shift in the carrier domain positional equilibrium towards the BC domain because the rate of re-equilibration to the CT domain at saturating ligand concentrations is reduced relative to Q891C/N1102C SaPC (Table 4-1). This agrees with the shift in carrier domain equilibrium, towards the BC domain, that was shown kinetically, through the increased rate of ADP phosphorylation (Zeczycki et al., 2011). The lower $k_{\text{min}}$ value, in a system with an equilibrium of the carrier domain shifted towards the BC domain, validates our
crosslinking system and shows that it can be used to report on relative changes in carrier
domain equilibrium.

**Simulated Experiment Predicts Microscopic Rate Constants of Crosslinking Reaction**

The global rate constants, $k_{\text{obs}1}$ and $k_{\text{obs}2}$, are macroscopic collections of rate
constants. They cannot be used to directly determine the individual rates of carrier
domain translocation. To gain a preliminary estimate of the individual microscopic steps,
an 8-step kinetic reaction scheme was developed that can adequately describe the
experimental observations (Figure 4-8A). These 16 microscopic rate constants represent
the individual kinetic steps that contribute to the global rate constants, $k_{\text{obs}1}$ and $k_{\text{obs}2}$
(Hakala and Laseke et al., 2022). Kintek Explorer was used to fit the observed data to this
multi-step scheme (Johnson, 2009). The simulated data closely resembled the
experimental data (Figure 4-8A). Using Kintek’s FitSpace Explorer, the upper and lower
bounds for each predicted microscopic rate constant was determined to offer a
preliminary estimate for each microscopic rate constant (Johnson et al., 2009). Due to the
complexity of the system and the limited number of experimental observations, the
individual microscopic rate constants cannot be accurately predicted, with ranges that
span 20 to 100-fold in magnitude (Table 4-2). Although the microscopic rate constants
are not precise, they nevertheless offer reasonable insights into the slow and fast kinetic
steps associated with this system. From the initial estimates of these microscopic rate
constants we can conclude that carrier domain translocation from the BC to the CT
domain is much faster than the rate of crosslinking ($k_1$ vs $k_2$ Table 4-2). To fully map out
the conformational energy landscape of PC, additional biophysical studies need to be
conducted, trapping the carrier domain in additional sites to be able to accurately predict these microscopic rate constants.

**Table 4-2. Estimated Microscopic Rate Constants.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rate Constant</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>k₁</td>
<td>340 ± 10</td>
<td>190</td>
<td>3400</td>
</tr>
<tr>
<td>k₂</td>
<td>370 ± 10</td>
<td>210</td>
<td>3700</td>
</tr>
<tr>
<td>k₃</td>
<td>0.03 ± 0.01</td>
<td>0.0045</td>
<td>0.048</td>
</tr>
<tr>
<td>k₄</td>
<td>65 ± 10</td>
<td>0.065</td>
<td>650</td>
</tr>
<tr>
<td>k₅</td>
<td>15 ± 4</td>
<td>1.3</td>
<td>130</td>
</tr>
<tr>
<td>k₆</td>
<td>0.05 ± 0.01</td>
<td>0.0041</td>
<td>0.48</td>
</tr>
<tr>
<td>k₇</td>
<td>10 ± 2</td>
<td>0.12</td>
<td>480</td>
</tr>
<tr>
<td>k₈</td>
<td>37 ± 2</td>
<td>9.8</td>
<td>900</td>
</tr>
<tr>
<td>k₉</td>
<td>3000 ± 200</td>
<td>710</td>
<td>82000</td>
</tr>
<tr>
<td>k₁₀</td>
<td>540 ± 30</td>
<td>71</td>
<td>5400</td>
</tr>
<tr>
<td>k₁₁</td>
<td>1100 ± 200</td>
<td>1.1</td>
<td>11000</td>
</tr>
<tr>
<td>k₁₂</td>
<td>0.27 ± 0.03</td>
<td>0.023</td>
<td>2.7</td>
</tr>
<tr>
<td>k₁₃</td>
<td>0.52 ± 0.02</td>
<td>0.022</td>
<td>3.5</td>
</tr>
<tr>
<td>k₁₄</td>
<td>0.07 ± 0.01</td>
<td>0.015</td>
<td>0.49</td>
</tr>
</tbody>
</table>
The previous studies that utilized ITF and determined that carrier domain translocation is governed by conformational selection utilized a crosslinking trap that crosslinked the carrier domain in the CT exo site. The exo site is near the periphery of the CT domain close to the AD. The carrier domain binds in the CT active site when that

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**Figure 4-8. Kintek Explorer simulated experiment of ADP+PPA titration in Q891C/N1102C SaPC.**

A. A kinetic scheme of the simulated experiment. A represents ADP+PPA ligands and X represents BMOE crosslinker. BCCP* represents the final crosslinked species that has a linear decay in fluorescence signal. B. The simulated experiment (solid lines) overlaid on the fluorescence signals from the ADP+PPA titration: 0mM (red), 0.5mM (green), 1mM (blue), 1.5mM (yellow) 2mM (cyan), 2.5mM (pink), 3mM (dark green), 3.5mM (purple), 4mM (sky blue) and 4.5mM (orange).

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**G938C/N1102C SaPC Construct Crosslinks Carrier Domain in CT Active Site**

The previous studies that utilized ITF and determined that carrier domain translocation is governed by conformational selection utilized a crosslinking trap that crosslinked the carrier domain in the CT exo site. The exo site is near the periphery of the CT domain close to the AD. The carrier domain binds in the CT active site when that
subunit is participating in catalysis. To be able to probe differences in carrier domain translocation, when the carrier domain binds in the CT domain active site versus the CT domain exo site, another double cysteine mutant, that can trap the carrier domain when it is bound in the active site, needed to be generated. A previous structure of SaPC with the carrier domain bound in the active site, PDB ID 3BG5, was used to design the crosslinking trap. Gly938, in the CT domain was chosen to be utilized in the crosslinking trap due to its proximity to Asn1102, a residue on the carrier domain that had already been mutated to a cysteine in the exo binding site crosslinking trap. When the carrier domain is bound in the CT exo site, Gly938 and Asn1102 are ~25 Å apart, which is a distance too far to facilitate crosslinking. When the carrier domain is bound in the CT domain active site, Gly938 and Asn1102 are ~5 Å apart, which is an ideal distance for a crosslink trap (Figure 4-10). Gly938 is not conserved in PC (Figure 4-9). This residue was selected for mutagenesis on the premise that altering this residue would be unlikely to significantly impact the enzyme activity. Consistent with this prediction, the G938C mutation retained 50% of the WT activity, which is very similar to what was observed for Q891C/N1102C SaPC (Table 4-3).
Figure 4-9. A Sequence alignment for residues on the surface V934-E940 helix-loop in SaPC. *Corynebacterium glutamicum* pyruvate carboxylase (CgPC), *Rhizobium etli* pyruvate carboxylase (RePC), *Homo sapiens* pyruvate carboxylase (HsPC), *Aspergillus nidulans* pyruvate carboxylase (AnPC), *Staphylococcus aureus* pyruvate carboxylase (SaPC) sequence alignment. The residue in each enzyme that corresponds to position 938 in SaPC is colored in red.

Figure 4-10. Design of the G938C/N1102C SaPC CT active site crosslinking trap. (PDB ID 3BG5) A. The carrier domain bound in the active site. The residues Gly938 and Asn1102 are shown in stick representation and have been mutated to cysteines and are labeled. The distance between residues is indicated by a dotted line. The active site in CT domain is labeled and indicated by dotted circle. Pyruvate and Mg$^{2+}$ are shown in the CT domain active site colored in magenta and purple, respectively. B. The carrier domain bound in the exo site. The residues Gly938 and Asn1102 are shown in stick representation and have been mutated to cysteines and are labeled. The distance between the residues is indicated by dotted line. The CT domain exo site is labeled and indicated by a dotted circle. Pyruvate and Mg$^{2+}$ are shown in the CT domain active site and are colored purple and green, respectively.
Table 4-3. Pyruvate carboxylation activity for SaPC and mutated constructs in the presence of acetyl-CoA.

<table>
<thead>
<tr>
<th>SaPC</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>% Compared to WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15 ± 2</td>
<td>-</td>
</tr>
<tr>
<td>Q891C</td>
<td>8.5 ± 0.8</td>
<td>57</td>
</tr>
<tr>
<td>G938C</td>
<td>8.4 ± 0.2</td>
<td>55</td>
</tr>
<tr>
<td>N1102C</td>
<td>11.5 ± 0.3</td>
<td>80</td>
</tr>
<tr>
<td>G938C/N1102C</td>
<td>7.8 ± 0.2</td>
<td>51</td>
</tr>
<tr>
<td>Q891C/N1102C</td>
<td>8.5 ± 0.1</td>
<td>57</td>
</tr>
</tbody>
</table>

Both a double cysteine mutant, G939C/N1102C SaPC, and a single cysteine mutant, G938C SaPC, were generated and confirmed by complete gene sequencing. The N1102C SaPC single cysteine mutant was generated previously by Dr. Joshua Hakala. The single and double cysteine mutants were reacted with crosslinker and evaluated through SDS-PAGE (Figure 4-11A). Similar to the Q891C/N1102C SaPC exo site trap, the G938C/N1102C SaPC active site trap was also crosslinked in the presence of multiple crosslinkers (Figure 4-11A and B). Crosslinkers of a wide variety of lengths were capable of crosslinking, ensuring that the observation of dimer formation on the SDS-PAGE gel was not dependent on the length of the crosslinker (Green et al., 2008). The single cysteine mutants, G938C SaPC and N1102C SaPC showed no dimer formation when crosslinked with 200 µM BMOE or 200 µM dibromobimane (bBBr) (Figure 4-11A). Both double cysteine mutant G938C/N1102C SaPC and single cysteine mutant G938C SaPC have a small fraction of crosslinked species at ~180 kDa when crosslinked with any crosslinker longer than 5 Å (Figure 4-11A and B). Both G938C/N1102C SaPC and G938C SaPC had a degradation product of ~90 kDa that appeared in the purification gels, and the crosslinked product at ~180 kDa could be the result of the degradation products being crosslinked (Figure 4-12A and B).
Figure 4-11. G938C/N1102C SaPC crosslinked with crosslinkers of various lengths. 
A. An 8% acrylamide SDS-PAGE gel with PC protein (1 mg/mL) reacted with 200 µM crosslinker. Q891C/N1102C SaPC (QN), G938C/N1102C SaPC (GN), G938C SaPC (G), and N1102C SaPC (N) were crosslinked with crosslinkers of various lengths. B. An 8% acrylamide SDS-PAGE gel with PC protein (1 mg/mL) reacted with 200 µM crosslinker. G938C SaPC (G) and G938C/N1102C SaPC (GN) were crosslinked with crosslinkers of various lengths. C. The structures of the crosslinkers Bismaleimidoethane (BMOE), 1,8-bismaleimido-diethyleneglycol (BM(PEG)₂), bismaleimidohexane (BMH), 1,4-bismaleimidobutane (BMB), and dibromobimane (bBBr), with their associated lengths denoted below. Monomeric SaPC is 129,370 kDa and dimeric SaPC is 258,740 kDa.
Stopped-Flow Fluorescence Signal SaPC is not a Function of Crosslinking

Mixing 0.175 mg/mL G938C/N1102C SaPC with 200 µM BMOE in a stopped-flow spectrometer resulted in a small decrease in ITF fluorescence intensity after excitation at 295 nm. This is in marked contrast to the increased ITF signal intensity observed in the Q891C/N1102C SaPC exo site trap (Figure 4-13A and B). Unfortunately, when G938C SaPC was mixed with 200 µM BMOE in a stopped-flow spectrometer, a decrease in fluorescence signal was observed. Therefore, this decrease in fluorescence is likely not a function of crosslinking, because G938C SaPC cannot be crosslinked and still has the same signal with just half the amplitude. The decrease in fluorescence signal is likely from the reaction between the side chain of the cysteine and the crosslinker.

Figure 4-12. The purification gels show a degradation product. A. A G938C/N1102C SaPC 8% acrylamide SDS-PAGE gel with samples from purification indicated at the top. Degradation product indicated with red arrow. B. A G938C SaPC 8% acrylamide SDS-PAGE gel with samples from purification indicated at the top. The degradation product indicated with red arrow. The nickel nitriloacetic acid (Ni-NTA) column samples were loaded in columns 4-7, and the Q-sepharose (Q-seph) column samples were loaded in columns 8-11. See the Materials and Methods for the full purification protocol.
Since ITF could not be used to observe crosslinking in G938C/N1102C SaPC, the use of a fluorescent crosslinker, dibromobimane (bBBr), was explored (Figure 4-12C). Dibromobimane is a shorter, more ridged crosslinker that emits fluorescence at ~480 nm when it crosslinks two cysteines (Green et al., 2008). Stopped-flow fluorescence experiments with bBBr were performed by setting excitation wavelength to 350 nm with a GG435 cutoff filter to observe the bBBr emission fluorescence at ~480 nm. This cutoff filter is designed to isolate the signal from bBBr by excluding the majority of the fluorescence signal coming from tryptophan, which has an emission wavelength of ~320 nm. We hoped that this fluorescent crosslinker would give a more robust change in signal intensity than the relatively weak ITF signal, whose intensity changed by only 0.2 units. Relative to Q891C/N1102C SaPC, G938C/N1102C SaPC had a greatly reduced signal intensity.

Figure 4-13 ITF in G938C/N1102C SaPC crosslinked with BMOE. A. The normalized fluorescence data plotted on a linear scale showing 0.175 mg/mL of Q891C/N1102C SaPC (blue), G938C SaPC (green) and G938C/N1102C SaPC (red) reacted with 200 µM BMOE. B. The same data from panel A was plotted and shown on a logarithmic scale.
intensity when crosslinked with bBBr. The signal resulting from the single mutant G938C SaPC was very similar to the double mutant G938C/N1102C SaPC.

G938C/N1102C SaPC, Q891C/N1102C SaPC and G938C SaPC stopped-flow fluorescence controls were conducted with monobromobimane (mBBr), which has only one reactive group, therefore will fluoresce when it reacts with cysteines but not crosslink the protein. Q891C/N1102C SaPC reacted with mBBr resulted in the same signal but with just half the amplitude, relative to when it was reacted with bBBr. This means that the fluorescence signal from bBBr is not a function of crosslinking but a result of the reactive group on the bimane compound. G938C/N1102C SaPC and G938C SaPC reacted with mBBr resulted in the same signal. We would expect G938C/N1102C SaPC and Q891C/N1102C SaPC reacted with mBBr to result in the same signal, but this was not seen, leading to the conclusion that the signal cannot report on crosslinking and/or carrier domain translocation.
Kinetics of Inactivation Reports on Crosslinking in the CT Active and Exo Site

In lieu of the stopped-flow fluorescence data to monitor crosslinking in the G938C/N1102C SaPC active site system not working, carrier domain equilibrium positioning was instead monitored using an established assay that monitors the kinetics of inactivation. When the carrier domain is crosslinked in either the active site or the exo site, the carrier domain is locked in place by the irreversible crosslinker. The inability of the crosslinked enzyme to catalyze intermediate transfer between active sites results in the inactivation of the enzyme. The rate of inactivation can be followed as a function time as the population of enzyme becomes increasingly crosslinked and inactivated.
The ultimate goal of these studies is to directly compare the traps at the exo binding site and the CT domain active site in order to gain insights into the PC catalytic cycle. The best comparison of these two systems requires a crosslinker that inactivates both systems on the same timescale. When the active site trap was crosslinked with BMOE, the rate of inactivation was almost instantaneous on the timescale of the experiment, precluding accurate measurements due to sample handling constraints (Figure 4-15A). When the active site trap was crosslinked with the smaller (~5 Å), more rigid crosslinker bBBr, the rate of inactivation could be observed on the timescale of the experiment (Figure 4-15A).

When the exo site trap was mixed with bBBr, very little inactivation was observed. This is consistent with the SDS-PAGE gels, which show less crosslinking of the exo site trap with bBBr than with the slightly longer (~8 Å) BMOE crosslinker (Figure 4-11A). When the exo site trap was mixed with BMOE, more dimer formation is observed (Figure 4-11) and there is a substantially higher degree of inactivation (Figure 4-15B). When the active site trap, G938C/N1102C SaPC, is crosslinked with bBBr, and the exo site trap, Q891C/N1102C SaPC, is crosslinked with BMOE, the rates of inactivation are comparable. Therefore, to gain interpretable and comparable data between the two systems, each system was crosslinked with the crosslinker that resulted in measurable inactivation on a comparable timescale. For both systems 30% of residual activity consistently remains, so the entire population of enzyme is never fully crosslinked, which is consistent with the SDS-PAGE gels that always show a small population of monomer.
Control experiments for the Q891C/N1102C SaPC, the exo site trap, were performed previously, demonstrating that the single mutants, Q891C SaPC and N1102C SaPC, were neither crosslinked nor inactivated by BMOE (Hakala and Laseke et al., 2022). Similar control experiments were conducted in G938C/N1102C SaPC, the active site trap, to ensure that G938C/N1102C SaPC was not crosslinked or inactivated by mBBr (Figure 4-16). Additionally, the single mutants, G938C SaPC and N1102C SaPC, were neither crosslinked nor inactivated by either bBBr or mBBr (Figure 4-11A and Figure 4-16C). To determine the concentration of bBBr that resulted in complete inactivation of G938C/N1102C SaPC, a titration with 0-500 µM bBBr was conducted. At 200 µM bBBr, the system achieves the maximum degree of inactivation, with no additional inactivation at concentrations of 200-500 µM bBBr (Figure 4-16D). Additionally, through SDS-PAGE analysis, the protein is crosslinked to its highest degree.
at 200 µM bBBr (Figure 4-16A). Both crosslinking and inactivation are complete after 2 minutes in the presence of 200 µM bBBr (Figure 4-16B and C).

Figure 4-16. G938C/N1102C SaPC crosslinking controls with bBBr and mBBr. A. An 8% acrylamide SDS-PAGE gel with 1 mg/mL G938C/N1102C SaPC reacted with 0, 10, 50 and 200 µM bBBr and mBBr. B. An 8% acrylamide SDS-PAGE gel 1 mg/mL G938C/N1102C SaPC reacted with 200 µM bBBr over a time course. All reactions were quenched with 13 mM DTT. C. A residual activity plot of 1 mg/mL G938C/N1102C SaPC (blue), G938C SaPC (green) and N1102C SaPC (orange) reacted with 200 µM bBBr (circles) or mBBr (squares) over a time course. The error bars represent the standard deviation of three independent measurements. D. A residual activity plot for bBBr titration with 1 mg/mL G938C/N1102C SaPC all reacted for 1 hour. The data was fit to Equation 4-6.
**Allosteric Effectors Influence Rate of Inactivation.** In order to determine the influence allosteric effectors had the positional equilibrium of the carrier domain, the two effector molecules were added to the crosslinking reaction and the rate of inactivation was monitored in both the active site and exo site trap. The allosteric inhibitor, L-aspartate, inhibits bicarbonate dependent ATP cleavage in the BC domain half reaction (Sirithanakorn et al., 2014). L-aspartate has no effect on the CT domain reaction, nor does it affect the coupling of the BC and CT domain reactions (Legge et al., 1996). The localization of L-aspartate inhibition to the BC domain suggests that it should have little impact on the positional equilibrium of the carrier domain and, consequently, little impact on the rate or degree of inactivation. In both the active site and exo site traps, the rate of inactivation decreased only slightly when 10 mM L-aspartate was added to the crosslinking reaction (Figure 4-17). For G939C/N1102C SaPC the $k_{obs}$ went from 0.016 ± 0.003 s$^{-1}$ to 0.007 ± 0.002 s$^{-1}$ in the absence and presence of L-aspartate, respectively. For Q891C/N1102C SaPC the $k_{obs}$ went from 0.012 ± 0.001 s$^{-1}$ to 0.009 ± 0.005 s$^{-1}$ in the absence and presence of L-aspartate, respectively.
Acetyl-CoA’s allosteric effect is much less localized than L-aspartate’s, and for this reason we did predict that acetyl-CoA would affect the rate of inactivation in both the active site and exo site systems. When acetyl-CoA is present the binding affinity for both BC domain substrates, bicarbonate and ATP, and CT domain substrate, pyruvate, increases, therefore it influences both catalytic domains (Westerhold et al., 2017). Acetyl-CoA also couples the BC and CT domain reactions, shown through decrease abortive ATP cleavage when it is present (Legge et al., 1996), which is indicative of influencing carrier domain motion. Prior to conducting kinetics of inactivation experiments in the presence of acetyl-CoA, the concentration at which acetyl-CoA is saturating, for both constructs, needed to be determined. This was conducted for acetyl-CoA and not L-aspartate because it is common for different constructs of PC to have variable $K_a$ values.

**Figure 4-17. Influence of allosteric inhibitor L-aspartate on rate of inactivation in active site and exo site traps.**

A. 1 mg/mL of G938C/N1102C SaPC incubated with 10 mM L-aspartate (red squares) and without (black circles) crosslinked with 200 µM bBBr. B. 1 mg/mL of Q891C/N1102C SaPC incubated with 10 mM L-aspartate (red squares) and without (black circles) crosslinked with µM BMOE. Both A and B were fit to Equation 4-6. The error bars represent the standard deviation of three independent measurements. When assaying in the presence of allosteric effectors, different amounts of protein need to be added to the assay due to inhibition/activation, therefore, $k_{i\text{cat}}/k_{\text{cat}}$ is plotted on the y-axis to control for amount of protein per reaction.
for acetyl-CoA, from 1 µM to 500 µM (Adina-Zada, Zeczycki, et al., 2012). The $K_i$ of L-aspartate is already in the mM range and the concentration of L-aspartate cannot be increased higher than 20 mM, due to enzyme stability in solution.

The rate of pyruvate carboxylation was determined as a function of acetyl-CoA concentrations for WT SaPC, G938C/N1102C SaPC, Q891C/N1102C SaPC, G938C SaPC and N1102C SaPC. Interestingly, the G938C/N1102C SaPC was inhibited at concentrations of acetyl-CoA greater than 250 µM (Figure 4-18A). This phenomenon was not observed in either of the single mutants, G938C SaPC or N1102C SaPC.

Therefore, I predicted that the loss of enzyme activity was a result of disulfide bond formation between the two cysteines at elevated concentrations of acetyl-CoA (Figure 4-18D). To test this prediction, TCEP was added as a reducing agent prior to the addition of acetyl-CoA to ensure that all cysteines remained fully reduced and to prevent the formation of any disulfide bonds. The addition of 100 µM TCEP eliminated the inhibition at high concentrations of acetyl-CoA. Both 100 µM TCEP and 10 mM TCEP were incubated with WT SaPC and G938C/N1102C SaPC to ensure the thiols were fully reduced and to control for any effect TCEP may have on the coupling enzyme for the pyruvate carboxylation assay. There was no difference when incubated with 100 µM or 10 mM TCEP (Figure 4-18C).

The fact that concentrations of acetyl-CoA greater than 250 µM cause the formation of disulfide bonds between G938C and N1102C indicates that the allosteric activator is promoting a conformation in which the V934-E940 helix-loop is shifted towards the carrier domain, bringing these two cysteines are within 3 Å of each other, but only when the concentration is above saturating levels (Figure 4-18D). This is only
occurring in G938C/N1102C SaPC, the active site trap, and not Q891C/N1102C SaPC, the exo site trap, indicating that no matter how much acetyl-CoA is present residues 891 and 1102 never come within 3 Å of each other and do not form disulfide bonds.

Figure 4-18. Acetyl-CoA titrated in active site and exo site systems. A. The pyruvate carboxylation reaction was monitored with increasing amounts of acetyl-CoA in Q891C/N1102C SaPC (blue), G938C/N1102C SaPC (red), G938C SaPC (green), N1102C SaPC (orange) and WT SaPC (black) while titrating acetyl-CoA. All data sets were fit to Equation 4-4, except G938C/N1102C SaPC was fit to Equation 4-5. B. The pyruvate carboxylation reaction was monitored with increasing amounts of acetyl-CoA in WT SaPC (black), G938C/N1102C SaPC (red) and G938C/N1102C SaPC with 100 µM TCEP (purple). C. The pyruvate carboxylation reaction was monitored with increasing amounts of acetyl-CoA in WT SaPC (black) and G938C/N1102C SaPC (red) with 10 mM TCEP (circles) and 100 µM TCEP (squares). Error bars represent the standard deviation of three independent measurements. D. PDB ID 3BG5. A representation of disulfide bond that forms in the presence of high concentrations of acetyl-CoA.
Having confirmed that 250 µM acetyl-CoA was saturating for both constructs, kinetics of inactivation experiments were conducted in the presence of saturating acetyl-CoA (250 µM). The rate of inactivation for Q891C/N1102C SaPC, the exo site system, increased when acetyl-CoA was present (Figure 4-19B). Surprisingly, when acetyl-CoA was added to the active site trap, an initial decrease in activity was seen followed by a gradual increase in activity after 2 minutes (Figure 4-19A). The recovery from inactivation increased with increasing concentrations of acetyl-CoA. This recovery in activity is thought to result from the slow remixing of partially crosslinked tetramers, where some proportion of the population reassorts in the presence of acetyl-CoA to increase the population of un-crosslinked, active tetramers. The activity recovers over 2 to 30 minutes, before slightly decreasing again beyond 30 minutes, a likely consequence of the newly formed tetramers becoming crosslinked and inactivated (Figure 4-20A). The presence of TCEP does not change the inactivation profile since recovery from inactivation was observed both in the presence and absence of TCEP (Figure 4-20B). The recovery from inactivation was not specific to the bBBbr crosslinker, since G938C/N1102C SaPC crosslinked with BMOE in the presence of acetyl-CoA also recovered activity over the same time period, albeit to a lesser degree (Figure 4-20C). This recovery from inactivation is observed only for the active site trap when acetyl-CoA is present, suggesting that acetyl-CoA specifically orders the system to free one face of the tetramer to dissociate, remix and reform a new, catalytically active tetramer.
Figure 4-19. Influence of allosteric activator acetyl-CoA on inactivation rate in active and exo site traps. A. 1 mg/mL of G938C/N1102C SaPC was crosslinked with 200 μM bBBr with 0 mM acetyl-CoA (circles), 0.01 μM acetyl-CoA (squares) and 1 mM acetyl-CoA (triangles) and the data was fit to Equation 4-7. B. 1 mg/mL of Q891C/N1102C SaPC was crosslinked with 200 μM BMOE with 0 mM acetyl-CoA (circles), 0.01 μM acetyl-CoA (squares) and 1 mM acetyl-CoA (triangles) and the data was fit to Equation 4-6. The error bars represent the standard deviation of 3 independent measurements.

Figure 4-20. Recovery of inactivation is observed when acetyl-CoA is present while crosslinking G938C/N1102C SaPC. A. 1 mg/mL G938C/N1102C SaPC crosslinked with 200 μM bBBr with 250 μM acetyl-CoA for a 1 hour time course. B. 1 mg/mL G938C/N1102C SaPC crosslinked with 200 μM bBBr with 250 μM acetyl-CoA without (circles) and with 100 μM TCEP (squares). C. 1 mg/mL G938C/N1102C SaPC crosslinked with 200 μM BMOE with 250 μM acetyl-CoA. Error bars represent the standard deviation of 3 independent measurements.
Inactivation kinetics were performed for G938C/N1102C SaPC at varying concentrations of acetyl-CoA and the data (not including the 0 second time point) were fit to a reciprocal biphasic exponential equation (Equation 4-7) (Figure 4-21). To determine the time it takes to reach the minimum \( \frac{k^{\text{cat}}}{k^{\text{cat}}} \), the first derivative of Equation 4-7 was taken (Equation 4-9) and the derivative was set to 0 and \( x \) was solved for (Equation 4-10). Constants \( b \) and \( c \) were then used to calculate the time, \( x \), to achieve a minimum value (where the slope is equal to zero). The observed rate constant, \( k_{\text{obs}} \), was estimated from the reciprocal of \( x \). Although the \( k_{\text{obs}} \) from calculated from fitting the data to Equation 4-7 and the \( k_{\text{obs}} \) from Equation 4-6 are not equivalent, they allow for comparisons in trends and are both on similar timescales and have the same units \((s^{-1})\).

In the active site trap, as acetyl-CoA concentration increased the \( k_{\text{obs}} \) values increased. The replotted \( k_{\text{obs}} \) values as a function of acetyl-CoA concentration exhibit a hyperbolic increase. The fact that the \( k_{\text{obs}} \) increases as the concentration of the allosteric activator increases indicates that acetyl-CoA increases the rate of inactivation, which is a function of crosslinking. This means that when acetyl-CoA is present the rate at which the population of enzyme becomes crosslinked and inactivated, which is dependent on carrier domain translocation, increases. As acetyl-CoA concentration increased, the slope of recovery also increased (Table 4-5). This indicates that acetyl-CoA promotes the population of enzyme to remix and reform catalytically active tetramers.
Substrates Influence Rate of Inactivation. To better understand the reaction conditions that can affect the positional equilibrium of the carrier domain, the influence BC and CT domain substrates had on the rate of inactivation was investigated in both the active site...
trap and the exo site trap. From the ITF data crosslinking the carrier domain in the exo
binding site, it was known that BC domain substrates, ADP+PPA, perturbed carrier
domain equilibrium, but we wanted to see if these substrates also altered carrier domain
equilibrium when the carrier domain was being crosslinked in the CT active site. When
G938C/N1102C SaPC, the active site trap, was crosslinked with bBBr in the presence of
increasing amounts of ADP+PPA the rate of inactivation decreased (Figure 4-22). This
means that as BC domain substrates become saturating, the rate at which the carrier
domain translocates and crosslinks in the CT domain active site decreases, because the
carrier domain positional equilibrium is shifted towards the BC domain. When the raw
data was fit to a one-phase decay equation (Equation 4-6) and the $k_{obs}$ values were
replotted as a function of ADP+PPA concentration, the trend was best described by a
linear regression (Figure 4-22B). The data is best fit by a linear regression, over the
concentrations investigated, but it is not likely that the $k_{obs}$ values go to zero, or becomes
negative, as the linear regression would predict because $k_{obs}$ is a function of crosslinking
which cannot be negative. Higher concentrations of ADP+PPA cannot be tested because
at concentrations higher than 3.5 mM G938C/N1102C SaPC was precipitating out of
solution.
When a similar experiment was performed by crosslinking Q891C/N1102C SaPC, the exo site trap, with BMOE in the presence of increasing amounts of ADP+PPA the rate of inactivation also decreased with increasing ligand concentration. This was consistent with prior experiments performed using ITF (Figure 4-5). A replot of the $k_{\text{obs}}$ values as a function of ADP+PPA concentration was best described by an inverse rectangular hyperbolic equation (Equation 4-2), which is also consistent with prior ITF experiments. In both cases, ITF and kinetics of inactivation, the $k_{\text{obs}}$ decreased with increasing ADP+PPA concentrations, consistent with a shift in carrier domain positional equilibrium towards the BC domain (Figure 4-23).
The influence CT domain substrate, pyruvate, had on inactivation rate was investigated to determine if CT domain substrates were also able to perturb the carrier domain positional equilibrium and whether the effect on the equilibrium was different when the carrier domain was crosslinked in the active site or exo site of the CT domain. Pyruvate was titrated from 0 mM to 15 mM. For SaPC, 12 mM pyruvate is considered saturating for the pyruvate carboxylation reaction (Westerhold et al., 2017). For both the active site and exo site crosslinking traps (G938C/N1102C SaPC and Q891C/N1102C SaPC), pyruvate did not affect the rate of inactivation (Figure 4-24). When the $k_{obs}$ values were replotted as a function of pyruvate concentration, no clear trend was observed.

**Figure 4-23. The influence of ADP+PPA on rate of inactivation in Q891C/N1102C SaPC.**

A. 1 mg/mL of Q891C/N1102C SaPC was crosslinked with 200 µM BMOE with 0 mM (black), 0.1 mM (blue), 0.3 mM (pink), 0.5 mM (green), 2 mM (purple) and 3.5 mM (cyan) ADP+PPA. The error bars represent the standard deviation from 3 independent measurements. The data were fit to Equation 4-6. B. A replot of $k_{obs}$ values as a function of ADP+PPA concentration. The data were fit to a simple linear regression. The error bars represent the standard errors for $k_{obs}$ calculated from iterative non-linear curve fitting in Graphpad Prism. The R² was 0.9702.
Substrates Influence Rate of Inactivation in the Presence of Acetyl-CoA. To determine if allosteric activator, acetyl-CoA, in combination with BC and CT domain substrates altered the positional equilibrium, kinetics of inactivation experiments titrating
substrates with 250 µM acetyl-CoA held constant were conducted. This was done to see whether the presence of acetyl-CoA altered the way the substrates effected the rate of inactivation and to see if the effect of the substrate is the same with and without allosteric activation. This can help define the role acetyl-CoA plays in altering carrier domain positioning and elucidate how allostery influences carrier domain translocation to the CT active site and exo site differently. I predicted that the trends seen in the ADP+PPA and pyruvate titrations would stay the same but the $k_{\text{obs}}$ values would increase, because the enzyme is being allosterically activated. The active site trap, G938C/N1102C SaPC, was crosslinked with bBBr at varying ADP+PPA concentrations in the presence of acetyl-CoA. As previously observed for the active site trap in the presence of acetyl-CoA (Figure 4-21), a recovery of inactivation was observed (Figure 4-25A). As ADP+PPA concentration increased the $k_{\text{obs}}$ decreased (Figure 4-25). The $k_{\text{obs}}$ values, as a whole, increased when acetyl-CoA was present. With the allosteric activator at saturating levels the $k_{\text{obs}}$ ranged from 0.02-0.15 s$^{-1}$, whereas without acetyl-CoA the $k_{\text{obs}}$ ranged from 0.005-0.015 s$^{-1}$. The slope of the replot of $k_{\text{obs}}$ as a function of ADP+PPA concentration is more negative than the slope of the replot in the absence of acetyl-CoA, due to the high $k_{\text{obs}}$ at low concentrations of ADP+PPA (Figure 4-22 and Figure 4-25). Increasing concentrations of ADP and PPA reduce the rate of recovery of inactivation, as seen in the decreased slope of recovery of inactivation (Table 4-5). This indicates that higher BC domain substrate concentration decreases the remixing of the enzyme population to reform catalytically active tetramers.

The exo site trap, Q891C/N1102C SaPC, was crosslinked with BMOE at varying ADP+PPA concentrations in the presence of acetyl-CoA (Figure 4-26). A similar trend
was observed as in the absence of acetyl-CoA (Figure 4-23), with a decrease in $k_{\text{obs}}$ as a function of increasing ADP+PPA concentrations. The replot of the $k_{\text{obs}}$ values as a function of ADP+PPA concentration is best described by a linear equation, as opposed to a hyperbolic equation, but this could be due to the higher errors. The standard errors of the curve fitting, or the errors of the one-phase decay fit, for the $k_{\text{obs}}$ values increased significantly when acetyl-CoA was present. Unlike the active site trap, the $k_{\text{obs}}$ values did not increase as a whole and the ADP+PPA titration done with and without acetyl-CoA both had $k_{\text{obs}}$ values that ranged from 0.005-0.03 s$^{-1}$. Additionally, when acetyl-CoA was present the minimum $k_{\text{cat}}/k_{\text{cat}}$ value increased as ADP+PPA concentration increased, suggesting that less of the population of enzyme is being crosslinked in the CT exo site.

**Figure 4-25. The influence of ADP+PPA on the rate of inactivation in the presence of acetyl-CoA in the G938C/N1102C SaPC** A. 1 mg/mL of G938C/N1102C SaPC was crosslinked with 200 µM bBr with 0 mM (black), 0.1 mM (blue), 0.3 mM (pink), 0.5 mM (green), 2 mM (purple) and 3.5 mM (cyan) ADP+PPA in the presence of 250 µM acetyl-CoA. The data were fit to Equation 4-7. The error bars represent the standard deviation of 3 independent measurements. B. The $k_{\text{obs}}$ values from panel B were replotted against ADP+PPA concentration. The $k_{\text{obs}}$ values were calculated by taking the inverse of the Time to Minimum. The data was fit to a linear regression and the equation was $Y = -0.03603X + 0.1341$ and the $R^2$ value was 0.93.
Table 4-5. Rate constants from kinetics of inactivation experiment for G938C/N1102C SaPC ADP+PPA titration in the presence of acetyl-CoA.

<table>
<thead>
<tr>
<th>[ADP + PPA]</th>
<th>a</th>
<th>c</th>
<th>b</th>
<th>Time to Minimum (s)</th>
<th>Slope of Recovery (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>14 ± 7</td>
<td>7.9 ± 2.0</td>
<td>-3.7 ± 0.9</td>
<td>7.5 ± 0.7</td>
<td>0.00042</td>
</tr>
<tr>
<td>0.1mM</td>
<td>14 ± 10</td>
<td>8.9 ± 2.5</td>
<td>-4.1 ± 1.1</td>
<td>7.8 ± 0.8</td>
<td>0.00051</td>
</tr>
<tr>
<td>0.3mM</td>
<td>16 ± 4</td>
<td>9.0 ± 0.8</td>
<td>-4.1 ± 0.4</td>
<td>7.8 ± 0.3</td>
<td>0.00041</td>
</tr>
<tr>
<td>0.5mM</td>
<td>35 ± 27</td>
<td>12 ± 3</td>
<td>-5.5 ± 1.3</td>
<td>7.9 ± 0.7</td>
<td>0.00052</td>
</tr>
<tr>
<td>2mM</td>
<td>1.1 ± 0.2</td>
<td>2.4 ± 0.6</td>
<td>-0.71 ± .27</td>
<td>27 ± 2</td>
<td>-0.000082</td>
</tr>
<tr>
<td>3.5mM</td>
<td>1.0 ± 0.6</td>
<td>2.4 ± 1.9</td>
<td>-0.61 ± 0.90</td>
<td>49 ± 6</td>
<td>0.00021</td>
</tr>
</tbody>
</table>

Figure 4-26. The influence of ADP+PPA on the rate of inactivation in the presence of acetyl-CoA in the Q891C/N1102C SaPC. A. 1 mg/mL of Q891C/N1102C SaPC was crosslinked with 200 µM BMOE with 0 mM (black), 0.1 mM (blue), 0.3 mM (pink), 0.5 mM (green), 2 mM (purple) and 3.5 mM (cyan) ADP+PPA in the presence of 250 µM acetyl-CoA. The data were fit to Equation 4-6. The error bars for panels A and B represent the standard deviation of 3 independent measurements. B. The $k_{obs}$ values from panel B were replotted against ADP+PPA concentration. The data was fit to a linear regression and the equation was $Y = -0.002056*X + 0.01813$ and the $R^2$ value was 0.9323. The error bars represent the standard errors calculated from iterative non-linear curve fitting in Graphpad Prism.
The active site trap, G938C/N1102C SaPC, was crosslinked with bBBr at varying pyruvate concentrations in the presence of acetyl-CoA (Figure 4-27). As previously observed for the active site trap in the presence of acetyl-CoA (Figure 4-21), a recovery of inactivation was observed (Figure 4-27A). As pyruvate concentration increased \(k_{\text{obs}}\) values decreased (Figure 4-27B), unlike the pyruvate titration done without acetyl-CoA, which exhibited no trend. With the allosteric activator at saturating levels the \(k_{\text{obs}}\) range from 0.07-0.13 s\(^{-1}\), whereas without acetyl-CoA the \(k_{\text{obs}}\) range from 0.025-0.035 s\(^{-1}\). The replot of the \(k_{\text{obs}}\) values as a function of pyruvate concentration was best described by a linear decrease. Additionally, the slope of recovery of inactivation decreases as pyruvate concentration increases. This indicates that both BC domain substrates, ADP+PPA, and CT domain substrate, pyruvate, decrease the recovery of inactivation, or the remixing of the population of enzyme to re-form active tetramers.
Figure 4-27. The influence of pyruvate on the rate of inactivation in the presence of acetyl-CoA in G938C/N1102C SaPC. A. 1 mg/mL of G938C/N1102C SaPC was crosslinked with 200 μM bBBr with 0 mM (black), 1 mM (blue), 3 mM (pink), 5 mM (dark green), 7 mM (cyan), 10 mM (lime green), 12 mM (orange) and 15 mM (magenta) pyruvate in the presence of 250 μM acetyl-CoA. The data were fit to Equation 4-7. The error bars represent the standard deviation of 3 independent measurements B. The $k_{obs}$ values were replotted as a function of pyruvate concentration. The $k_{obs}$ values were calculated by taking the inverse of the Time to Minimum. The error bars represent the standard errors calculated from iterative non-linear curve fitting in Graphpad Prism. The data was fit to a linear regression with an equation of $Y = -0.003915*X + 0.1307$ and an $R^2$ of 0.8116.
Table 4-6. Rate constants from kinetics of inactivation experiment for G938C/N1102C SaPC pyruvate titration in the presence of acetyl-CoA.

<table>
<thead>
<tr>
<th>[Pyruvate]</th>
<th></th>
<th></th>
<th></th>
<th>Time to Minimum (s)</th>
<th>Slope of Recovery (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>71 ± 50</td>
<td>14 ± 3</td>
<td>-6.5 ± 1.2</td>
<td>8.0 ± 0.4</td>
<td>0.00053</td>
</tr>
<tr>
<td>1 mM</td>
<td>9.5 ± 7.8</td>
<td>7.1 ± 3.1</td>
<td>-3.2 ± 1.4</td>
<td>8.2 ± 0.8</td>
<td>0.0006</td>
</tr>
<tr>
<td>3 mM</td>
<td>9.4 ± 5.6</td>
<td>8.2 ± 2.2</td>
<td>-3.6 ± 1.0</td>
<td>8.6 ± 0.5</td>
<td>0.00041</td>
</tr>
<tr>
<td>5 mM</td>
<td>12 ± 9</td>
<td>7.5 ± 2.9</td>
<td>-3.4 ± 1.3</td>
<td>8.1 ± 0.7</td>
<td>0.00043</td>
</tr>
<tr>
<td>7 mM</td>
<td>3.5 ± 2.1</td>
<td>5.0 ± 2.2</td>
<td>-2.1 ± 1.0</td>
<td>9.8 ± 0.8</td>
<td>0.00021</td>
</tr>
<tr>
<td>10 mM</td>
<td>7.0 ± 4.3</td>
<td>7.0 ± 2.2</td>
<td>-3.0 ± 1.0</td>
<td>9.0 ± 0.6</td>
<td>0.00025</td>
</tr>
<tr>
<td>12 mM</td>
<td>1.0 ± 0.8</td>
<td>2.7 ± 1.6</td>
<td>-1.0 ± 0.7</td>
<td>13 ± 1</td>
<td>0.0002</td>
</tr>
<tr>
<td>15 mM</td>
<td>1.5 ± 1.1</td>
<td>3.5 ± 2.7</td>
<td>-1.2 ± 1.3</td>
<td>16 ± 1</td>
<td>0.00019</td>
</tr>
</tbody>
</table>

The exo site trap, Q891C/N1102C SaPC, was crosslinked with BMOE at varying pyruvate concentrations in the presence of acetyl-CoA. When acetyl-CoA was present the $k_{obs}$ values increased as pyruvate concentration increases, unlike the pyruvate titration done without acetyl-CoA, which exhibited no trend. The $k_{obs}$ values, as a whole, increased slightly. With the allosteric activator at saturating levels the $k_{obs}$ range from 0.01-0.05 s$^{-1}$, whereas without acetyl-CoA the $k_{obs}$ range from 0.01-0.02 s$^{-1}$. An increase in $k_{obs}$ as pyruvate concentration increases is consistent with a shift in the positional equilibrium of the carrier domain towards the CT domain.
Studying Conformational Dynamics Requires the use of Biophysical Tools

The carrier domain of PC undergoes a large translocation during catalysis, which is essential to its function. The mechanism governing the translocation is undefined and different models have been applied to this system. Some models of PC catalysis propose that carrier domain movement is governed by an induced conformational change, where the carrier domain remains in proximity to the BC domain until a CT domain ligand binds (Attwood & Wallace, 1986; Goodall et al., 1981; Wallace et al., 1985). A logical consequence of this model is that a CT domain ligand is required for translocation of the carrier domain to the CT domain. Conversely, several x-ray structures of PC with bound

Figure 4-28. The influence of pyruvate on the rate of inactivation in the presence of acetyl-CoA in Q891C/N1102C SaPC. A. 1 mg/mL of Q891C/N1102C SaPC was crosslinked with 200 µM BMOE with 0 mM (black), 1 mM (blue), 3 mM (pink), 5 mM (dark green), 7 mM (cyan), 10 mM (lime green), 12 mM (orange) and 15 mM (magenta) pyruvate in the presence of 250 µM acetyl-CoA. The data were fit to Equation 4-6. The error bars represent the standard deviation of 3 independent measurements. B. The $k_{obs}$ values were replotted as a function of pyruvate concentration. The data was fit to Equation 4-2. The error bars represent the standard errors calculated from iterative non-linear curve fitting in Graphpad Prism.
ligand(s) lack defined electron density for the carrier domain, suggesting that the carrier domain can sample multiple conformations, even in the presence of ligand (St. Maurice et al., 2007; Xiang & Tong, 2008; Zeczycki et al., 2011). This is consistent with a conformational selection model where substrates and effectors are not required to induce carrier domain movement and serve only to shift the conformational equilibrium of the enzyme.

This chapter outlines several methods to directly observe carrier domain positioning, independent of catalytic turnover. Multiple biophysical techniques were employed to report on carrier domain crosslinking at two different binding sites in the CT domain to probe conformational dynamics in PC. In all cases, the observed rates are one to two orders of magnitude lower than the $k_{cat}$ value for the PC-catalyzed reaction and, therefore, do not estimate the rates of carrier domain translocation during catalytic turnover. Nevertheless, the observed rate constants represent the re-equilibration of the carrier domain within an ensemble population. A comparison of these observed rate constants in the presence of ligands can, therefore, inform on the degree to which ligands influence the enzyme conformational equilibrium and the resulting equilibrium position of the carrier domain.

**ITF Studies Reveal Conformational Selection Governs Carrier Domain Translocation**

Based off the ITF studies in Q891C/N1102C SaPC, substrates and ligands shift the carrier domain positional equilibrium as expected. The BC domain ligands (ADP+PPA) reduced the rate of crosslinking and shifted the carrier domain equilibrium away from the CT domain exo site, consistent with proposals that BC domain ligands
shift the BCCP carrier domain towards the BC domain (Attwood & Wallace, 1986; Goodall et al., 1981; Wallace et al., 1985).

While these BC domain substrate analogues shifted the carrier domain equilibrium as expected, it is important to note that saturating concentrations of ADP+PPA did not completely prevent crosslinking in the CT domain (Figure 4-5). Thus, the translocation of the carrier domain to the CT domain does not require the binding of a CT domain ligand, nor is its translocation prevented by the presence of a BC domain ligand. I conclude that the carrier domain samples multiple conformations in both the presence and absence of ligand. While substrates and effectors alter the equilibrium positioning of the carrier domain, they are not required for BCCP-CT domain positioning. This is fully consistent with a conformational selection model for the carrier domain.

Crosslinking of the carrier domain near the CT domain exo site results in a change in the ITF intensity, offering new opportunities to directly probe the kinetics of carrier domain equilibration independent of catalytic turnover. The double exponential burst and the resulting non-linear response of both \( k_{obs1} \) and \( k_{obs2} \) against [ADP+PPA] indicates a multi-step process (Figure 4-5). Since the ligand is pre-equilibrated with both the enzyme and the crosslinker prior to mixing, the observed change in ITF intensity is a response to crosslinking and not to ligand binding. Thus, both \( k_{obs1} \) and \( k_{obs2} \) represent kinetic steps in crosslinking between the BCCP and CT domains. It is likely that \( k_{obs1} \) represents the initial intersubunit capture of the BCCP domain while \( k_{obs2} \) represents a slow transition to a lower energy state (Figure 4-6). Because of the complexity of the system, the current data cannot accurately determine the individual microscopic rate constants that comprise
$k_{\text{obs1}}$ and $k_{\text{obs2}}$, though the data are adequately described by an 8-step kinetic scheme (Figure 4-8). More accurate estimates of the individual microscopic rate constants will require future studies that trap the carrier domain in additional conformational states.

To demonstrate the ability of this crosslinking system to report on relative changes in the carrier domain equilibrium, we layered the crosslink trap onto the R346M mutation in SaPC. This mutation behaves similarly to an equivalent mutation in RePC, which was proposed to favor an enhanced BCCP-BC domain interaction compared to the WT enzyme (Zeczycki et al., 2011). Such a system, with an equilibrium favoring the BCCP-BC domain interaction, is predicted to have a larger percentage of the BCCP carrier domain population positioned at the BC domain in the absence of substrate (represented by a smaller value for $k_{\text{max}}$), a greater shift towards the BC domain at saturating substrate concentrations (represented by a smaller value for $k_{\text{min}}$) and an altered $K_{0.5}$ if the mutation impacts ligand binding. This is exactly what was observed when the crosslink trap was applied in the presence of the R346M mutation (Figure 4-7 and Table 4-1). This validates the interpretation of the data and demonstrates that this, and other, designed crosslink traps can be used to investigate how mutations impact the carrier domain equilibrium in PC.

**CT Exo Site is Catalytically Relevant**

The carrier domain accesses two binding sites within the CT domain: the active site and the exo site. The carrier domain has been observed to occupy both of these sites in crystal and cryo EM structures (Chai et al., 2022; Lasso et al., 2010, 2014; López-Alonso et al., 2022; Yu et al., 2009). When the carrier domain is bound in the CT active site, the biotin cofactor attached is positioned in close proximity to the CT domain
substrate, pyruvate, facilitating carboxyl group transfer to pyruvate during catalysis (Figure 4-10A). When the carrier domain is bound in the CT domain exo site, the biotin cofactor is located distant from the active site at the periphery of the CT domain, near the AD. This site is believed to only accommodate biotin, not carboxybiotin, due to N1 atom of biotin hydrogen bonding to Phe588 (Xiang & Tong, 2008). The exo binding site was first identified in SaPC and was postulated to be a feature unique to bacterial PC enzymes (Xiang & Tong, 2008). However, a recently reported cryo EM structure of human PC also revealed the carrier domain bound in the exo binding site, suggesting that this is a universal feature of PC enzymes (Chai et al., 2022). It has been postulated that the exo binding site contributes an additional regulatory mechanism (Xiang & Tong, 2008), but a precise role for the exo binding site in catalysis has not been demonstrated. The work described in this chapter serves to directly investigate the carrier domain positional equilibrium when the carrier domain is trapped in the CT domain exo site. The carrier domain positional equilibrium at the exo binding site is influenced by substrate concentrations, supporting a role for this exo site in the catalytic cycle. Structural data repeatedly captures the carrier domain bound in the exo binding site, consistent with a stable, low energy conformation. I propose that the specific role of the exo binding site is to hold the carrier domain in place when that specific subunit in the homotetramer is not undergoing catalysis. Carrier domain translocation is governed by conformational selection, where each individual subunit can sample multiple conformations independent of substrate, but there is tetrameric communication that coordinates the movement of the carrier domains so that they are not all actively turning over substrate at once.
**Investigating Carrier Domain Translocation to CT Active Site and Exo Site**

Comparing differences in the carrier domain positional equilibrium when it is translocating to the CT active site versus the CT exo site can help elucidate the catalytic cycle of the carrier domain in PC. To investigate carrier domain positional equilibrium, not only in the CT exo site, but also in the CT active site a new double cysteine crosslinking trap, G938C/N1102C SaPC, was generated. This active site trapping system traps the carrier domain only when it is bound in the CT active site (Figure 4-10A). Due to the location of the mutated cysteines and the length of the homobifunctional crosslinker, the carrier domain will not be trapped and crosslinked when the carrier domain is bound in the CT exo site, but only crosslinked when bound in the active site (Figure 4-10). We know this because of SDS-PAGE analysis, where the WT and both single mutants, Q891C SaPC and N1102C SaPC, do not form any crosslinked species and Q891C/N1102C SaPC shifts from the monomeric molecular weight to the dimeric molecular weight when crosslinker is added. Unfortunately, ITF could not be used to probe the positional equilibrium of the carrier domain when it translocates and crosslinks in the active site because the fluorescence signal was not a function of crosslinking (Figure 4-13). Stopped-flow fluorescence with the fluorescent crosslinker, bBBr, was attempted, but the signal was also not a function of crosslinking therefore this technique could not be used (Figure 4-14).

Alternatively, a technique that monitors the rate of inactivation as the population of enzyme becomes crosslinked, termed “kinetics of inactivation”, was used to investigate changes in conformational dynamics of PC when the carrier domain is trapped in either the CT active site or exo site. When CT active site trap, G938C/N1102C SaPC,
is crosslinked with the ~8 Å BMOE crosslinker, the rate of inactivation is too fast and
this technique is not sufficiently sensitive to capture a rate of inactivation when
crosslinking with BMOE (Figure 4-15A). When G938C/N1102C SaPC is crosslinked
with the ~5 Å bBBr crosslinker, the rate of inactivation is slow enough to be measured.
When the CT exo site trap, Q891C/N1102C SaPC, is crosslinked with bBBr, only a small
decrease in activity is observed. This is likely because the bBBr crosslinker is too short
to effectively span the distance between the two engineered cysteines when the carrier
domain is bound in the exo site (Figure 4-15B). Consistent with this interpretation, less
crosslinked dimer is formed when Q891C/N1102C SaPC is crosslinked with bBBr
compared to when it is crosslinked with BMOE (Figure 4-11A). G938C/N1102C SaPC,
the active site trap, crosslinked with bBBr and Q891C/N1102C SaPC, the exo site trap,
crosslinked with BMOE have similar rates of inactivation 0.023 ± 0.004 s⁻¹ and 0.027 ±
0.003 s⁻¹ respectively. Since the two systems are crosslinked on the same timescale and
with the same crosslinking efficiency, we can then compare the shift in the rate of
inactivation in both systems when various substrates and effector molecules are added to
the crosslinking reaction. This will help better define the positional equilibrium of the
carrier domain when it is bound in active site versus the exo site.

Acetyl-CoA Coordinates Carrier Domain Translocation

The allosteric activator, acetyl-CoA, effectively couples the BC and CT half
reactions, and orders the tetramer, which is shown through a reduction in transition state
activation entropy when acetyl-CoA is present versus when it is absent (Adina-Zada,
Zeczycki, et al., 2012; Legge et al., 1996; Westerhold et al., 2017). Acetyl-CoA was
titrated in both active site and exo site crosslinking trap systems, and pyruvate
carboxylation was observed, to confirm that 250 μM acetyl-CoA was saturating for both constructs. Surprisingly, G938C/N1102C SaPC was being inhibited at high concentrations of acetyl-CoA, but the addition of a reducing agent eliminated this inhibition (Figure 4-18). This implies that super-saturating concentrations of acetyl-CoA promotes a conformation where G938C and N1102C are close enough to form a disulfide bond. While acetyl-CoA does not cause a large structural change in either BC or CT domain active sites, this data demonstrates that the allosteric activator does alter the tetrameric structure so that the carrier domain is bound more tightly in the CT active site.

A saturating amount of acetyl-CoA, in the active site trap, causes a recovery from inactivation after ~120 seconds (Figure 4-19). The only way for this phenomenon to occur is if the population of enzyme is remixing, and remixing can only occur if both faces of the tetramer are not crosslinked. If one or two carrier domain(s) are crosslinked on one face of the tetramer, but the carrier domains on the other face of the tetramer are not crosslinked, if given enough time (after 120 seconds) the population of enzyme can remix, and the un-crosslinked subunits can form a new tetramer with other free subunits (Figure 4-29). This phenomenon only occurs in the active site crosslinking trap in the presence of acetyl-CoA. We predict this is not occurring in the exo site crosslinking system because with and without acetyl-CoA present both faces of the tetramer are crosslinked, therefore there are no un-crosslinked subunits to remix. This indicates that acetyl-CoA is coordinating the translocation of the carrier domain to the CT active site, so that only one face of the tetramer has a carrier domain(s) bound in the active site at a time. This technique cannot distinguish between one or two carrier domains bound in the CT active site on the same face of the tetramer because if either one or two subunits were
crosslinked both subunits on that face would be trapped. Recovery could not occur if three carrier domains were crosslinked in CT active sites, because that would trap both faces of the tetramer.

![Diagram](image)

**Figure 4-29. Model of G938C/N1102C SaPC enzyme population remixing that enables recovery from crosslinking inactivation.** A model of the active site crosslinking trap with (bottom) and without (top) acetyl-CoA. The black bar represents crosslinking. PC model is simplified by only showing one BCCP domain on each face of the tetramer.

Taking the kinetics of inactivation data, in the presence of acetyl-CoA, from both the exo site and the active site trap together, we can determine that only one carrier domain is bound in the CT active site at once. From the exo site trap, Q891C/N1102C SaPC, kinetics of inactivation data in the presence of 250 µM, it can be concluded that at
least one carrier domain is bound in the exo site on each face of the tetramer, because no
recovery of inactivation is seen, therefore both faces must have a carrier domain
crosslinked in the exo site. From the active site trap, G938C/N1102C SaPC, kinetics of
inactivation data in the presence of 250 µM, it can be concluded that only one face of the
tetramer has a carrier domain bound and crosslinked in the CT active site. Through the
process of elimination, it can be concluded that one face of the tetramer has one carrier
domain bound in the exo site and the other carrier domain bound in the active site,
whereas the other face of the tetramer has just one carrier domain bound in the exo site.
The fourth carrier domain is likely transient and unbound (Figure 4-30). This agrees with
the crystal structure, PDB ID 3BG5, where three carrier domains are modeled with two
bound in the exo site and one bound in the active site, with the fourth carrier domain
unresolved (Xiang & Tong, 2008). While crystal structures are just snapshots of the
enzyme, taking this structural data in combination with our biophysical data can give
catalytic significance to this particular conformation.
Acetyl-CoA coordinates carrier domain movement in the tetramer, which is why recovery of inactivation is seen in the active site trap. As acetyl-CoA concentration increases the slope of the recovery phase increases, and the $k_{\text{obs}}$ increases (Figure 4-21 and Table 4-4). The fact that acetyl-CoA increases the $k_{\text{obs}}$, indicates that allosteric activation increases the rate of inactivation, which is a function of crosslinking and translocation. The fact that acetyl-CoA increases the slope of the recovery demonstrates that allosteric activation promotes the remixing and formation of catalytically active tetramers. As acetyl-CoA concentration increased in the exo site trap, the $k_{\text{obs}}$ did not vary, but the minimum $k_{i\text{cat}}/k_{o\text{cat}}$ decreased, indicating that more of the population of enzyme was crosslinked and inactivated.

**Figure 4-30. Ball and stick representation of the homotetramer.** The top face is shown on the left, with one carrier domain bound in the exo site, and the other in the active site. The bottom face is shown on the right with one carrier domain bound in the exo site with the other carrier domain unresolved (faded red).
**BC and CT Domain Ligands Shift the Carrier Domain Positional Equilibrium**

In both the active site and exo site traps, as ADP+PPA concentration increased the $k_{\text{obs}}$ values decreased. This is consistent with the carrier domain positional equilibrium shifting towards the BC domain. The active site trap requires more ADP+PPA to perturb the equilibrium in comparison to the exo site trap. When ADP+PPA concentration was varied in the presence of saturating acetyl-CoA, the trend in $k_{\text{obs}}$ as the ligand concentration increased stayed the same, but in the active site trap the $k_{\text{obs}}$ values as a whole increased (Figure 4-22 and Figure 4-25). In both the active and exo site traps when acetyl-CoA was present as ADP+PPA were titrated the minimum $k_{\text{cat}}/k_{\text{cat}}$ increased, which is an indication that the minimum rate of re-equilibration of the carrier domain is increased. Therefore, in the presence of saturating BC domain substrates, the amount of activity retained in the ensemble population is increased by allosteric activation.

In both the active and exo site traps when pyruvate concentration was varied, the $k_{\text{obs}}$ values did not change. When pyruvate concentration was varied in the presence of saturating acetyl-CoA the $k_{\text{obs}}$ values decreased in the active site trap and the $k_{\text{obs}}$ values increased in the exo site trap. This is the first difference seen in carrier domain translocation to the CT active site versus the exo site (Figure 4-31). This suggests that in the exo site BC substrates shift the carrier domain positional equilibrium towards to BC domain and CT domain substrates shift the equilibrium towards the CT domain, which intuitively makes sense and was what we predicted.
In the active site trap both BC and CT domain substrates shift the equilibrium towards to BC domain. This could be because the carrier domain is only bound in the CT domain active site briefly, to transfer the carboxyl group, whereas the carrier domain is held in the exo site for a longer period. Both BC and CT domain substrates may shift the positional equilibrium so that the carrier domain does not occupy the CT domain active site for too long, because if biotin cofactor remains in the active site for too long it can facilitate the decarboxylation of the reaction product, oxaloacetate. In the active site system, ADP+PPA have a greater influence than pyruvate on the positional equilibrium seen by the slope of the replotted $k_{obs}$ values being more negative, -0.036 and -0.004 s$^{-1}$ respectively (Figure 4-25 and Figure 4-27). The $k_{obs}$ values calculated from the reciprocal biphasic curve fit (Equation 4-7) and the one-phase decay curve fit (Equation 4-6) are not equivalent, but they are in the same units and in relatively the same timescale, therefore can be used to compare trends.

**Figure 4-31. Representation of the positional equilibrium of the carrier domain when it is translocating to the active site and exo site of the CT domain.**

A. The positional equilibrium when the carrier domain is being trapped in the CT active site is represented by the red arrow. B. The positional equilibrium when the carrier domain is being trapped in the CT exo site is represented by the red arrow.
In the active site system, as previously mentioned, when acetyl-CoA is present a recovery of inactivation is observed. BC domain substrates, ADP+PPA, and CT domain substrate, pyruvate, both decrease the recovery of inactivation, as seen by the decrease in the slope of the recovery (Table 4-5 and Table 4-6). As ADP+PPA or pyruvate concentration increases the slope of the recovery of inactivation decreases. Substrates bound in either active site reduces the remixing of the population of enzyme, likely because when substrate is bound, the tetramer is in a conformation in which it is harder for an individual subunit to disassociate.

Pyruvate, CT domain substrate, shifted the positional equilibrium less than BC domain substrates in both active and exo site systems. In all structures of SaPC the carrier domain is either disordered or in the CT active site or exo site, but never in the BC domain, suggesting that the interaction between BCCP-CT is more stable than BCCP-BC (Lasso et al., 2010, 2014; St. Maurice et al., 2007; Xiang & Tong, 2008). Recently the carrier domain was positioned in the BC domain in a cryo EM structure, but it occupied this conformation much less than it occupied the CT domain (López-Alonso et al., 2022). It is likely that pyruvate effects the positional equilibrium of the carrier domain less than ADP+PPA because the carrier domain prefers the CT domain to the BC domain and thus its positional equilibrium is already shifted towards the CT domain.

The current study leads to an updated model of conformational selection for carrier domain positioning in PC. The carrier domain exists in an ensemble of conformations, where ligand binding shifts the free energy landscape and alters the conformational equilibrium to favor those conformations that have a higher affinity for the ligand (Boehr et al., 2009). Prior structural data suggest how these equilibrium shifts
can occur. BC domain substrates promote the closing of the B-subdomain lid, enhancing interactions with the carrier domain and perturbing the free energy landscape to promote enzyme conformations that favor BC-BCCP domain interactions. Similarly in the CT domain, the presence of a CT domain substrate or substrate analogue results in the formation of a biotin-binding pocket that favors the positioning of biotin in the CT domain active site (Lietzan & St. Maurice, 2013a). This perturbs the free energy landscape to promote enzyme conformations that favor CT-BCCP domain interactions. Acetyl-CoA increases the observed rate constant, $k_{obs}$, in both active and exo site traps, which indicates allosteric influences the rate at which the carrier domains in the ensemble population are translocating and becoming crosslinked in either the CT active or exo sites. Acetyl-CoA also coordinates carrier domain movement at a tetrameric level, so that all subunits do not have their carrier domain in the CT active site at the same time.

Through this study and other recent reports, a revised view of carrier domain translocation in PC is emerging: ligands and allosteric effectors shift the conformational equilibrium in PC to appropriately favor carrier domain positioning during catalytic turnover, but they neither impede nor rigidly control the ability of the carrier domain to regularly sample a wide range of conformations (Liu et al., 2018a; Westerhold et al., 2017). This study also reveals the role allostery plays in tetrameric coordination and while the carrier domains do not undergo a conformational change in direct response to ligand binding, they do translocate in an ordered fashion.
CHAPTER 5. DISCUSSION

Main Conclusions

Here, I have defined the molecular basis of allosteric regulation in SaPC by defining the acetyl moiety binding site at the BC dimer interface. This site was probed through mutagenesis and visualized through X-ray crystallography. Residues Arg21, Lys46, and Glu418 all interact with the acetyl moiety. These residues are essential for both allosteric activation by acetyl-CoA and allosteric inhibition by L-aspartate. I also demonstrated that acetyl-CoA elicits a long range effect to the distantly CT domain. This long range effect is not mediated by the BC dimer interface or the acetyl moiety but likely from the nucleotidyl portion of acetyl-CoA.

Another major conclusion of this work is that microbial PC’s can also catalyze the hydrolysis the acetyl moiety of acetyl-CoA. Due to the slow hydrolysis rate that only occurs at extremely high concentrations of acetyl-CoA, this is likely an in vitro artifact. The acetyl moiety binding site in the BC domain is not solely responsible for this hydrolysis, and there is likely another binding site for acetyl-CoA in the CT domain.

Lastly, it has been determined that carrier domain translocation is governed by conformational selection. The carrier domain exists in a positional equilibrium that is perturbed by the presence of substrates and effector molecules. BC domain substrate analogs ADP+PPA shift the positional equilibrium towards the BC domain. The CT domain exo site is likely a part of the catalytic cycle because carrier domain translocation to the exo binding site is dependent on BC domain substrate concentration. Through the kinetics of inactivation studies, using both the exo site and active site crosslinking traps, I
determined that carrier domain positioning in the active site is coordinated by the allosteric activator acetyl-CoA. This data also enabled the prediction of a catalytic cycle of the tetramer in the presence of acetyl-CoA.

**Acetyl-CoA Allosteric Activation is Physiologically Relevant**

Acetyl-CoA is an extremely important metabolite for a multitude of reasons one being that it serves as an allosteric activator of PC. This allosteric regulation provides a way for PC to respond to changing levels of acetyl-CoA and impact the relative flux of carbon entering the TCA cycle. Allosteric activation by acetyl-CoA increases the anaplerotic activity of PC without expending any cellular energy. The cells’ ability to adjust anaplerotic input into the TCA cycle ensures homeostasis. When allosteric regulation of PC is impaired, metabolic homeostasis is compromised. The R451C HsPC mutation in humans, which impairs allosteric activation, causes a hereditary form of PC deficiency that can be deadly, therefore allosteric regulation is essential to PC function *in vivo* (Wexler et al., 1998). The R451C HsPC mutation can be deadly because it impairs acetyl-CoA binding, which results in lower PC activity that causes a myriad of physiological problems including lactic acidosis, hypoglycemia and decreased cognitive function. This disease-causing mutation further amplifies the physiological significance of allosteric activation of PC by acetyl-CoA. Although allosteric activation by acetyl-CoA is imperative to PC’s function, the molecular interactions between the activator and protein and the complete allosteric mechanism has not been characterized.

**Entropically Driven Allostery**

When allostery was first discovered in the early 1960’s, the binding of an effector molecule was always thought to be accompanied by a conformational change. The
allosteric effector molecule was thought to induce a change in the energy landscape of the enzyme so that a different conformation was more energetically favorable (Figure 5-1A). When these conformational changes were observed, they were typically centered on the active site, where the side chains in and around the active site became less flexible and more catalytically competent (Akke et al., 1993). In the late 1970’s the first instance of allosteric regulation in the absence of a conformational change was discovered in glyceraldehyde-3-phosphate dehydrogenase (GPDH) (Niekamp et al., 1977).

Calorimetric studies of nicotinamide adenine dinucleotide (NAD+) binding to GPDH revealed that the binding was cooperative and the binding of a second NAD+ molecule was more thermodynamically favorable than the first because the entropy ($\Delta S$) was less negative. This form of allostery was termed “dynamic allostery” because the allosteric communication from effector binding site to active site(s) is mediated by the dynamic frequencies and amplitudes of conformational fluctuations, and not by obvious structural changes (Cooper & Dryden, 1984). The cooperative interaction, in a dynamic allostery scenario, is driven by entropy.
Another example of entropically driven allostery is in the molecular chaperone heat shock protein 70 (Hsp70). ATP allosterically inhibits Hsp70 by decreasing the enzyme’s affinity for exposed hydrophobic regions of unfolded substrate proteins. ATP binds in the nucleotide binding domain (NBD) which is distinct from where the unfolded protein binds in the C-terminal substrate binding domain (SBD). ATP binding/hydrolysis elicits a subtle structural change, but this conformational change cannot account for the allosteric effect seen in the SBD. The conformational dynamics involved with ATP binding/hydrolysis were explored through molecular dynamics simulations and NMR relaxation experiments to uncover the allosteric mechanism (Zhuravleva & Gierasch, 2015). The simulation identified residues that could be implicated in the allosteric network and these residues were subject to mild mutations and the chemical shift
perturbation (CSP) was measured to analyze the flexibility of these residues with and without the allosteric inhibitor ATP. Although ATP binds in the NBD the change in dynamics was seen in the SBD. The SBD increases in flexibility and conformational dynamics when the allosteric inhibitor is present, which effectively lowers the substrate affinity. In Hsp70 the allosteric effector does not change which conformation is preferred but changes the flexibility of the SBD which decreases the stability of the bound substrate. The allosteric mechanism is mediated through changes in dynamics not a change in preferred conformation.

In the majority of cryo EM and crystal structures of full length PC the BCCP domain is bound in the CT domain (Table 1-1). The presence of the carrier domain in the CT domain in all of these structures implies that this conformation is more thermodynamically favorable than when the carrier domain is bound in the BC domain. The BCCP domain is bound in the CT domain in structures both with and without acetyl-CoA. Therefore, the effector molecule does not change which conformation is more energetically favorable but likely lowers the energy minimum of the CT-BCCP conformation even further, just like the Hsp70 example (Figure 5-1). Allostery in both Hsp70 and PC is mediated through changes in conformational dynamics. When acetyl-CoA binds to PC, no conformational change in either the BC or CT domain active site occurs (Adina-Zada, Zeczycki, et al., 2012). The recent cryo EM study conducted in L/PC showed that while acetyl-CoA does not cause a conformational change in the active sites, it does alter the angle between the BC and CT domains in the same subunit. When acetyl-CoA is present, the BC domain tilts towards the center of the tetramer so that the BC domain active site is oriented 1.4° more towards the CT domain (López-Alonso et al.,
The structural change is subtle although the activation can drastically alter the kinetics of PC. Therefore, the allosteric effect is likely driven by conformational dynamics and conformational entropy.

**Acetyl-CoA Modulates the Free Energy Basin of Specific Conformations**

As previously mentioned in Chapter 4, allosteric activation by acetyl-CoA only causes subtle conformational changes in the tetramer. The G891C/N1102C SaPC mutant was designed to trap the carrier domain in the CT domain active site. When acetyl-CoA was titrated in G891C/N1102C SaPC it fully activated the protein but became inhibitory at about 300 µM (Figure 4-18). When a reducing agent was added, this inhibition at high concentrations of acetyl-CoA disappeared leading to the conclusion that the inhibition was due to the formation of disulfide bonds between the two cysteines. The formation of disulfides occurred because high concentrations of acetyl-CoA tightened the CT active-BCCP conformation. The carrier domain must be positioned tightly in the CT domain active site funnel region in order for these two residues to come within 3 Å of each other and form a disulfide bond. Entropically driven allostery can not only change the conformational dynamics of the system but also change the free energy basin of a specific conformation. This can be thought of as the conformational space that the BCCP domain occupies when it is in the CT domain active site. This space is narrowed when acetyl-CoA concentration rises above saturation. The shape of the free energy basin associated with a specific conformation determines the conformational entropy (Guo & Zhou, 2016). Conformational entropy is technically hard to measure but NMR relaxation studies and molecular dynamics simulations can approximate the conformational entropy of a system/conformation (Wand & Sharp, 2018). The Gibbs free energy of a system is
described by a classic thermodynamic relationship: \( \Delta G = \Delta H - T\Delta S \). A negative change in Gibbs free energy is energetically favorable. Therefore, a more positive change in entropy contributes to a more favorable (negative) \( \Delta G \). The entropy, which is a measure of the number of energetically equivalent states, increases as the side chains in a region increase in flexibility or increase in translational degrees of freedom (Wand & Sharp, 2018). Although increasing dynamics of side chains increases entropy, often, in an enzyme system, increasing the rigidity of a region of the system can increase the flexibility of another area of the enzyme, and ultimately increase the conformational entropy of the whole system. This is how a reduction in flexibility can be more energetically favorable (Li et al., 2014).

**Figure 5-2. The free energy landscape of SaPC with and without allosteric activation.** The Gibbs free energy of PC plotted as a function of conformational state. The free energy landscape in the presence of allosteric activator acetyl-CoA is represented by the green trace and without activation by the black trace.
BC Domain Causes Conformational Variability

As previously mentioned, the first structure of PC, RePC, revealed an asymmetric tetramer, where the ADs are in close proximity on one face of the tetramer and far apart on the other (St. Maurice et al., 2007). A later crystal structure of ΔBCΔBCCP RePC revealed that the CT and ADs were positioned symmetrically in the tetramer, suggesting that the asymmetric tetramer configuration in RePC was caused by the BC domains (Lietzan & St. Maurice, 2013a).

One of the ways acetyl-CoA allosterically activates PC is through stabilizing the BC domain dimer. In the recent HsPC cryo EM study, when acetyl-CoA was absent the BC domains were too flexible to be modeled into the structure and were unresolved. When acetyl-CoA was added, two of the BC domains that formed a BC dimer, were stabilized and could be visualized in the structures (Chai et al., 2022). Even at saturating amounts of acetyl-CoA only two molecules of acetyl-CoA were bound to the tetramer in these cryo EM structures, allowing for only one BC dimer to be modeled (Figure 5-3B). The number of allosteric activating molecules that can bind to a tetramer at once may be species-specific. In the structure of RePC, two activating molecules were also bound to the tetramer, but on the same face of the tetramer, whereas in the HsPC structure they were on opposite faces (St. Maurice et al., 2007). SaPC and LlPC structures typically have four activating compounds bound (López-Alonso et al., 2022; Yu et al., 2009) (Figure 5-3).
Figure 5-3. Activating compounds bound to tetrameric PC. CoA/acetyl-CoA molecules are shown as spheres in all panels and PC is shown in cartoon representation. A. PDB 2QF7. Two activating compounds are bound to asymmetric RePC. B. PDB 7WTD. Two activating compounds are bound to symmetric HsPC. C. PDB 3HO8. Four activating compounds are bound to symmetric SaPC. D. PDB 7ZY8. Four activating compounds are bound to symmetric LlPC.
Previously it was thought that asymmetric tetramers could only bind two activators, whereas symmetric tetramers could bind four. This has since been disproven, because structures of symmetrical HsPC with two molecules bound have been observed (Chai et al., 2022). In the structure I obtained of WT SaPC with acetyl-CoA analog (Xtal455), acetyl-carba(dethia), four compounds were bound to the tetramer, whereas R21A SaPC co-crystallized with acetyl-CoA revealed two binding sites with well-defined electron density corresponding to the activator, but the other two sites had extremely weak density. R21A SaPC has a lower affinity for acetyl-CoA than WT SaPC does, which could be the reason for low occupancy in two of the binding sites. Alternatively, the crystal structure could represent a snapshot of the tetramer after two activating compounds have been released. The symmetry between the ADs on opposite faces of the tetramer is no longer a focus because over the past decade of PC research it has been elucidated that it only occurs in PC with low intrinsic activity (Chai et al., 2022).

**Allosteric Activation Coordinates Domain Flexibility**

X-ray crystal structures are snapshots of enzymes, and it is hard to gain dynamic information from this technique. A crude dynamic parameter that can be obtained from crystallographic data, B-factors, represent the attenuation of x-ray scattering caused by thermal motion (Sun et al., 2019). B-factors, also referred to as the temperature factors or atomic displacement parameters, can be used to identify regions in the crystal structure that deviate in their flexibility. Each atom in an X-ray crystal structure is refined with a unique B-factor, and in the B-factor for a residue can be estimated simply by averaging the B-factors of its constituent atoms. Similarly, the B-factors from each residue in a
chain are averaged and these averaged subunit B-factors can be compared (Figure 5-5). Since the overarching degree of order (the Wilson B-factor) in a crystal differs between crystals, absolute B-factor values cannot be compared between structures. However, trends in B-factor values can be used to identify local differences in flexibility between similar structures with different ligands.

There is a wealth of structural information available for PC, especially SaPC (Table 1-1). Within a single subunit of PC, individual regions typically display higher B-factors. This is most notable for the highly flexible B-subdomain lid and the conformationally dynamic BCCP domain. These regions are often undefined in the crystal structures due to poorly defined electron density caused by this high degree of flexibility (Figure 5-4).

![Figure 5-4. B-factors vary throughout a subunit of PC. B-factors of chain A from PDB 3HO8 with the corresponding domain on the X-axis. B-subdomain lid and BCCP domain are not modeled in this structure.](image-url)
When comparing structures of SaPC with and without an allosteric activator present there is a clear difference in B-factor trend. Structure 3HO8, which includes hydrolyzed acetyl-CoA, has two subunits of the tetramer with lower B-factor values in the CT domain (Xiang & Tong, 2008; Yu et al., 2009). The two subunits of 3HO8 that have reduced B-factors are chains B and C, which comprise one CT dimer. Structure 3BG5, which was crystallized in the absence of acetyl-CoA, had similar B-factor profiles in all four subunits (Figure 5-5A and B). The B-factors in the BC domain of 3BG5 are higher relative to the CT domain, than in 3HO8. As previously mentioned, one way acetyl-CoA allosterically activates PC is through BC domain stabilization. Consistent with this notion, the structure of SaPC without acetyl-CoA present has greater relative flexibility in the BC domain than the structure with CoA (Figure 5-5B).

The structure of WT SaPC (Xtal455) with the non-hydrolysable acetyl-CoA analog, acetyl-carba(dethia) CoA, (Chapter 2) revealed the same trend in B-factors as was observed in 3HO8, the structure of SaPC with CoA bound. Two subunits, B and C, of Xtal455 had lower B-factors in the CT domain and these subunits come into contact at their CT dimer interfaces (Figure 5-5D). The structure of R21A SaPC with acetyl-CoA/CoA, 8GK8, (Chapter 3) showed a more subtle trend. As described in Chapter 2, residues Arg21, Lys46, and Glu418 are all implicated in the allosteric mechanism. These residues affect both the degree of activation and the binding affinity of the allosteric activator. The B-factors of 8GK8 also show more flexibility in the BC domain, compared to 3HO8 and Xtal455. The B-factors for subunits B and C in 8GK8 are slightly lower than in A and D. Therefore, the same trend is seen but is not as apparent as in 3HO8 and Xtal455, likely due to the disrupted allosteric mechanism. The correlation between B-
factors only occurs when acetyl-CoA is present, indicating that it is promoting tetrameric coordination and modulating the flexibility of the CT dimers in opposite corners of the tetramer.

The B-factors in the structure of K46A SaPC, Xtal447, are overall very high, because this crystal structure has a high Wilson B-factor. This is due to poor data quality and was one of the reasons this structure was not deposited. The same slight trend was seen in K46A SaPC, similar to the R21A SaPC structure, where subunits B and C had lower B-factor values, but no large conclusions can be made from this data because of the poor data quality.

Both Xtal455 and 3HO8 show the same trend in B-factor values. Both structures are of WT SaPC with an activator bound. Conversely, R21A SaPC (PDB 8GK8) has a disrupted allosteric mechanism and the B-factors from that structure fall somewhere between the trends seen in 3HO8 (with CoA) and 3BG5 (without CoA). The BC domain of 8GK8 has higher B-factor values indicating increased flexibility, like 3BG5, but the B-factors for subunits B and C in the CT domain are lower, like 3HO8. While B-factors are a crude measure of dynamics within a crystal structure, these trends point towards allostery modulating the flexibility of certain domains in a coordinated manner. The presence of acetyl-CoA reduces the flexibility in the CT dimer at one corner of the tetramer, while the other CT dimer displays an increase in flexibility. This change in conformational dynamics likely impacts carrier domain positioning. Acetyl-CoA mediates the allosteric mechanism through introducing asymmetric flexibility to the tetramer.
Figure 5-5. B-factors for SaPC structures both with and without an allosteric activator present. A. B-factors for 3HO8 which is SaPC with CoA. B. B-factors for 3BG5 which is SaPC with ATP and pyruvate present but with no allosteric activator present. C. B-factors for 8GK8 which is R21A SaPC with acetyl-CoA/CoA. D. B-factors for Xtal455 which is SaPC with acetyl-carba(dethia)-CoA. For all panels chain A is in red, chain B is in blue, chain C is in yellow and chain D is in green.
Table 5-1. Wilson B-factors and average subunit B-factors for full length SaPC crystal structures.

<table>
<thead>
<tr>
<th>Crystal Structure</th>
<th>Wilson B-Factor</th>
<th>Chain</th>
<th>Avg Chain B-Factor</th>
</tr>
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<tbody>
<tr>
<td>3HO8 WT SaPC CoA</td>
<td>67.9</td>
<td>A</td>
<td>78.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>54.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>54.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>85.59</td>
</tr>
<tr>
<td>3BG5 WT SaPC</td>
<td>60.71</td>
<td>A</td>
<td>65.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>73.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>68.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>64.24</td>
</tr>
<tr>
<td>8GK8 R21A SaPC CoA</td>
<td>50.4</td>
<td>A</td>
<td>53.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>45.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>42.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>52.2</td>
</tr>
<tr>
<td>Xtal455 WT SaPC acetyl-carba(dethia)-CoA</td>
<td>64.08</td>
<td>A</td>
<td>70.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>45.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>45.16</td>
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<tr>
<td></td>
<td></td>
<td>D</td>
<td>70.06</td>
</tr>
<tr>
<td>Xtal447 K46A SaPC CoA</td>
<td>71.05</td>
<td>A</td>
<td>69.89</td>
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<tr>
<td></td>
<td></td>
<td>B</td>
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<td>C</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>D</td>
<td>96.45</td>
</tr>
</tbody>
</table>

Allosteric Activation Coordinates Carrier Domain Positioning in the Tetramer

The B-factor trends seen in SaPC with allosteric activation show a clear trend and suggest that there is tetrameric coordination in domain flexibility. Another indication that acetyl-CoA allosteric activation elicits tetrameric coordination is the recovery of inactivation phenomena seen in the kinetics of inactivation data. As described in Chapter
4, when the carrier domain is crosslinked in the CT domain active site, in the presence of acetyl-CoA, a recovery of crosslinking inactivation is seen after 120 seconds. This phenomenon only occurs when the BCCP domain is being crosslinked in the CT domain active site when acetyl-CoA is present. This phenomenon is only possible if one face of the tetramer is irreversibly crosslinked, and the other face is un-crosslinked and therefore can remix and form new catalytically active tetramers. I propose that this only happens when acetyl-CoA is present because allostERIC activation is coordinating BCCP positioning so that only one BCCP is in a CT domain active site at a time.

In the exo site crosslinking trap, acetyl-CoA increased the $k_{\text{obs}}$ value, but recovery of inactivation was never observed. This means that at least one carrier domain on each face of the tetramer is crosslinked in the CT exo site, both in the presence and absence of acetyl-CoA. In both the active site and exo site crosslinking systems, about 30% of activity is always retained. At saturating concentrations of crosslinker, the population of enzyme is never fully crosslinked, as observed in both SDS-PAGE and kinetics of inactivation experiments. This could be due to one of the four BCCP domains being sequestered and unable to be crosslinked. SaPC has consistently been observed with only three carrier domains resolved likely because the fourth is too flexible to be seen in the electron density (Xiang & Tong, 2008).

Combining the crosslinking data from both the CT active and CT exo site kinetics of inactivation experiments in the presence of acetyl-CoA we can deduce that on one face of the tetramer, one carrier domain is bound in the active site and the other is bound in the exo site. The other face of the tetramer has at least one carrier domain bound in the exo site, and the fourth carrier domain could be transient and unable to be crosslinked.
(Chapter 4). The 2022 cryo EM study utilizing L/PC conducted a global conformational analysis that binned a large dataset of PC structures with each domain categorized by their catalytic state BC/CT\textsubscript{react} or BC/CT\textsubscript{empty} (López-Alonso et al., 2022). The highest positive correlation in the analysis was between BC\textsubscript{react} and BC\textsubscript{react} for BC domains in the same dimer. This indicates that BC domains on opposite faces of the tetramer, but within the same corner of the tetramer, are simultaneously active. CT domains in the same dimer were not correlated indicating that multiple BCCP domains are not occupying multiple CT active sites at once, in agreement with the kinetics of inactivation data in the presence of acetyl-CoA I have described.

Combining recent cryo EM studies with my own work, I can predict a probable tetrameric catalytic cycle, in the presence of allosteric activation (Figure 5-6). When acetyl-CoA is present, on one face of the tetramer, one carrier domain gets carboxylated in the BC active site, and on the other face both carrier domains interact with the BC domains and become carboxylated. Once three carrier domains have carboxybiotin covalently attached, they translocate across the face of the tetramer to the CT domain on the same face of the tetramer. BCCP domains of chains A and D, on opposite faces of the tetramer, translocate to the CT domains of B and C, respectively. From the B-factor data, we can see that the CT domains of subunits B and C are more rigid, and therefore I predict that two carrier domains translocate to this corner of the tetramer simultaneously. One carrier domain binds in the CT active site and the other binds in the CT exo site. The carrier domain from subunit C also translocates to the CT domain exo site of chain D.

In the presence of acetyl-CoA, two subunits have correlated B-factors, subunits A&D and B&C, and these subunits share CT dimer interfaces. This indicates that while
the allosteric activator binds in the BC dimer interface, allosteric communication is being transferred to the CT dimer interface and the CT dimer interface likely participates in intersubunit communication. As described in Chapter 3, the CT domain also likely has an allosteric site, because ΔBCΔBCCP RePC/SaPC can still catalyze the hydrolysis of acetyl-CoA. The CT dimer interface in other microbial PC’s has an allosteric binding site so it is probable that SaPC does too (López-Alonso et al., 2022; Sureka et al., 2014). The allosteric mechanism is mediated through changes in conformational dynamics in the two corners of the tetramer. Changes in flexibility/rigidity to the corners of the tetramer likely aids in the coordination of the BCCP domains (Figure 5-6).
Allosteric Inhibition by L-Aspartate does not Alter Dynamics or BCCP Positioning

Both acetyl-CoA and L-aspartate primarily effect the rate of the BC domain half reaction, the bicarbonate dependent ATP cleavage that results in the formation of carboxybiotin (Sirithanakorn et al., 2014). Acetyl-CoA and L-aspartate binding is mutually exclusive and at high concentrations of acetyl-CoA, L-aspartate inhibition is overcome (Yu et al., 2009). This implies that a portion of their binding sites overlap, but

Figure 5-6. The predicted tetrameric catalytic cycle of SaPC. A. A scheme of carrier domain positioning during the BC domain half reaction, the bicarbonate dependent ATP cleavage. B. A scheme of carrier domain positioning during the CT domain half reaction, the carboxylation of pyruvate. The carrier domain colored in faded red on the top face in panels A and B represents the carrier domain that is unresolved.
mutations near the nucleotidyl portion that affect acetyl-CoA activation had no effect on L-aspartate inhibition (Adina-Zada, Sereeruk, et al., 2012; Sirithanakorn et al., 2014, 2016). Mutations to residues in the BC dimer interface that interact with the acetyl moiety eliminated L-aspartate inhibition. R21A, K46A and E418A SaPC all lost their ability to be inhibited by L-aspartate (Chapter 2). This demonstrates that the mutual exclusivity of acetyl-CoA and L-aspartate binding is due to an overlap in binding sites at the BC dimer interface where the acetyl moiety of acetyl-CoA binds.

From a metabolic standpoint, the competitive binding of allosteric effectors acetyl-CoA and L-aspartate at the acetyl moiety binding site is highly logical. L-Aspartate serves as a feedback inhibitor of PC when the cell is in the metabolic “fed” state, which contributes to metabolic homeostasis. The acetyl-CoA/CoA ratio serves as a way for the cell to sense the metabolic state of the cell. The most direct way for this feedback inhibitor to alleviate activation in the fed state is to compete at the acetyl moiety binding site that is directly responsive to the acetyl-CoA/CoA ratio.

Unlike allosteric activation, allosteric inhibition is much more localized to the BC domain half reaction. L-Aspartate has no effect on the CT domain reaction and when 5 mM L-aspartate is present the IC$_{50}$ values of CT domain inhibitors do not change (Chapter 2). This implies that L-aspartate has no effect on the CT domain. Additionally, L-aspartate does not change the inactivation profile of the CT active site or CT exo site crosslinking traps (Chapter 4). Allosteric inhibition does not alter carrier domain translocation but likely inhibits PC through inhibiting carboxybiotin formation in the BC domain half reaction.
A Model of Allostery in SaPC

In the past, PC research has been focused on asymmetry in the two layers of the enzyme. In my research and in recent cryo EM studies, a different type of asymmetry has been revealed. Instead of the asymmetry altering opposing layers of the tetramer, the asymmetry is centered at the corners of the tetramer and involves both faces of the tetramer. Although half-the-sites reactivity likely does not occur in the BC domains (Adina-Zada et al., 2019; López-Alonso et al., 2022), and the BC dimer interface is not mediating the half-the-sites activity between the two faces, the BC dimer is still an extremely important locus of allosteric regulation and intersubunit communication. Mutations in residues that form intersubunit contacts disrupt both allosteric activation by acetyl-CoA and allosteric inhibition by L-aspartate. The acetyl moiety of acetyl-CoA binds in the BC dimer interface and interacts with Arg21, Lys46, and Glu418. The binding of this allosteric activator elicits a subtle structural change and activates the pyruvate carboxylation reaction by catalyzing the bicarbonate dependent ATP cleavage in the BC domain while also increasing the binding affinity of both BC and CT domain substrates. Additionally, acetyl-CoA decreases the binding affinity of CT domain inhibitors. Allosteric activation is mediated through changes in conformational dynamics. The rate of carrier domain translocation increases, and carrier domain positioning is coordinated in the tetramer. These changes in conformational dynamics, mediated by acetyl-CoA, could be due to changes in flexibility that promotes dissociation of the carrier domain and subsequent translocation. The acetyl-CoA allosteric mechanism is entropically driven through asymmetric dynamic changes in the corners of the tetramer that increases rigidity in one corner while flexibility is increased in the other. This leads to a change in
conformational entropy is energetically favorable. The movement of the carrier domain is governed by conformational selection, where the carrier domain exists in a dynamic equilibrium that is shifted by substrates and effector molecules. While substrates do not induce conformational changes, the dynamic movement of the carrier domain is regulated by allosteric activation and acetyl-CoA promotes the coordination of carrier domain movement in the tetramer.

**Impact of Characterizing Allostery and Conformational Dynamics in *SaPC***

Describing the underlying molecular mechanisms of allosteric control in *SaPC* has broader implications for numerous other PCs, including *HsPC*. The allosteric site responsible for binding acetyl-CoA, situated at the BC dimer interface, exhibits remarkable conservation. Key residues, namely Arg21, Lys46, and Glu418, which my analysis has identified as pivotal for allosteric regulation in *SaPC*, are conserved in *HsPC*. The ionic interactions occurring between these residues and acetyl-CoA could be harnessed for designing inhibitors aimed at reducing PC activity in scenarios where its hyperactivity contributes to cancer progression and metastasis.

The highly conserved nature of the allosteric site is problematic when designing inhibitors specifically for *SaPC* to interfere with the virulence mechanism of *Staphylococcus aureus*. Due to the high conservation, an inhibitor that binds to the allosteric site in *SaPC* would likely also interfere with allosterity in *HsPC*, which as can result in severe physiological issues (Wexler et al., 1998). The CT domain of *SaPC* is not highly conserved therefore targeting this catalytic domain in inhibitor design would be preferred.
Investigating the conformational dynamics and mechanisms of regulation that modulate carrier domain positioning not only impacts PC but can also be applied to other carrier domain enzymes. The work conducted here demonstrates that carrier domain movement can be influenced by allostery and that not all subunits are simultaneous active.

From a basic science standpoint, this work contributes to a clearer understanding of dynamic allostery that can be applied to any enzyme that exhibits little to no structural change when the allosteric effector is present. The biophysical tools established in this work, and previous work conducted in our lab, can be applied to other carrier domain enzymes to probe changes in dynamics.
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