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Conformational Selection Governs Carrier Domain Positioning in *Staphylococcus aureus* Pyruvate Carboxylase

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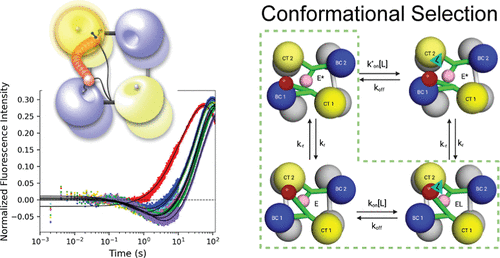
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# Abstract



Biotin-dependent enzymes employ a carrier domain to efficiently transport reaction intermediates between distant active sites. The translocation of this carrier domain is critical to the interpretation of kinetic and structural studies, but there have been few direct attempts to investigate the dynamic interplay between ligand binding and carrier domain positioning in biotin-dependent enzymes. Pyruvate carboxylase (PC) catalyzes the MgATP-dependent carboxylation of pyruvate where the biotinylated carrier domain must translocate ∼70 Å from the biotin carboxylase domain to the carboxyltransferase domain. Many prior studies have assumed that carrier domain movement is governed by ligand-induced conformational changes, but the mechanism underlying this movement has not been confirmed. Here, we have developed a system to directly observe PC carrier domain positioning in both the presence and absence of ligands, independent of catalytic turnover. We have incorporated a cross-linking trap that reports on the interdomain conformation of the carrier domain when it is positioned in proximity to a neighboring carboxyltransferase domain. Cross-linking was monitored by gel electrophoresis, inactivation kinetics, and intrinsic tryptophan fluorescence. We demonstrate that the carrier domain positioning equilibrium is sensitive to substrate analogues and the allosteric activator acetyl-CoA. Notably, saturating concentrations of biotin carboxylase ligands do not prevent carrier domain trapping proximal to the neighboring carboxyltransferase domain, demonstrating that carrier domain positioning is governed by conformational selection. This model of carrier domain translocation in PC can be applied to other multi-domain enzymes that employ large-scale domain motions to transfer intermediates during catalysis.

# Introduction

Conformational changes accompanying ligand binding in biological macromolecules are often described using two contrasting models. The induced fit model states that ligand binding induces a conformational change in the macromolecule,(1,2) while the conformational selection model states that the macromolecule pre-exists in multiple conformational states, with ligand binding shifting the population toward the ligand-bound conformation(3,4) (Figure 1). These models continue to be updated; modern interpretations consider perturbed free energy landscapes on equilibrium populations and recognize that induced fit and conformational selection are not mutually exclusive.(5,6) Nevertheless, the tendency of a system to follow induced fit versus conformational selection has important mechanistic implications and remains an active area of investigation. While these conformational change models have been tested and elaborated in many systems, they have not been thoroughly evaluated in the so-called “swinging domain enzymes,” which are large, multi-domain enzymes that employ carrier domains to transfer intermediates between remotely located active sites.(7)

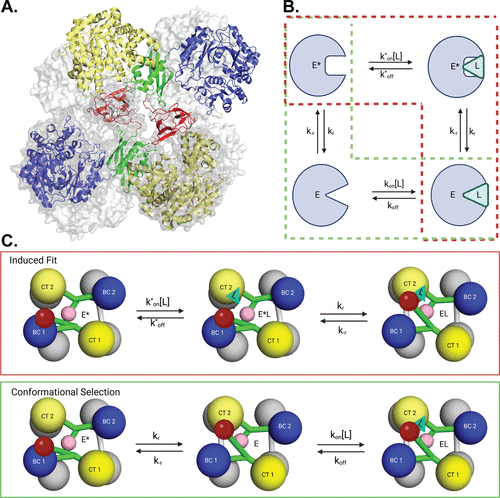


Figure 1. *Staphylococcus aureus* PC structure and dynamic conformational changes. (A) Overall structure of the *S. aureus* PC tetramer. BC domains are colored in blue, CT in yellow, BCCP in red, and the allosteric domain in green. A single, individual subunit is contoured in gray. The two subunits on the top face of the tetramer are illustrated with secondary structure, while the two subunits on the bottom face are illustrated in surface representation and colored in white (PDB ID: 3BG5). (B) Simple representation of the induced fit model (outlined in red) and the conformational selection model (outlined in green) for ligand binding. E\* represents the enzyme in a conformation not optimally suited for ligand binding, while E represents the enzyme in a conformation suitable for ligand binding. The triangle L represents the ligand (adapted from ref(14)). (C) Representations of induced fit and conformational selection in the context of PC carrier domain movement, where triangle L represents a CT domain ligand. Note the key difference between these models: in the conformational selection model, the ligand is not required for the carrier domain to access the CT domain. Conversely, in the induced fit model, the ligand is required for carrier domain translocation. (Created using Biorender.com).

Swinging domain enzymes offer an intriguing and challenging system to study how ligands influence large-scale protein conformational changes. It is not clear how carrier domain conformational changes facilitate intermediate transfer in a coordinated, unidirectional manner in these enzymes. For example, assembly line polyketide synthases are built on an array of multiple modules and domains, requiring exquisite coordination as intermediates are transferred between active sites. Vectorial synthesis in multi-modular enzymes is proposed to depend on conformational changes among the catalytic and carrier domains,(8) but it is not known if these conformations interchange freely at equilibrium or whether conformational changes are induced by ligand binding and catalytic turnover events. For swinging domain enzymes undergoing large-scale conformational changes, this is a particularly relevant question. In these systems, the movement of the carrier domain between distant active sites is expected to be slow relative to the functionally relevant rates of ligand binding and dissociation, consistent with a conformational selection model.(9) Despite this, induced fit has often been invoked as the dominant mechanism governing carrier domain movement in many of these enzymes.

Conformational changes are difficult to directly investigate in large and unwieldy swinging domain enzymes for which conventional structural and functional approaches do not easily apply. Instead, smaller and more tractable swinging domain enzyme systems can serve as structurally and biophysically accessible paradigms to study the relationship between ligand binding and large-scale protein conformational changes. Pyruvate carboxylase (PC) is a homotetrameric, swinging domain enzyme that has been extensively structurally and functionally characterized.(10−13) During catalysis, the carrier domain of PC undergoes a conformational change that spans more than 70 Å, offering an ideal system in which to study the influence of ligand binding on large-scale conformational changes.

PC catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate, with bicarbonate serving as the carboxyl group donor. The reaction proceeds through two connected half-reactions catalyzed in distinct and remote active sites (Figure 2). The biotin carboxylase (BC) domain catalyzes the first half-reaction, where the biotin cofactor on the biotin carboxyl carrier protein (BCCP) domain is carboxylated with the concomitant cleavage of MgATP. The carboxyltransferase (CT) domain catalyzes the second half-reaction, where the carboxyl group is transferred from the biotin cofactor on the BCCP domain to pyruvate, generating oxaloacetate. These two half-reactions are coupled through the intersubunit translocation of the biotinylated carrier domain, which traverses a distance greater than 70 Å to transfer the carboxyl group between active sites. (15−17)

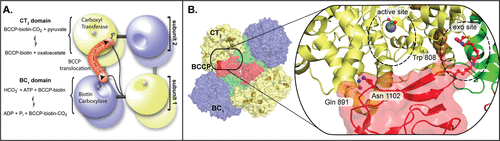


Figure 2. Catalysis and carrier domain translocation in PC. (A) PC catalysis occurs in two distinct active sites connected by carrier domain translocation. In the primary translocation pathway, the biotin cofactor is carboxylated in the biotin carboxylase domain of subunit 1 (BC1) and is carried to the carboxyltransferase domain of subunit 2 (CT2) by the BCCP carrier domain. (B) A cross-linking trap was established by mutating both Gln891 and Asn1102 to cysteine residues in *S. aureus* PC. A cross-linker captures the BCCP domain of subunit 1 when it is positioned in close proximity to the CT domain of subunit 2, with the biotin cofactor bound at the nearby exo-binding site. The residue numbering corresponds to the amino acid sequence in *S. aureus* PC. Note that the amino acid residues in the deposited crystal structure (PDB ID: 3BG5) were renumbered according to the human sequence. Consequently, Gln891 and Asn1102 in *S. aureus* PC are identified by their human equivalent residue numbers Gln 923 (chain A) and Asn 1134 (chain B), respectively, in the deposited 3BG5 crystal structure.

Prior efforts to study the influence of ligand binding on carrier domain translocation in PC have largely relied on interpretations of kinetic data. For example, detailed studies of carboxybiotin decarboxylation in sheep liver PC(18,19) and chicken liver PC(20) 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.(21,22) 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 best account for conformational change in the carrier domain.

In contrast to the interpretations described above, snapshots of PC from X-ray crystallography and cryo-electron microscopy have revealed that, consistent with conformational selection, multiple conformational states and carrier domain positions appear to pre-exist in PC and that these are independent of the identity or bound state of the ligand.(15,16,23) These structural studies suggest that the carrier domain may sample multiple positions in a ligand-independent dynamic equilibrium.(24,25) Ultimately, neither kinetic measurements nor structural studies are suitable to determine whether carrier domain positioning exists in a dynamic equilibrium (conformational selection) and/or is governed by ligand-induced conformational changes. This question can best be addressed by directly following the positioning of the carrier domain.

Carrier domain translocation in PC likely plays a role in coordinating and regulating catalytic turnover. Thermodynamic linkage analysis of *Staphylococcus aureus* PC (*Sa*PC) demonstrated that acetyl-CoA promotes strong coupling between pyruvate binding in the CT domain and MgATP binding in the BC domain; this coupling is abolished in the absence of acetyl-CoA, leading to the proposal that acetyl-CoA constrains the movement of the highly flexible BCCP carrier domain to facilitate coordination and communication between distant active sites.(26) Our recent work has also demonstrated that the carrier domain accesses a wide range of catalytically productive positions and that this conformational sampling is restricted in the presence of acetyl-CoA.(27) These recent studies suggest that the BCCP carrier domain contributes to coordinating catalysis between remote active sites, but the mechanism by which the carrier domain movement is controlled, if at all, remains unexplored. To explore this question, methods are required that directly investigate how changes in the conformational equilibrium of PC reposition the BCCP carrier domain in the absence of catalytic turnover.

Here, we present several approaches to directly observe changes in the conformational equilibrium of *Sa*PC that reposition the BCCP carrier domain. Using an intersubunit cross-linking trap, combined with a variety of analytical approaches, the positioning of the BCCP carrier domain is directly observed as a function of substrates and allosteric effectors. These data demonstrate that carrier domain positioning in PC is governed by conformational selection. This work clarifies the mechanism of carrier domain positioning in PC and may be extended to other carrier domain enzyme systems that function in vectorial biosynthesis.

# Materials and Methods

Acetyl-CoA was purchased from Crystal Chem, Inc. (Elk Grove Village, IL) and CoALA Biosciences (Austin, TX). All other materials were obtained from standard commercial sources. *S. aureus* PC (UniProtKB─A0A0E8G8A7) was previously cloned into a modified pET-27b vector and was generously supplied by Dr. Liang Tong, Columbia University.(17)

## SaPC Expression and Purification

All *Sa*PC genes were expressed and purified in an identical manner. Mutations in *Sa*PC were generated according to the Agilent QuikChange II site-directed mutagenesis protocol. Primers were obtained from Integrated DNA Technologies (Coralville, IA). All constructs used in this study were confirmed by DNA sequencing of the gene in its entirety.

All PC 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 of kanamycin and 30 μg/mL of 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 and l-arabinose to a final concentration of 0.5 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 h before harvesting by centrifugation.

All *Sa*PC enzymes were purified using sequential Ni2+-affinity and anion-exchange chromatography. Harvested cell paste (20–30 g) was re-suspended in 200 mL of 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 min at a temperature between 4 and 10 °C and pelleted by centrifugation. The supernatant was loaded on a 5 mL Ni2+-nitrilotriacetic acid profanity 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 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 1,4-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 in dialysis buffer with a gradient from 50 mM to 1 M NaCl. *Sa*PC typically elutes between 400 and 800 mM of 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 Tris(2-carboxyethyl)phosphine (TCEP)] for three successive changes of 4 h or more. The protein was concentrated to a final concentration of 4–10 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 Cross-Linking

Bismaleimidoethane (BMOE) was prepared in 100% dimethyl sulfoxide (DMSO) at 20× the desired reaction concentration. *Sa*PC was reacted at room temperature at a final concentration of 1 mg/mL (premixed with substrates for a minimum of 10 min when applicable) in a buffer containing 0.1 M Tris-HCl (pH 7.8), 7 mM MgCl2 with the desired concentration of BMOE, and identical concentrations of the substrate, when applicable. After a defined incubation period, DTT was used to quench the cross-linking reaction at a final concentration of 13 mM for 15 min at room temperature. Samples were then either assessed for activity using the MDH-coupled assay described below or mixed with 4× Laemmli buffer under reducing conditions and heated to 100 °C for 3 min 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)).

Densitometric analyses were performed using the LabWorks software developed by UVP, Inc. (Upland, CA). The coloration intensity of each band was determined from images of the SDS-PAGE gels. The normalized density of the monomer band was determined by dividing the monomer band density by the total band density for both the monomer and dimer bands. Plots of normalized density against time were fitted to eq 1, where *a*1 represents the initial normalized band density at *t* = 0, *a*0 describes the limiting normalized band density, *t* is the time in seconds, and *k*obs is the observed rate constant of cross-link formation.

(1)

## 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). 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 to 10 μg/mL per reaction. Data from cross-linking inactivation assays were fit to eq 2 using a least-squares regression in GraphPad Prism (version 8.1.1 for Windows, GraphPad Software, San Diego, California, USA). The observed rates of inactivation were determined by fitting the data to a single exponential decay (eq 1) or a double exponential decay (eq 2), where *a*1 and *a*2 represent the amplitudes,  and  represent the observed rates, *t* represents the time in seconds, and *a*0 represents the limiting value.

(2)

## 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. *Sa*PC (0.35 mg/mL) was premixed with substrates for a minimum of 10 min when applicable and was loaded in one drive syringe and mixed 1:1 with the substrate/BMOE mixture in the second drive syringe. The final concentration of substrates or ligands used was 2.5 mM of ATP, 5 mM of ADP, 5 mM of phosphonoacetate (PPA), 0.25 mM of acetyl-CoA, and 12 mM of pyruvate. 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 4–5 replicates. Reported rates are the average value of three independent acquisitions of these replicates. Where the data were normalized to enable comparative visualization, 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 fitted to a double exponential burst (eq 3), where *a*1, and *a*2 represent the amplitudes,  and  represent the observed rates, *t* represents the time in seconds, *c* represents the slope of the linear phase, and *a*0 represents the initial value at *t* = 0. The values for  and  were replotted against the concentration of ADP + PPA and fit to eq 4, which describes an inverse rectangular hyperbola where  is the observed rate constant in the absence of the ligand,  is the limiting value for the observed rate constant at the saturating ligand, and *K*0.5 is the concentration of ligand at which .

(3)

(4)

## 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 eight-step kinetic scheme, with the observable indicated in eq 5, where *a* and *b* are scaling factors and F = BCCP-CT and FX = BCCP-CT-X.

(5)

# Results

## Cross-Linkers Trap an Intersubunit Carrier Domain Conformation in SaPC

To directly observe how *Sa*PC carrier domain positioning is altered with changes in the *Sa*PC conformational equilibrium, we designed a trap for the BCCP carrier domain using maleimide-based homobifunctional cross-linkers. Specifically, the system was designed to capture the BCCP carrier domain as it accesses an intersubunit interaction with the CT domain on the opposing subunit of the *Sa*PC tetramer (Figure 2). In crystal structures of *Sa*PC, the carrier domain has been observed to interact with the CT domain on the opposing subunit in two different conformations. In one conformation, *Sa*PC re-equilibrates to position the carrier domain with biotin inserted into the active site, while in the second conformation, the carrier domain is positioned with biotin in the so-called “exo-binding site,” located immediately adjacent to the CT domain active site. We chose to trap *Sa*PC with the carrier domain positioned in the exo-binding site to minimize introducing mutations that might directly interfere with catalytic turnover. Moreover, the carrier domain has been observed in the intersubunit exo-binding site conformation in all *Sa*PC crystal structures, implying that this conformation is readily accessed as a low-energy conformational state. Two specific residues were targeted for mutation to cysteine based on X-ray crystal structures of *Sa*PC(17) (PDB ID: 3BG5): Gln891 on the CT domain and Asn1102 on the BCCP domain were both mutated to cysteine to generate single mutant controls (Q891C *Sa*PC and N1102C *Sa*PC) and the double mutant (Q891C/N1102C *Sa*PC; Figure 2B). The Q891C/N1102C double mutant and the corresponding single mutants retained 60–80% of the wild-type pyruvate carboxylation activity, indicating that mutations at these residues minimally impact catalytic turnover (Table S1).

To characterize the amenability of this system to cross-linking, Q891C *Sa*PC, N1102C *Sa*PC, and Q891C/N1102C *Sa*PC were incubated with bismaleimidoethane (BMOE) for 15 min and analyzed by SDS-PAGE (Figure 3A). Cross-linking was predicted to occur between the BCCP carrier domain of one subunit and the CT domain of the adjacent subunit, resulting in cross-linked samples that would run at twice the subunit molecular weight (*Sa*PC monomer = 131 kDa) to yield a ∼260 kDa cross-linked dimer. No significant cross-linked dimers were observed in the Q891C and N1102C single mutants across a wide range of BMOE concentrations. Compared to these controls, a high percentage of cross-linked dimers were observed in the Q891C/N1102C double mutant at 300 μM and 1 mM. BMOE was selected as the cross-linker for its relatively short spacer arm, but there was no discernible difference observed for longer cross-linkers (Figure S1). A small percentage of high molecular-weight species was observed at all BMOE concentrations in both the single mutants and the Q891C/N1102C double mutant. Analytical ultracentrifugation of cross-linked wild-type *Sa*PC and Q891C/N1102C *Sa*PC confirmed the presence of high molecular-weight species composed of large, aggregated proteins (data not shown). These species presumably arose from nonspecific cross-linking between individual protomers in pre-existing high molecular-weight soluble aggregates that are present in all purified samples of recombinant PC. Since a similar baseline level of high molecular-weight, cross-linked species were present for all variants incubated with BMOE, they were considered to be nonspecific and were not included in our subsequent analysis of Q891C/N1102C cross-linking kinetics.

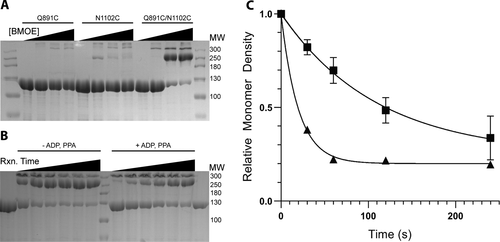


Figure 3. Q891C, N1102C, and Q891C/N1102C *Sa*PC cross-linked with BMOE. A. Representative 8% acrylamide SDS-PAGE gel of *Sa*PC mutants (5 μg) reacted with either 0 μM, 10 μM, 300 μM, or 1 mM BMOE for 15 min. Q891C *Sa*PC and N1102C *Sa*PC did not form cross-linked dimers at BMOE concentrations greater than 300 μM. Q891C/N1102C *Sa*PC was primarily cross-linked into dimers at BMOE concentrations of 300 and 1000 μM. B. Representative 8% acrylamide SDS-PAGE gel showing time-dependent cross-linking Q891C/N1102C *Sa*PC with BMOE ± [5 mM ADP + 5 mM phosphonoacetate (PPA)] for various reaction times. Q891C/N1102C *Sa*PC (5 μg) was incubated with 0 or 300 μM BMOE for the indicated times in the absence/presence of 5 mM ADP + 5 mM PPA and then quenched with 13 mM DTT for 15 min. Lane 1: Q891C/N1102C *Sa*PC reacted with 0 μM BMOE. Lanes 2–7: Q891C/N1102C *Sa*PC reacted with 300 μM BMOE for 30 s, 1 min, 2 min, 4 min, 8 min, or 15 min. Lane 8: molecular weight ladder. Lanes 9–14: Q891C/N1102C *Sa*PC pre-incubated with 5 mM ADP + 5 mM PPA for 15 min, then reacted with 300 μM BMOE for 30 s, 1 min, 2 min, 4 min, 8 min, and 15 min. Lane 15: wild-type *Sa*PC reacted with 300 μM of BMOE. (C) Densitometric analysis of monomer bands in Figure 3B as a function of time fitted to a single exponential decay described by eq 1. Error bars represent the standard deviation of densitometry measurements from three separate gels. Either no substrates were added (▲) or 5 mM ADP + 5 mM PPA (■) were present during cross-linking. The y-axis represents the normalized monomer density determined as described in the Methods section.

To initially assess the rates of cross-linked dimer formation, the mutants were analyzed via SDS-PAGE. In the absence of ligands, the percentage of cross-linked dimers rapidly approached equilibrium (Figure 3B). However, in the presence of a BC domain intermediate analogue, ADP + phosphonoacetate (PPA), the rate of approach to equilibrium was greatly reduced (Figure 3B). The combination of ADP and PPA (ADP + PPA) was chosen because it mimics the carboxyphosphate reaction intermediate that forms in the BC domain following ATP cleavage. A quantitative densitometric analysis was performed to determine the change in monomer density as a function of time (Figure 3C). These data, best described by a single exponential decay (eq 1), revealed that ADP + PPA dramatically reduced the observed rate constant for cross-linked dimer formation from *k*obs = 0.051 ± 0.003 s–1 in the absence of ligands to *k*obs = 0.009 ± 0.001 s–1 in their presence, illustrating that BC domain ligands can substantially perturb the rate of carrier domain equilibration. Unfortunately, the rapid rate of equilibration observed in the absence of substrates (Figure 3C, triangles) prevented an accurate determination of *k*obs, and consequently, a comparison of the equilibration kinetics in the presence and absence of the ligand could only be qualitatively assessed using this technique.

To complement and confirm the SDS-PAGE densitometric analysis, inactivation kinetics were also measured as a reporter of carrier domain cross-linking. Cross-links between the BCCP and CT domains will immobilize the carrier domain, thereby inactivating the enzyme by preventing subsequent rounds of carboxyl group transfer between active sites. To evaluate enzyme inactivation and confirm that it was specific to carrier domain cross-linking, initial velocities were measured for Q891C *Sa*PC, N1102C *Sa*PC, Q891C/N1102C *Sa*PC, and wild-type *Sa*PC as a function of cross-linker incubation time (Figures 4 and S2). The native pyruvate carboxylation activities of these mutants are shown in Table S1. Wild-type *Sa*PC retained its full enzymatic activity over 15 min of incubation with 300 μM BMOE. The activity of the Q891C mutant increased slightly upon incubation with BMOE, perhaps as a consequence of masking the cysteine mutation at that position. The N1102C mutation exhibited a slight loss of activity (∼8% over 15 min), which coincides with the small amount of dimers observed by SDS-PAGE (Figure 3A). Compared with the wild-type and individual mutants, the Q891C/N1102C mutant exhibited a rapid and significant loss of activity upon incubation with BMOE, consistent with specific cross-linking between the BCCP carrier domain and the CT domain.

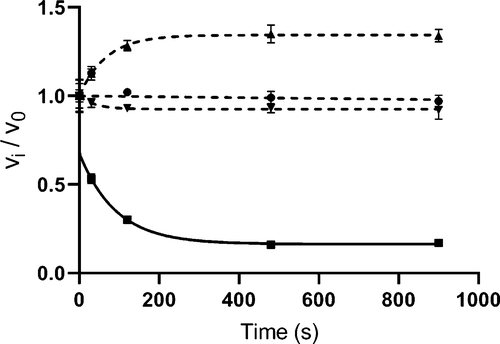


Figure 4. Inactivation kinetics for wild-type *Sa*PC and three mutated constructs of *Sa*PC. Wild-type (●), Q891C (▲), N1102C (▼), or Q891C/N1102C (■) *Sa*PC were reacted with 300 μM BMOE in 5% DMSO for 30 s, 2 min, 8 min, or 15 min. All reactions were then quenched with 13 mM DTT for 15 min, which directly stops the cross-linking reaction. The initial velocity (*v*i) was divided by the initial velocity measured after incubating the same enzyme with 5% DMSO for 15 min (*v*o). Each sample was measured in triplicate using the MDH-coupled enzyme assay. The error bars represent the standard deviation from three independent measurements. Error bars for Q891C/N1102C are included but obscured by the data markers.

## Intrinsic Tryptophan Fluorescence Reports on Carrier Domain Conformational States

The rate measurements of cross-linking by SDS-PAGE densitometry and inactivation kinetics were constrained by manual sample handling and were unable to capture rapid equilibration events. To gain greater time-resolution and accuracy, we explored changes in intrinsic tryptophan fluorescence (ITF) intensity observed through stopped-flow spectroscopy as a reporter on carrier domain positioning. In *Sa*PC, all five tryptophan residues are located in the CT domain (Figures 2B and S3). We predicted that the local environment of the five CT domain tryptophan residues will be impacted by the proximity of the biotinylated BCCP carrier domain. Consistent with this prediction, wild-type *Sa*PC yielded distinct ITF signals in the presence of different ligands (Figure 5A). When wild-type *Sa*PC was mixed with ATP, a BC domain substrate, a rapid decrease in the ITF signal intensity was observed. Conversely, when wild-type *Sa*PC was mixed with acetyl-CoA, an increase in the ITF signal intensity was observed.

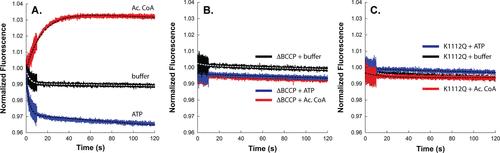


Figure 5. Role of substrates and the biotinylated carrier domain in generating the intrinsic tryptophan fluorescence signal. (A) Wild-type *Sa*PC ITF in the presence of a substrate/effector. Wild-type *Sa*PC (0.35 mg/mL) was mixed with 2.5 mM of ATP (blue), 0.25 mM of acetyl-CoA (red), or buffer (black) at 0 s in a stopped-flow instrument with tryptophan excitation at 295 nm and ITF emission measured at 350 nm. (B) Q891C/ΔBCCP *Sa*PC ITF in the presence of a substrate/effector. Q891C/ΔBCCP *Sa*PC (final concentration of 0.175 mg/mL) was mixed with buffer (black) or final concentrations of 2.5 mM ATP (blue) or 0.25 mM acetyl-CoA (red) at 0 s in a stopped-flow instrument with tryptophan excitation at 295 nm and ITF emission measured at 350 nm. C. Q891C/N1102C/K1112Q *Sa*PC ITF in the presence of a substrate/effector. Q891C/N1102C/K1112Q *Sa*PC (final concentration of 0.175 mg/mL) was mixed with buffer (black) or final concentrations of 2.5 mM ATP (blue) or 0.25 mM acetyl-CoA (red) at 0 s in a stopped-flow instrument with tryptophan excitation at 295 nm and ITF emission measured at 350 nm. Dashed lines represent the fit of the data to eq 3.

We reasoned that the presence of ATP favors a conformational state for the BCCP carrier domain that is reported as a decrease in ITF signal intensity, while the presence of acetyl-CoA favors a different state for the carrier domain that is reported as an increase in ITF signal intensity. We hypothesized that the ITF signal intensity increases when the biotinylated BCCP carrier domain is positioned in close proximity to the CT domain. Consistent with this hypothesis, we first confirmed that the biotinylated carrier domain mediates the observed ITF signal by constructing a C-terminal truncation of *Sa*PC that eliminates the BCCP domain through the introduction of a premature STOP codon in place of amino acid 1062 (Q891C/ΔBCCP *Sa*PC). This ΔBCCP *Sa*PC mutant was mixed with either ATP or acetyl-CoA, and no change in ITF intensity was observed (Figure 5B). This result confirmed that changes in the ITF intensity are a consequence of interactions mediated by the biotinylated BCCP carrier domain and that they are not simply a result of ligand-binding events. The specific contribution of biotin to the ITF signal was also investigated by mutating the biotinylated lysine 1112 to glutamine (Q891C/N1102C/K1112Q) to produce an unbiotinylated *Sa*PC. Again, the fluorescence intensity did not change in the presence of either ATP or acetyl-CoA (Figure 5C), indicating that the biotin cofactor is required to produce the change in ITF signal intensity. Thus, the ITF signal intensity is dependent on both the carrier domain and the biotin cofactor. Consequently, the observed changes in ITF signal intensity must result from a change in the position of the carrier domain.

We next sought to determine how the ITF signal intensity changes in response to carrier domain re-equilibration by evaluating the change in ITF signal intensity in the presence of a BMOE cross-linker, which traps the BCCP carrier domain when it is proximal to the CT domain. While wild-type *Sa*PC and the single mutants displayed minimal changes in ITF signal intensity in the presence of BMOE (Figure 6A), the cross-linking competent Q891C/N1102C *Sa*PC displayed a large increase in ITF intensity in the presence of BMOE. Thus, when the *Sa*PC carrier domain equilibrium shifts to favor an interaction with the CT domain, the ITF signal increases.

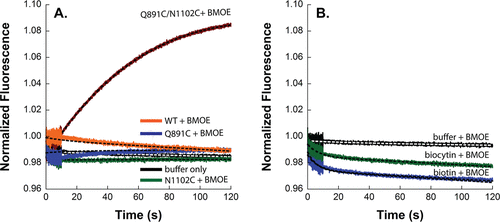


Figure 6. *Sa*PC ITF changes observed in the presence of a cross-linker. (A) Q891C/N1102C mixed with buffer (black), Q891C *Sa*PC reacted with 200 μM BMOE (blue), N1102C *Sa*PC reacted with 200 μM BMOE (green), Q891C/N1102C *Sa*PC reacted with 200 μM BMOE (maroon), and wild-type *Sa*PC reacted with 200 μM BMOE (orange). All mixing occurred at 0 s in a stopped-flow instrument with excitation at 295 nm and ITF emission measured at 350 nm. B. Q891C/N1102C/K1112Q *Sa*PC ITF changes observed when cross-linked in the presence of biotin or biocytin. Q891C/N1102C/K1112Q *Sa*PC (0.175 mg/mL final concentration) was incubated with buffer (black), 5 mM final concentration of biotin (blue), or 5 mM final concentration of biocytin (green) for 15 min before being mixed with an equal concentration of biotin or biocytin and 200 μM final concentration of BMOE at 0 s in a stopped-flow instrument with excitation at 295 nm and ITF emission measured at 350 nm. Q891C/N1102C/K1112Q *Sa*PC mixed with buffer (black) is also shown for reference. Dashed lines represent the fit of the data to eq 3.

## Tethered Biotin Cofactor is Required to Produce the ITF Signal

Consistent with our experiments in the absence of the cross-linker, no ITF signal change was observed when a cross-linking competent unbiotinylated mutant (Q891C/N1102C/K1112Q) was reacted with 200 μM of BMOE (Figure 6B). These results confirm that the tethered biotin cofactor is necessary for the ITF signal, but its effect could be mediated either by producing the ITF signal itself or by contributing to BCCP carrier domain positioning. To distinguish these possibilities, we evaluated the cross-linking of the unbiotinylated *Sa*PC in the presence of BMOE (Figure S4). While the response to substrates was dramatically diminished in unbiotinylated *Sa*PC, the unbiotinylated carrier domain did not lose the ability to position itself for cross-linking. Furthermore, when Q891C/N1102C/K1112Q was reacted with 200 μM of BMOE in the presence of exogenously added biotin or biocytin, the ITF signal intensity did not replicate what was observed with the biotinylated Q891C/N1102C (Figure 6B). These data indicate that tethered biotin itself is responsible for the ITF signal. Given that biotin is necessary for mediating changes in the ITF signal intensity, we sought to determine which Trp residue(s) in the CT domain contributes to the ITF signal change. The crystal structure of *Sa*PC (PDB ID: 3BG5) reveals that the tethered biotin cofactor is positioned near Trp808 in the exo-binding site of *Sa*PC (Figure 2B). This residue was mutated to a phenylalanine (W808F) to assess the sensitivity to changes in ITF intensity. Unfortunately, W808F was aggregation-prone and could not be purified, precluding a clear confirmation of the contribution from Trp808 to the ITF signal.

## Substrates and Effectors Alter the Carrier Domain Positioning Equilibrium

Having validated three different approaches to monitor conformational changes in the BCCP carrier domain of *Sa*PC using a cross-link trap (SDS-PAGE densitometry, enzyme inactivation, and ITF), we next sought to employ these combined approaches to assess the contribution of substrates and effectors to carrier domain positioning. First, the cross-linking-competent Q891C/N1102C *Sa*PC was incubated with 300 μM of BMOE for 2 min in the presence of various substrates and effectors (Figure S5). Both pyruvate and acetyl-CoA increased the amount of the cross-linked dimer, while ADP + PPA reduced the amount of dimer formation, indicating that pyruvate and acetyl-CoA shift the carrier domain equilibrium toward the CT domain, while ADP + PPA shift carrier domain positioning away from the CT domain.

Next, we evaluated the rate of inactivation for Q891C/N1102C *Sa*PC reacted with the BMOE cross-linker in the presence of ADP + PPA, in combination with pyruvate and acetyl-CoA (Figure 7). Since the rate of inactivation was too fast to monitor in the absence of ADP + PPA, it was necessary to include ADP + PPA in our measurements to determine the impact of pyruvate and acetyl-CoA on the rate of inactivation. The data in the absence of substrates were best described by a double exponential decay (eq 2). The data in the presence of ADP + PPA were adequately described by a single exponential decay (eq 1; Figure S6). Consistent with the densitometry analysis, the fastest rates for  were observed in the absence of substrates (Figure 7; Table 1). The presence of ADP + PPA reduced the rate of inactivation ∼30-fold, consistent with BC domain substrates drawing the carrier domain equilibrium position away from the CT domain. Conversely, when acetyl-CoA was added in the presence of ADP + PPA, the rate of inactivation increased, consistent with an equilibrium shift of the carrier domain from the BC to the CT domain. In the presence of ADP + PPA, the combination of acetyl-CoA and pyruvate produced the fastest rates of inactivation, whereas pyruvate alone had no significant effect on the observed rate constant (Figure 7; Table 1).

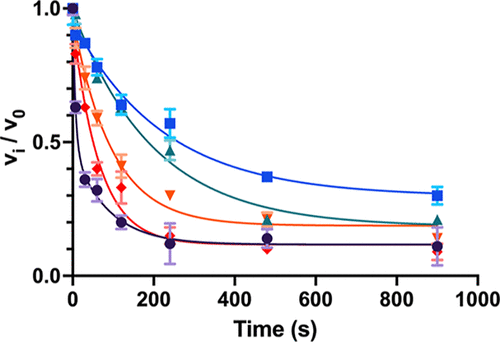


Figure 7. Inactivation kinetics for Q891C/N1102C *Sa*PC cross-linked with BMOE in the presence of various substrates. Q891C/N1102C *Sa*PC was reacted with 300 μM BMOE in 5% DMSO for 10 s, 30 s, 1 min, 2 min, 4 min, 8 min, or 15 min and then quenched with 13 mM DTT for 15 min. Each sample was then assayed in triplicate for catalytic activity using the malate dehydrogenase coupled enzyme assay. The initial velocity (*v*i) was determined and divided by the initial velocity determined after incubating the same enzyme with 5% DMSO for 15 min (*v*o). The error bars represent the standard deviation obtained from each sample assayed in triplicate. Rates were measured in the absence of added ligands (purple circles); in the presence of 5 mM ADP and 5 mM phosphonoacetate (blue squares); in the presence of 5 mM ADP, 5 mM phosphonoacetate, and 12 mM pyruvate (green triangles); in the presence of 5 mM ADP, 5 mM phosphonoacetate, and 0.25 mM acetyl-CoA (orange inverted triangles); or in the presence of 5 mM ADP, 5 mM phosphonoacetate, 12 mM pyruvate, and 0.25 mM acetyl-CoA (red diamonds). The data set in the absence of ADP + PPA (purple) was fit to a double exponential decay with a limiting residual value (eq 2). All other data sets were adequately described by a single exponential decay with a limiting residual value (eq 1).

Table 1. Observed rates of Q891C/N1102C *Sa*PC Inactivation in the Presence of Various Substrates

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **ADP (mM)** | **phosphonoacetate (mM)** | **pyruvate (mM)** | **acetyl-CoA (mM)** | **(s–1)a** | **(s–1)a** |
| 0 | 0 | 0 | 0 | 0.15 ± 0.03 | 0.012 ± 0.004 |
| 5 | 5 | 12 | 0.25 | 0.015 ± 0.002 |  |
| 5 | 5 | 0 | 0.25 | 0.010 ± 0.001 |  |
| 5 | 5 | 12 | 0 | 0.0051 ± 0.0007 |  |
| 5 | 5 | 0 | 0 | 0.0045 ± 0.0008 |  |

aReported errors represent the standard errors calculated from iterative non-linear curve fitting in Graphpad Prism. The data were fit to data, where each data point was plotted as the average ± standard deviation of three independent determinations. Data in the absence of ADP and phosphonacetate were fit to a double exponential decay (eq 2), while all data in the presence of ADP and phosphonoacetate were fit to a single exponential decay (eq 1).

As a final measure, we evaluated the effects of substrates on carrier domain positioning by the following changes in ITF intensity. The rapid acquisition of ITF data using a stopped-flow instrument permitted a direct analysis of the effect of substrates and effectors on carrier domain positioning without requiring the addition of ADP + PPA to reduce the overall rates. Q891C/N1102C *Sa*PC was mixed with various substrates, and the change in ITF intensity was monitored continuously for 120 s. The presence of ADP + PPA initially decreased the observed ITF intensity, as shown in the first 120 s of Figure S7, consistent with the data presented for ATP in Figure 5A and with a shift in the carrier domain equilibrium away from the CT domain. After the system was allowed to reach equilibrium, the cross-linker was subsequently added to observe the impact of the ligand on the rate at which the system re-equilibrated to the trapped conformation (Figure S7). The ITF signal intensity was observed for 120 s following the addition of cross-linkers and was very well described by a double exponential burst (eq 3), best represented on a logarithmic scale (Figure 8). In these experiments, both the Q891C/N1102C *Sa*PC and the cross-linker were pre-incubated with ligand prior to mixing. Since the concentration of ligand was constant before and after mixing, the observed change in ITF intensity was attributed to the presence of the cross-linker and not to the ligand. Additional control experiments in the presence and absence of substrates and cross-linkers confirmed that the ITF signal intensity changed only in the presence of the cross-linker (data not shown). The presence of ADP + PPA substantially reduced the rate of change in the ITF intensity relative to buffer (Figures S8 and 8A─blue trace vs yellow trace). Notably, while the observed rate constants measured by ITF were ∼10-fold faster than the *k*obs values measured from inactivation kinetics, the overall trends observed in the presence and absence of ligand were consistent across all techniques employed in this study.

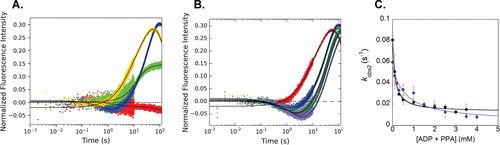


Figure 8. ITF signal intensity change as a function of BC domain substrates. (A) Q891C/N1102C *Sa*PC (0.175 mg/mL) cross-linked at *t* = 0 with 200 μM BMOE. Both the enzyme and the cross-linker were pre-incubated in the presence of 5 mM ADP + 5 mM PPA (blue), 0.25 mM acetyl-CoA (green), 12 mM pyruvate (red), or buffer (yellow). (B) Q891C/N1102C *Sa*PC (0.175 mg/mL) cross-linked at *t* = 0 with 200 μM BMOE. Both the enzyme and the cross-linker were pre-incubated with ADP and PPA at 0 mM (red), 0.5 mM (green), 1 mM (blue), 1.5 mM (yellow), 2 mM (cyan), 2.5 mM (pink), 3 mM (green), or 3.5 mM (purple). (C) Plot of  for Q891C/N1102C (black) and for R346M/Q891C/N1102C (blue) versus ADP + PPA concentration. Both curves were fit to eq 4. The error bars represent the standard deviation resulting from three independent measurements.

To gain further insights into the kinetics of the ITF system, we evaluated the dependence of the observed rate constants ( and ) on ligand concentration. The addition of either acetyl-CoA or pyruvate fundamentally changed the kinetic profile, complicating comparisons in the presence and absence of ligands (Figure 8A). However, the kinetic profile in the presence of 5 mM ADP + 5 mM PPA was the same as that observed in the absence of substrates. We, therefore, examined the dependence of the observed rate constants on the concentration of ADP + PPA. Both a faster phase () and a slower phase () were observed in this system. The amplitude of the faster phase decreased with ligand concentration, precluding accurate measures of  at low concentrations of ADP + PPA (<0.5 mM). Nevertheless, a clear dependence on concentration was observed in replots of both  and  versus [ADP + PPA]. Both replots are described by an inverse rectangular hyperbola (eq 4). A replot of  (Figure S9) resulted in a mM with a  approaching 0 at saturation, although the limiting value could not be accurately determined ( s–1). The replot of the slower  resulted in a  mM with a limiting value of  s–1 (Figure 8). These experiments demonstrate that the observable rate constants are saturable with ADP + PPA. At saturation,  approached a minimum non-zero value. Since  is a function of carrier domain cross-linking in proximity to the CT domain, this indicates that the carrier domain accesses the CT domain even when BC domain ligands are saturating.

The  value from the replot of  versus [ADP + PPA] is expected to represent the residual cross-linking of the carrier domain in the presence of saturating BC domain ligands. We predicted that a mutation with altered carrier domain equilibrium in PC would show altered  values in a replot of  against ADP + PPA. To test this prediction, we evaluated the R346M mutation in *Sa*PC, which was previously kinetically shown to promote the interaction between the carrier domain and the BC domain in *Rhizobium etli* PC.(28) We reasoned that, if the rate of cross-linking is dependent on the equilibration of the carrier domain between the BC and CT domains, the  value would be reduced in the R346M mutant, which has enhanced BCCP-BC domain interactions.(28,29) The R346M mutation, employed in the Q891C/N1102C *Sa*PC background, exhibited very similar relative kinetic behavior to what was observed for the equivalent mutation in *R. etli* PC (Table S3). As predicted, the  value from the replot of  decreased in the R346M/Q891C/N1102C system (Figure 8C; Table S3). This is consistent with the previously described shift in the dynamic equilibrium of the carrier domain toward the BC domain in the R346M mutation.(28) The  value, representative of the relative binding affinity of the combined ligands, increased to 0.63 ± 0.25 mM in the R346M mutation, compared with 0.11 ± 0.01 mM in the unmutated system.

# Discussion

The BCCP carrier domain of PC undergoes a large translocation during catalysis, moving primarily from an intrasubunit interaction with the BC domain to an intersubunit interaction with the opposing CT domain (Figure 2A).(15−17,27) 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.(18−20) 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 ligand(s) lack defined electron density for the carrier domain, suggesting that the carrier domain can sample multiple conformations even in the presence of the ligand.(16,17,29) These structural assemblies are 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 study represents one of the first attempts to directly observe BCCP carrier domain positioning in PC, independent of catalytic turnover. Using a range of techniques, we have measured rates of conformational re-equilibration in *Sa*PC by trapping the BCCP carrier domain when it is positioned in an intersubunit conformation with the neighboring CT domain. We note that the observed rates of carrier domain re-equilibration are consistent across multiple methods of observation. The general cross-linking approach, therefore, provides multiple ways to independently measure carrier domain re-equilibration absent of catalytic turnover. In all cases, the observed rates are 1 to 2 orders of magnitude lower than the  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 BCCP domain within an ensemble population. A comparison of these observed rate constants in the presence of ligands can, therefore, provide information on the degree to which ligands influence the enzyme conformational equilibrium and the resulting equilibrium position of the carrier domain.

Substrates and ligands shift the carrier domain positional equilibrium as expected. The BC domain ligands (ADP + PPA) reduced the rate of cross-linking and shifted the carrier domain equilibrium away from the CT domain, consistent with the proposal that BC domain ligands shift the BCCP carrier domain toward the BC domain.(18−20) X-ray crystal structures offer a structural explanation for this observation: the active site lid of the BC domain partially closes when Mg2+ and ATP are bound in the active site.(30) The carrier domain is positioned directly against this tightly closed lid of the BC domain, producing a new binding interface that serves to stabilize the BCCP-BC domain conformation.(29) The CT domain ligand (pyruvate) increased the rate of intermolecular cross-linking and shifted the carrier domain equilibrium toward the CT domain, consistent with observations that high concentrations of pyruvate inhibit the biotin-dependent ATPase reaction in PC.(21) X-ray crystal structures offer a structural explanation for this observation: a biotin-binding pocket is formed in the CT domain in the presence of CT domain substrates strengthening the BCCP-CT domain interaction.(25)

While substrates and substrate analogues shifted the BCCP carrier domain equilibrium as expected, it is important to note that saturating concentrations of ADP + PPA, a BC domain ligand, did not completely prevent cross-linking in the CT domain (Figure 8). 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. We conclude that the carrier domain samples multiple conformations in both the presence and absence of ligands. 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.

Similar conformational selection models have been proposed in other carrier domain enzymes. In the nonribosomal polypeptide synthase gramicidin synthetase I from *Aneurinibacillus migulanus*, positional changes of the carrier domain correlate with the presence of bound ligands that shift the carrier domain conformations in a dynamic equilibrium.(31) The carrier domains of polyketide synthases, such as 6-deoxyerythronolide B synthase (DEBS), may also be governed by conformational selection, with the carrier domain positional equilibrium shifted by a turnstile mechanism that ensures synchronization of the assembly line–style reactions.(32) Similar interactions are thought to govern carrier protein positioning of SoxYZ with SoxB. In the substrate-unbound state, a mobile loop clashes with the SoxYZ binding site, whereas in the presence of the substrate, the loop shifts to favor the SoxYZ interaction with SoxB.(33) These systems offer clear, simple examples of how substrates can locally shift carrier domain positioning through conformational selection without invoking long range-induced conformational changes to control carrier domain positioning.

Cross-linking of the carrier domain near the CT domain 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  and  against [ADP + PPA] indicate a multi-step process. Since the ligand is pre-equilibrated with both the enzyme and the cross-linker prior to mixing, the observed change in ITF intensity is a response to cross-linking and not to ligand binding. Thus, both  and  represent kinetic steps in cross-linking between the BCCP and CT domains. It is likely that  represents the initial intersubunit capture of the BCCP domain, while  represents a slow transition to a lower energy state. Because of the complexity of the system, the current data cannot accurately determine the individual microscopic rate constants that comprise  and , although the data are adequately described by an eight-step kinetic scheme (Figure S10). 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 cross-linking system to report on relative changes in the BCCP carrier domain equilibrium, we layered the cross-link trap onto the R346M mutation in *Sa*PC. This mutation behaves similar to an equivalent mutation in *R. etli* PC, which was proposed to favor an enhanced BCCP-BC domain interaction compared to the wild-type enzyme.(28) 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 the substrate (represented by a smaller value for ), a greater shift toward the BC domain at saturating substrate concentrations (represented by a smaller value for ), and an altered  if the mutation impacts ligand binding. This is exactly what was observed when the cross-link trap was applied in the presence of the R346M mutation (Figure 8C, Table S3). This validates the interpretation of the data and demonstrates that this, and other designed cross-link traps, can be used to investigate how mutations impact the carrier domain equilibrium in PC.

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.(3) 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 BCCP 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.(25) This perturbs the free energy landscape to promote enzyme conformations that favor CT-BCCP domain interactions. Through this study and other recent reports,(26,27) a revised view of carrier domain translocation in PC is emerging: ligands shift the conformational equilibrium in PC to appropriately favor BCCP carrier domain positioning during catalytic turnover, but they neither impede nor rigidly control the ability of the BCCP carrier domain to regularly sample a wide range of conformations.

# Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00298.

* Pyruvate carboxylation activity for *Sa*PC and mutated constructs of *Sa*PC in the presence of acetyl-CoA; effect of ligands on the observed rates of ITF intensity change; macroscopic rate constants determined for Q891C/N1102C and R346M/Q891C/N1102C; Q891C/N1102C *Sa*PC incubated with bismaleimidoethane (BMOE), bismaleimidobutane (BMB), and bismaleimidohexane (BMH); initial velocities for incubated WT *Sa*PC and Q891C/N1102C *Sa*PC ; X-ray crystal structure of *Sa*PC showing the location of tryptophans; Q891C/N1102C *Sa*PC cross-linked in the presence of various substrates and effectors; inactivation kinetics for Q891C/N1102C *Sa*PC cross-linked with BMOE in the presence of various substrates; *Sa*PC ITF intensity in the presence of substrates/effectors; Q891C/N1102C *Sa*PC cross-linked in the presence of ligands and resulting ITF changes;  for Q891C/N1102C *Sa*PC as a function of ADP+PPA ; and KinTek Explorer simulated experiment of ADP+PPA titration in Q891C/N1102C *Sa*PC (PDF)

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## Notes

The authors declare no competing financial interest.

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