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Co-catalytic metallopeptidases as pharmaceutical targets

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Abstract

Understanding the reaction mechanism of co-catalytic metallopeptidases provides a starting point for the design and synthesis of new molecules that can be screened as potential pharmaceuticals. Many of the enzymes that contain co-catalytic metallo-active sites play important roles in cellular processes such as tissue repair, protein maturation, hormone level regulation, cell-cycle control and protein degradation. Therefore, these enzymes play central roles in several disease states including cancer, HIV, stroke, diabetes, bacterial infections, neurological processes, schizophrenia, seizure disorders, and amyotrophic lateral sclerosis. The mechanism of AAP, an aminopeptidase from *Aeromonas proteolytica*, is one of the best-characterized examples of a metallopeptidase containing a co-catalytic metallo-active site, although this enzyme is not a specific pharmaceutical target at this time. As a large majority of co-catalytic metallopeptidases contain active sites that are nearly identical to the

one observed in AAP, the major steps of their catalytic mechanisms are likely to be very similar. With this in mind, it is possible to propose a general catalytic mechanism for the hydrolysis of amino acid substrates.

Abbreviations

ADEPT, antibody-directed enzyme pro-drug therapy; **ALS**, amyotrophic lateral sclerosis; **CPG₂**, carboxypeptidase G₂; **CPG-II**, carboxypeptidase G-II; **FGCP**, foylpolypoly-γ-glutamate carboxypeptidase; **MDAP**, meso-diaminopimelate; **MetAP**, methionyl aminopeptidase; **NAAG**, N-acetyl-l-aspartyl-l-glutamate; **PSMA**, prostate-specific membrane antigen

Introduction

Peptidases that contain co-catalytic metallo-active sites are key players in carcinogenesis, tissue repair, neurological processes, protein maturation, hormone-level regulation, cell-cycle control and protein-degradation processes [1](#), [2](#), [3](#), [4](#). The importance of understanding their mechanism of action is underscored by their central role in several disease states including stroke, diabetes, cancer, HIV, bacterial infections and neuropsychiatric disorders associated with the dysregulation of glutamatergic neurotransmission, such as schizophrenia, seizure disorders and amyotrophic lateral sclerosis (ALS) [5](#), [6](#), [7](#). For these reasons, several co-catalytic metallopeptidases have become the target of intense efforts in inhibitor design [1](#), [2](#), [3](#), [8](#), [9](#), [10](#). However, a limiting factor in the design of tight-binding, highly specific inhibitors is the paucity of mechanistic information available for this class of metallopeptidase.

This review focuses on recently reported mechanistic aspects of co-catalytic metallopeptidases [11](#), [12](#). The group can be sub-divided into endometallopeptidases and exometallopeptidases. No endometallopeptidase containing a co-catalytic active site has been reported; therefore, only exometallopeptidases will be discussed [13](#), [14](#), [15](#). Because two very detailed reviews on metalloaminopeptidases with co-catalytic active sites have recently appeared [8](#), [16](#), only less-well characterized but physiologically important metallopeptidases with co-catalytic active sites are discussed.

Aminopeptidases

Aminopeptidases catalyze the hydrolysis of N-terminal amino acid residues from proteins and polypeptide chains. Several aminopeptidases have been shown to have co-catalytic metallo-active sites and these can be split into two distinct groups on the basis of their active-site structures. The first group includes the leucine aminopeptidases from the bovine lens (BLAP), the porcine kidney, tomato and *Escherichia coli* (PepA) ([Figure 1](#)); the second group contains the leucine aminopeptidases from *Aeromonas (Vibrio) proteolytica* (AAP) and from *Streptomyces griseus* (SAP) ([Figure 1](#)) [[17](#)]. X-Pro aminopeptidase, AP-P, has also been classified as having a co-catalytic Mn(II) active site [18](#), [19](#); however, it is structurally similar to methionyl aminopeptidases (MetAPs) and X-Pro dipeptidases (prolidases), which have been suggested to contain co-catalytic Co(II) active sites on the basis of X-ray crystallography data [20](#), [21](#), [22](#), [23](#). Recently, MetAPs were reclassified as mononuclear Fe(II)-dependent metallopeptidases, suggesting that AP-P and prolidase may also be mononuclear Fe(II) enzymes [24](#), [25](#), [26](#). In fact, AP-P was very recently shown to be fully active with only one divalent metal ion, indicating that its metal-binding properties are similar to those of MetAP [[27](#)]. Detailed reviews discussing each of these enzymes have recently appeared [8](#), [9](#), [16](#), [28](#), and therefore they are not discussed further.

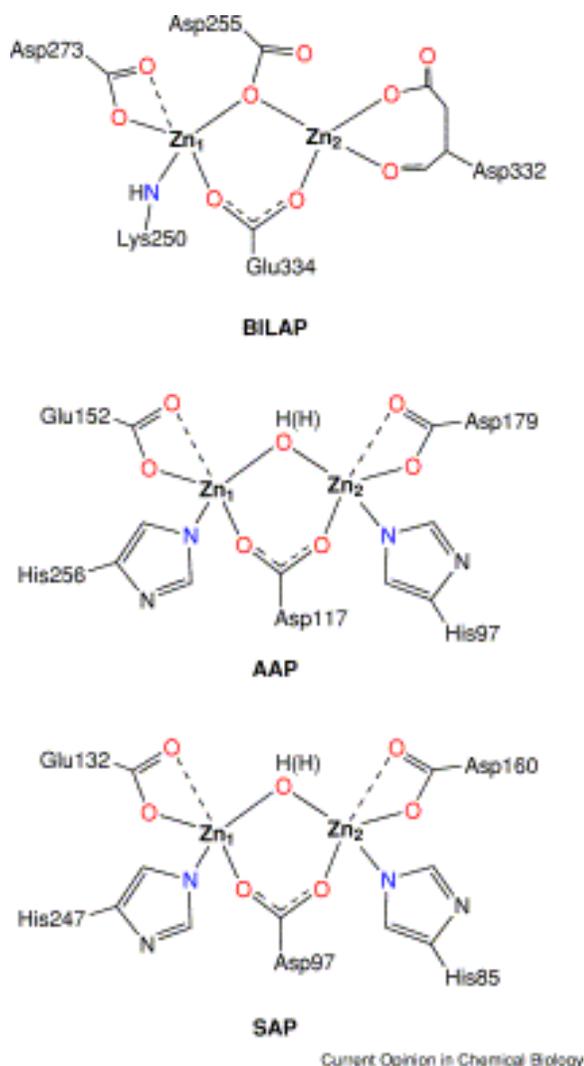


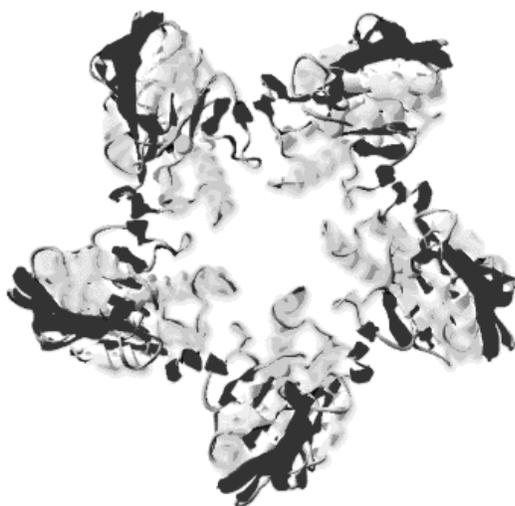
Figure 1. Drawings of the active sites of the leucine aminopeptidases BILAP (Protein Data Bank [PDB]: 1LAM), AAP (PDB: 1AMP) and SAP (PDB: 1CP7) based on X-ray crystallography.

DppA

Aminopeptidases that are stereospecific for D-amino acids have been shown to play a central role in peptidoglycan biosynthesis and, consequently, have emerged as potential pharmaceutical targets [29]. Only a few D-aminopeptidases have been described and most of these are serine proteases [30, 31, 32, 33]; however, there are a growing number of co-catalytic metallopeptidases that show specificity towards D-amino acids [29], such as the D,D-carboxypeptidase from *Streptomyces albus* G, Dpd [34], and both VanX and VanY, which are present in many enterococci [35]. The last two enzymes are important in the vancomycin resistance pathway [35]. The D-aminopeptidase from *Bacillus subtilis* (DppA) exhibits specificity towards D-aminoacyl- β -naphthylamides but not L-aminoacyl- β -naphthylamides and is capable of hydrolyzing di- and tri-D-alanine peptides [33]. DppA has been overexpressed in *Escherichia coli* and was suggested to be an octameric enzyme with a molecular weight of 30 kDa/subunit [29]. The enzyme is active over a large pH range, with maximum activity observed between pH 9.0 and pH 11.0 and a catalytic efficiency (k_{cat}/K_m) of $100\,000\text{ M}^{-1}\text{ s}^{-1}$ [29]. DppA is inactivated by EDTA but can be reconstituted by the addition of two equivalents of Zn(II).

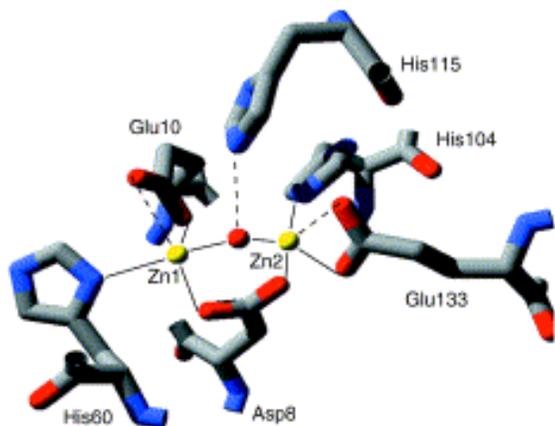
Recently, the X-ray crystal structure of DppA was determined at 2.4 Å resolution and revealed that DppA crystallizes as a decamer (Figure 2) [36]. The C-terminal domains are involved in electrostatic and hydrophobic interactions between the two pentamers in the decamer, whereas the N-terminal domains are responsible for

inter-subunit contacts between the pentamers [36]. A co-catalytic Zn(II) binding site resides in the N-terminal domain of each monomer, which places the sites at the center of a 20Å channel that forms a 50Å cavity. Like AAP, DppA's active site consists of a (μ-aquo)(μ-carboxylato)dizinc(II) core and has a Zn–Zn distance of 3.1 Å (Figure 3). The two Zn(II) ions are coordinated by Glu10, His60, Glu133 and His104, with Asp8 serving as the bridging ligand. Surprisingly, there are no glutamic acid residues near the co-catalytic Zn(II) active site that can function as the general acid/base during catalysis. However, His115 forms a hydrogen bond to the bridging water molecule suggesting that, in DppA, a histidine residue takes the place of the glutamic acid residues observed in AAP, SAP and carboxypeptidase A [8, 37, 38, 39]. Interestingly, ~5% of the crystallized protein is cleaved between His60 and Ser61 [29], but the significance of this cleavage and its effect on enzyme activity remain unclear. The active site does not appear to sterically discriminate against large substrates as it resides in a cleft 8.5 Å wide and 10 Å deep. The large active-site cleft, together with DppA's preference for D-Ala-D-Ala, suggests that DppA may be compartmentalized, thus preventing larger substrates from being degraded. This suggestion is consistent with the fact that the highest activity is observed with (D-Ala)₂ as the substrate, which is released upon direct cross-linking of diaminopimelate residues in the peptidoglycan layer [40, 41]. Consequently, it has been proposed that DppA may use (D-Ala)₂ during periods of L-amino acid starvation.



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Figure 2. Ribbon diagram of the X-ray crystal structure of DppA from *Bacillus subtilis* based on the coordinates from the PDP (PDP: 1HI9). Zinc ions shown in pale blue.



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Figure 3. Schematic of the native DppA active site based on the X-ray crystallographic coordinates (PDB: 1HI9). The zinc ions are depicted as yellow spheres. His115, also shown, is not a metal ligand but forms a hydrogen bond to the bridging water molecule and is thought to participate in the hydrolytic reaction.

Carboxypeptidases

Carboxypeptidases are exopeptidases that selectively cleave C-terminal amino acid residues from polypeptides and proteins. The vast majority of metallo-carboxypeptidases contain mononuclear Zn(II) active sites in which the Zn(II) ion resides in a classic HEXXH motif [42.](#), [43.](#). The detailed catalytic mechanism of mononuclear Zn(II) carboxypeptidases has been determined on the basis of X-ray crystallographic, kinetic and spectroscopic studies [38.](#), [44.](#).

CPG₂

Carboxypeptidase G₂ (CPG₂) from *Pseudomonas* sp. strain RS-16 (EC 3.4.17.11) also utilizes Zn(II) ions for catalytic activity but requires two equivalents for full enzymatic activity [\[45\]](#). CPG₂ is a dimeric enzyme with a mass of 42 kDa/subunit that catalyzes the hydrolytic cleavage of reduced and non-reduced folates to pterates and L-glutamate [45.](#), [46.](#). The X-ray crystal structure of CPG₂ has been determined at 2.5 Å resolution; each monomer contains a co-catalytic Zn(II) site and a dimerization domain consisting of four anti-parallel β-sheets flanked by two α-helices [\[46\]](#).

Examination of the co-catalytic Zn(II) active site of CPG₂ reveals a striking similarity to the active sites of AAP, SAP and DppA [36.](#), [37.](#), [39.](#). CPG₂ contains two metal ions bound in its active site with a (μ-aquo)(μ-carboxylato)dizinc(II) core and a Zn–Zn distance of 3.3 Å ([Figure 4](#)). The coordination geometry of each Zn(II) ion is tetrahedral with Zn1 being liganded by a carboxylate oxygen of Glu176 and an imidazole nitrogen of His385. Similarly, Zn2 is coordinated by a carboxylate oxygen of Glu200 and an imidazole nitrogen His112. Near the co-catalytic Zn(II) active site of CPG₂ resides a glutamic acid residue (Glu175) that forms a hydrogen bond to the bridging water molecule. Glu175 probably functions as a general acid/base during catalysis by assisting in the activation of the water molecule, in a similar manner to Glu151 and Glu270 in AAP and CP-A, respectively [8.](#), [38.](#). In contrast to AAP, CPG₂ must accommodate the pterate moiety of folic acid. Arg324 is also located near the active site and may be involved in the enzyme–substrate interaction. Mutation of Arg324 to alanine resulted in an enzyme with low activity towards methotrexate [\[46\]](#). These data indicate that Arg324 is important in catalysis but its exact role remains unknown.

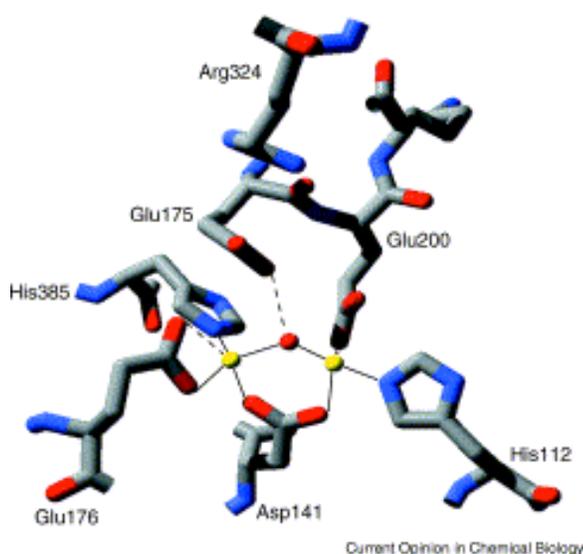


Figure 4. Schematic of the active site of CPG₂ from *Pseudomonas* sp. strain RS-16 based on the X-ray crystallographic coordinates (PDB: 1CG2). The zinc ions are depicted as yellow spheres. Glu175, also shown, is not a metal ligand but forms a hydrogen bond to the bridging water molecule and is thought to participate in the hydrolytic reaction. Arg324 is also shown and is believed to participate in substrate recognition.

One of the potential medical uses of CPG₂ involves the development of specific inhibitors for use in antibody-directed enzyme pro-drug therapy (ADEPT) [47]. In this procedure, a conjugate of the enzyme and an antibody specific to proteins present in tumor cells is administered, followed by the administration of a pro-drug with decreased toxicity compared with the actual drug. The pro-drug is converted to an active form of the drug by the enzyme and, thus, its action is limited to cells with the conjugate attached to them [46]. As the mechanism of action of CPG₂ has not been determined, the spectrum of inhibitors that are used in medicine is limited. General features that appear to be important include a free α -carboxylate moiety on the L-glutamate residue and a benzene ring close to the carbonyl carbon of the amide bond [48, 49]. Thiolate-containing inhibitors based on a thiocarbamate moiety attached to a benzene ring have also been reported for CPG₂ but were only modestly effective competitive inhibitors ($K_i=0.30\text{--}165\ \mu\text{M}$) [48]; however, these compounds exhibit little toxicity towards LS174T cells, potentially enabling their use as inhibitors in ADEPT.

GCP-II

Glutamate carboxypeptidase II (GCP-II; EC 3.4.17.21), sometimes referred to as N-acetylated- α -linked-acidic dipeptidase, is a membrane-bound enzyme that cleaves glutamate from the neuropeptide *N*-acetyl-L-aspartyl-L-glutamate (NAAG) [50, 51]. Immunocytochemical studies show that GCP-II is primarily localized in the brain and kidney; however, it is widely distributed and is abundant in neuropil, although absent from neuronal cytoplasm [52]. GCP-II gene expression occurs mainly in astrocytes [53], and displays significant regional heterogeneity [54]. Recently, it was shown that deletion of the gene encoding GCP-II in mice reveals a second enzyme activity that hydrolyzes NAAG, which is consistent with GCP-II's regional heterogeneity [55]. Because of GCP-II's location in the central and peripheral nervous system, it is believed to play a critical role in modulating the release of glutamate [50, 56]. The role of NAAG has been extensively studied; among other functions, it serves as a negative modulator of glutamatergic neurotransmission [57, 58, 59, 60]. There is also mounting evidence that NAAG is involved in neuropsychiatric disorders associated with the dysregulation of glutamatergic neurotransmission, such as schizophrenia, seizure disorders, Parkinson's disease and ALS [5].

GCP-II is a trans-membrane enzyme whose activity is localized on the external surface of the cell, suggesting that NAAG acts as a glutamate precursor and that the liberated glutamate probably acts directly on glutamate receptors [61]. The molecular weight of GCP-II ranges from 94–100 kDa, depending on the source and on whether the membrane-spanning sequence is present. Recently, the recombinant form of human GCP-II was reported and the substrate specificity and kinetic properties of this enzyme were examined [62]. NAAG has a very high affinity for GCP-II with K_m values of 540 nM for the membrane-bound form and 140 nM for the soluble enzyme. Monovalent anions such as Cl^- , Br^- , I^- , NO_3^- and, to a lesser degree, F^- are also required for full activity. Chloride is likely to be the physiological activator because of its high concentration in the brain. Other oxoanions such as phosphate and sulfate are competitive inhibitors of GCP-II with IC_{50} values of 100 μM and 1 mM, respectively. GCP-II exhibits remarkable thermal stability in that the enzyme from rat brain retains 56% of its activity after 15 min at 55°C. GCP-II also requires the presence of two equivalents of Zn(II) ions for full catalytic activity [50, 62, 63]. Only one Zn(II) ion appears to be tightly bound to GCP-II while dithiothreitol inhibits the enzyme. Apo-enzyme, obtained after incubation with EGTA, can be reconstituted with several first-row transition metal ions but higher activity was observed with Mn(II) (71%) and Ni(II) (42%) than with Zn(II)-loaded GCP-II.

No X-ray crystallographic data has been reported for GCP-II. However, on the basis of sequence comparisons with CPG₂, AAP and SAP, GCP-II has been classified as a co-catalytic metallopeptidase. Recently, the three-dimensional structure of the GCP-II extracellular domain was modeled using a homology modeling approach

[64]. The proposed model is consistent with site-directed mutagenesis studies based on the crystallographically characterized CPG₂ and AAP enzymes [65]. Combined, these data suggest that the Zn(II) ligands in human GCP-II are His377, Glu425, Asp453 and His553, with Asp387 functioning as the bridging ligand. Sequence alignments with CPG₂ and AAP as well as site-directed mutagenesis studies suggest that Glu424 is a catalytically important residue and may function as the general acid/base during catalytic turnover [65]. In addition, Arg463, Lys499, Lys500, Arg536 and Lys545 were suggested to constitute the recognition pocket for NAAG [64., 65.]. By analogy to AAP and SAP, Tyr552 may also be involved in the interaction with a phosphate group as phosphate inhibition was perturbed in a Y552F mutant.

Recently, several potent and selective inhibitors of GCP-II have been designed, synthesized and used to demonstrate that inhibition of GCP-II prevents neurodegeneration in animal models [57., 66., 67., 68., 69.]. GCP-II is thought to be more strongly inhibited by dipeptide analogs than tripeptide analogs on the basis of the observation that L-Glu-L-Glu inhibits GCP-II activity 100 times more than L-Glu-L-Glu-L-Glu. The enzyme active site also exhibits some stereospecificity — D-glu-D-glu is only a weak inhibitor of GCP-II. In general, N-acetylated compounds are the most potent inhibitors of GCP-II, whereas amidation of the γ -carboxyl group reduces the potency [50]. However, an N-acetyl group is not an absolute requirement for entry into the GCP-II active site, as N-succinyl-glutamate also shows high affinity for GCP-II [70]. One of the most potent inhibitors of GCP-II is 2-(phosphonomethyl)pentanedioic acid (2-PMPA), which exhibits a K_i of 0.28 nM and has shown robust neuroprotective activity in both *in vitro* and *in vivo* models of ischemia [57., 67., 68., 69.]. Interestingly, the aspartate analog of 2-PMPA is ~300 times less potent. In addition, 2-[[2-carboxyethyl]hydroxyphosphinoyl]methyl]pentanedioic acid, which has a group similar to the aspartate present in NAAG, does not exhibit increased potency [71]. Quisqualate, an amino acid derivative, is one of most potent inhibitors with an IC₅₀ value of 480 nM [62].

PSMA

One enzyme that is closely related to GCP-II and that possesses hydrolytic activity towards NAAG is human prostate-specific membrane antigen (PSMA), whose cDNA shares 86% identity with rat brain GCP-II cDNA [60., 72.]. In addition, folylpoly- γ -glutamate carboxypeptidase (FGCP; EC 3.4.19.9), which is found in the pig and human jejunum, is yet another gene product encoding an enzyme that is similar to GCP-II and PSMA and that is 91% identical to the latter [73]. FGCP's primary function is to cleave dietary folylpoly- γ -glutamate that is further absorbed at the jejunal brush border membrane.

PSMA is a 100 kDa transmembrane glutamate carboxypeptidase that removes terminal carboxy glutamates from both neuronal NAAG and γ -linked folate polyglutamate [74]. PSMA, which is highly expressed in prostate cancer and in the vasculature of most solid tumors, is probably involved in prostatic metastasis to lymph nodes. PSMA is the target of several diagnostic and therapeutic strategies [74., 75., 76.]. It has activity in both the membrane and cytosolic fractions and, thus, the two enzymes are termed PSMA and PSMA' (also called PSM'), respectively [76., 77.]. Prostate carcinogenesis is associated with elevated levels of PSMA and PSMA' enzyme activity; by contrast, no such enhancement in PSMA activity is observed for the neoplastic changes in benign prostatic hyperplasia. Thus, the PSMA activity enhancement observed in prostate cancer is not simply related to a generalized prostatic hyperplasia but is specific to its malignancy.

Polysulfonated naphthylurea suramin has been shown to possess significant antitumor activity in patients with hormone-refractory metastatic prostate cancer [78]. The mechanism by which suramin exerts this effect is unknown; however, suramin is a competitive inhibitor of PSMA, having K_i values of 15 nM and 68 nM for the membrane-associated and soluble forms, respectively. This is one of the most potent activities yet described for suramin and suggests that its pharmacologic and/or toxicological mechanism of action may involve PSMA binding.

Desuccinylases

The *meso*-diaminopimelate (mDAP)/lysine biosynthetic pathway offers several potential anti-bacterial targets that are yet to be explored [79](#), [80](#), [81](#), [82](#), [83](#), [84](#), [85](#). One of the products of this pathway, lysine, is required in protein synthesis and is also used in the peptidoglycan layer of Gram-positive bacterial cell walls. A second product, the amino acid mDAP, is an essential component of the peptidoglycan layer for Gram-negative bacteria, providing a link between polysaccharide strands. One of the enzymes in this pathway [86](#), the DapE-encoded *N*-succinyl-L,L-diaminopimelic acid desuccinylase (DapE; EC 3.5.1.18), catalyzes the hydrolysis of *N*-succinyl-L,L-diaminopimelic acid to L,L-diaminopimelic acid and succinate [87](#). It has been shown that deletion of the gene encoding DapE is lethal to *Helicobacter pylori* and *Mycobacterium smegmatis* [88](#), [89](#). Even in the presence of lysine-supplemented media, *H. pylori* was unable to grow. Therefore, DapE proteins are essential for cell growth and proliferation.

DapE proteins have been purified from *E. coli* and *Haemophilus influenzae*, and the genes that encode them have been sequenced from *Corynebacterium glutamicum*, *H. pylori* and *Mycobacterium tuberculosis*. The DapE proteins from *E. coli* and *H. influenzae* have been overexpressed in *E. coli* and purified to homogeneity [87](#), [90](#). They are both small, dimeric enzymes (mass 42 kDa/subunit) and require two Zn(II) ions per mole of polypeptide for full enzymatic activity. Alignment of all of the known gene sequences of DapE enzymes with CPG₂ and AAP sequences [39](#), [46](#), [87](#), [91](#), indicated that all of the amino acids that function as metal ligands are strictly conserved. These data suggest that, like CPG₂ and AAP, DapE should be assigned to the peptidase family M28 [17](#). Like AAP and GCP-II, the purified DapE enzyme contains only one tightly bound Zn(II) ion and exhibits ~80% of its total activity [92](#). Substitution of Zn(II) with Co(II) provides an enzyme that is hyperactive by a factor of ~2 [87](#). For both the Zn(II)- and Co(II)-bound enzymes, the K_m and k_{cat} values were determined over the pH range 6–9. A bell-shaped curve was observed and two pK_a values were found at pH 6.5 and pH 8.3 with the pK_a at pH 6.5 corresponding to a single proton-transfer step. Similar data have been reported for the active site water/hydroxide nucleophile (pK_a ~7.0) of AAP [93](#). Solvent kinetic isotope effect studies revealed an inverse isotope effect that was explained by the attack of a Zn(II)-bound hydroxide on the amide carbonyl [87](#).

N-acetylornithine deacetylase

L-Glutamate can undergo spontaneous cyclization and conversion to L-proline via two consecutive intermediates; however, acetylation prevents this cyclization reaction and initiates an eight-step biosynthetic pathway for arginine [94](#), [95](#). This arginine biosynthetic pathway is found in several bacteria including *Enterobacteriaceae* [96](#), *Myxococcus* [97](#) and *Vibrionaceae* [98](#) and also in the thermophilic archaeon *Sulfolobus* [99](#). The enzyme that catalyzes the fifth step in this pathway is the *N*-acetyl-L-ornithine deacetylase, ArgE [86](#). ArgE catalyzes the conversion of *N*-acetylornithine to ornithine, which can then be incorporated into the urea cycle. The ArgE from *E. coli* has been cloned, expressed and purified with a high yield [100](#), [101](#). The substrate specificity of ArgE is quite broad in that several α -*N*-acyl-L-amino acids can be hydrolyzed, including α -*N*-acetylmethionine and α -*N*-formylmethionine. In fact, ArgE exhibits higher activity on these two substrates than on α -*N*-acetyl-L-ornithine [100](#).

ArgE was shown to be a homodimer of mass 42 kDa/subunit and is activated by Zn(II) ions. Purified ArgE contains only a single Zn(II) ion per monomer but the observed activity levels increase by a factor of ~2 upon the addition of excess Zn(II). Addition of Co(II) ions to apo-ArgE increases enzyme activity levels by a factor of ~8 when compared with Zn(II)-loaded enzyme. In addition, ArgE shares significant sequence homology and biochemical features with *aacI*-encoded pig aminoacylase I [102](#), CPG₂ and DapE. Alignment of the ArgE gene sequence with CPG₂ and AAP indicates that all the amino acids that function as metal ligands are strictly conserved [102](#). The pH dependence of K_m and k_{cat} were determined for Co(II)-loaded ArgE over the pH range 5–9. A bell-shaped curve was observed and two pK_a values were found at pH 5.6 and pH 7.7. A large solvent kinetic isotope effect was also reported for ArgE for V_{max} (2.1) and a smaller solvent kinetic isotope effect of 1.3 was observed for V/K . A linear proton inventory at pH 7.0 was also observed, suggesting that a single proton transfer

occurs in a partially rate-limiting step. No inhibitors have been reported for ArgE, but fluoride was shown to be an uncompetitive inhibitor with a K_i of 3.4 mM.

Mechanistic insights and conclusions

Several metallopeptidases containing co-catalytic metallo-active sites are widely regarded as promising targets for drug discovery, but the efficiency of this has been hampered by the lack of detailed mechanistic information for this class of enzymes. However, one enzyme in this class, AAP, although not a specific pharmaceutical target, is mechanistically one of the best-characterized co-catalytic metallopeptidases. As nearly all co-catalytic metallopeptidases (except BILAP and enzymes related to BILAP) contain a $(\mu\text{-aquo})(\mu\text{-carboxylato})\text{dizinc(II)}$ core with one terminal carboxylate and one histidine residue at each metal site, the major steps of their catalytic mechanisms are likely to be very similar. With this in mind, the proposed catalytic mechanism for the hydrolysis of N-terminal amino acid residues by AAP can be generalized and applied to all metallopeptidases containing similar co-catalytic metallo-active sites ([Figure 5](#)) [8•].

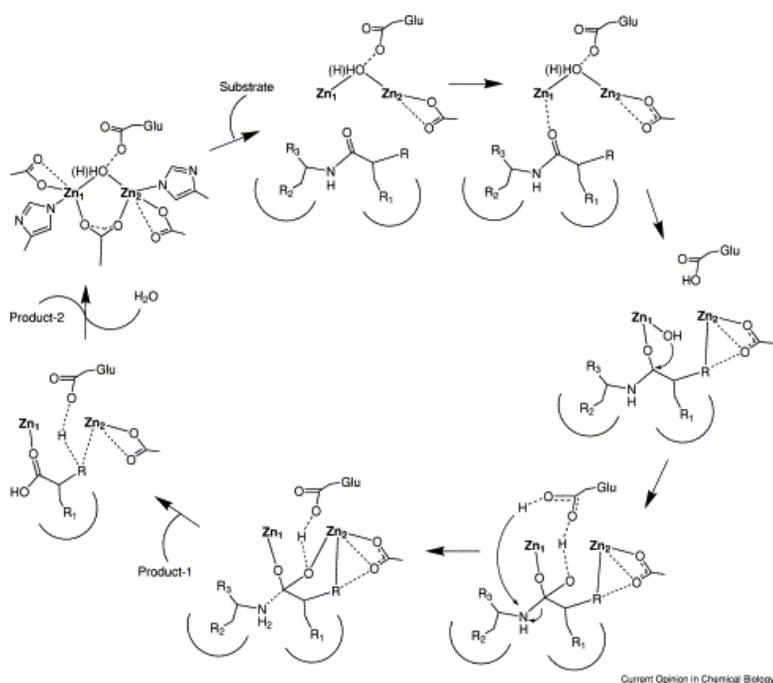


Figure 5. Proposed general mechanism for the hydrolysis of a peptide, catalyzed by a metallopeptidase with a co-catalytic active site where R1, R2, R3 are substrate side chains and R is an N-terminal amine or a C-terminal carboxylate. This mechanism is based on the proposed mechanism for the aminopeptidase from *Aeromonas proteolytica* [8•].

The first step in catalysis is probably the recognition of the N- or C-terminal R-group of the incoming substrate by either a hydrophobic or a hydrophilic pocket adjacent to the co-catalytic metallo-active site. Substrate recognition pockets have been observed or predicted for all metallopeptidases containing similar co-catalytic metallo-active sites. The second step is proposed to be the binding of the carbonyl oxygen atom of the incoming substrate to Zn1, which can polarize the carbonyl group, rendering it susceptible to nucleophilic attack. The bridging water/hydroxide, upon substrate binding, becomes terminal and is coordinated to Zn1. A terminal water/hydroxide on Zn1 is the arrangement that is most consistent with the fact that nearly all enzymes in this class are active (75–90%) with only one Zn(II) ion bound, which suggests that the substrate and the nucleophile reside on the same Zn(II) ion. The breaking of the Zn2-OH(H) bond is probably assisted by N-terminal amine binding in aminopeptidases and C-terminal carboxylate binding in carboxypeptidases to Zn2 whose role is simply to position the substrate correctly in the active site. Next, a glutamic acid residue (or a histidine) located near the catalytic active site assists in the deprotonation of the terminal water molecule, giving a nucleophilic

hydroxo moiety similar to that of Glu270 in carboxypeptidase A [38]. Once the metal-bound hydroxide has formed, it can attack the activated carbonyl carbon, forming a gem-diolate intermediate that is stabilized by coordination of both oxygen atoms to the co-catalytic Zn(II) site. The amide nitrogen must also be stabilized, via a hydrogen bond, to make it a suitable leaving group. This hydrogen bond would also facilitate the collapse of the transition state. The active site glutamate (histidine) probably supplies the additional proton to the penultimate amino nitrogen, returning it to its ionized state. Finally, the co-catalytic Zn(II) site releases the cleaved peptides and adds a water molecule that bridges the two metal ions. Thus, both metal ions are required for full enzymatic activity in metallopeptidases containing co-catalytic metallo-active sites, but their individual roles appear to differ markedly.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

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