Metalloaminopeptidases: Common Functional Themes in Disparate Structural Surroundings

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I. Introduction

Metalloaminopeptidases remove the N-terminal amino acid from proteins or small oligopeptides. This modification or truncation process can occur either co-translationally or posttranslationally after the action of an endoproteinase. The action or inaction of these exopeptidases is instrumental for many normal and pathophysiological processes.

The metalloaminopeptidases utilize conserved amino acid residues to generate a scaffold capable of binding one or two metal ions. The metal centers can be described as [M1M2(enzyme)], where M1 and M2 indicate the type of metal ion in site 1 and site 2 for a particular enzyme. Spectroscopic and kinetic analyses indicate that for some of the enzymes two metal ions are required for full activity. In other instances, only a single metal ion is essential for catalysis while the second metal ion modulates activity either positively or negatively. These mononuclear and cocatalytic,[1] dinuclear metal centers mediate catalysis by providing sites for substrate binding, by activating the nucleophile of the reaction, and by stabilizing the transition state.

Structural analyses of representatives of the different metalloaminopeptidase classes indicate that the site 1 metal ion is closest to the mouth of the active site while the site 2 ion is relatively buried and adjacent to the binding pocket for the P_1 side chain (nomenclature of Schechter and Berger[2]). The enzymes can be categorized based on their substrate specificity or preferences.[3] Cleavage of the scissile peptide bond is dependent upon the chemical structure and composition of the amino acid residues that flank the cleavage site (P_1*P_1'). The broad-range aminopeptidases are typified by the bovine lens leucine aminopeptidase (bLeuAP) and the aminopeptidases from *Aeromonas proteolytica* (ApAP) and *Streptomyces griseus* (SgAP). These enzymes are capable of cleaving hydrophobic amino acids from the N-terminus, usually without strict requirements for the penultimate (P_1') residue. In contrast, the "pita-bread" enzymes methionine aminopeptidase (MetAP), aminopeptidase P (APP), and prolidase process substrates restricted to particular N-terminal and penultimate residues.

This review describes the contributions of many laboratories as they have sought to understand the molecular basis for substrate specificity and catalysis. An effort has been made to give sufficient historical background to appreciate the current state of knowledge. The structure−function relationships for each

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enzyme system are presented based on the analysis of substrate specificity, metal dependence, and the comparison of ligand complexes determined by X-ray crystallography, when available. To facilitate comparisons, new figures have been prepared that present the ligand complexes and mechanistic descriptions in a mode that is consistent across all enzymes. Despite the differences in the three-dimensional folds and the metal centers used by these aminopeptidases, common motifs for substrate recognition and catalysis are readily discerned.

II. Leucine Aminopeptidase (LeuAP)

A. Physiological Roles and Substrate Specificity

LeuAPs (E.C. 3.4.11.1) are ubiquitous enzymes that process or degrade peptides.[4−6] In humans, LeuAP processes antigenic peptides for presentation by the major histocompatibility complex class I molecules.[7,8] This group of enzymes has also been implicated in a variety of pathophysiological states including HIV infection,[9] cataracts,[10,11] systemic lupus erythymetosus,[12] and cancer.[13−15]

As the name of this enzyme family implies, the LeuAPs were originally found to cleave substrates containing L-Leu in the P_1 position.[16−19] Substrates with an N-terminal amino acid that is blocked or in the D-configuration are not cleaved.[19−21] The enzymes are capable of cleaving a wide variety of di- and tripeptide substrates as well as chromophoric substrate analogues.[22−27] A comparison of the activities for LeuAPs from tomato, *Escherichia coli* (PepA) and pig against di- and tripeptide substrates shows that some preferences can be generalized for all organisms.[28] For example, substrates of the form Xaa−Leu, where Xaa represents Met, Arg, Ile, Leu, or Val, are hydrolyzed the most efficiently. Leu and Met are still the best N-terminal residues for the tomato and eukaryotic enzymes. In contrast, PepA prefers Ala, Phe, and Arg at the N-terminus and cleaves the Leu-containing peptide at a 50% slower rate. Asp and Gly in the P_1 position are uniformly the worst substrates.

The P_1' position of the substrate was investigated by analyzing substrates of the form Leu−Xaa, Gly−Xaa, and Arg−Xaa. The inability to cleave peptides containing Pro in the P_1' position confirmed the historical data for the bovine and kidney bean LeuAPs.[18,29,30] The substitution of the large hydrophobic residues, Phe and Tyr, in the P_1' position of Gly−Xaa resulted in accelerated rates, thus compensating for what is normally a poor substrate. The analysis of several tripeptides also suggested that the enzyme could accommodate all residues in the P_2' position, although the substitution of Arg resulted in a 3- to 6-fold reduction in cleavage rate.

B. Metal Dependence and Modulation of Activity

The LeuAP from bovine lens (bLeuAP) is a hexameric enzyme of molecular mass 324 000 Da, consisting of six identical subunits.[31−33] Atomic absorption spectra have confirmed that each 54 000 Da subunit of the native enzyme contains two Zn^{2+} ions.[31,34,35] The necessity of the metal ions for the related porcine kidney enzyme (pkLeuAP) was initially supported by the loss of activity upon dialyzing the protein against Cd^{2+}-containing buffer.[34] Activity for the bovine enzyme is also lost when treated with stoichiometric amounts of the metal chelator 1,10-phenanthroline.[31] The dialysis of the Cd^{2+}-containing enzyme against buffer containing Zn^{2+} or the direct addition of Zn^{2+} to apoenzyme results in the full restoration of enzymatic activity.[31,34]

Prior to understanding that the native enzyme contained Zn^{2+} ions, several studies showed that the addition of Mn^{2+} and Mg^{2+} activated the protein in a time-dependent manner.[19,36−40] Further metal ion analyses have shown that the activation is a consequence of replacing one of the Zn^{2+} ions.[31,41] Moreover, enzyme with full or enhanced activity can only

Table 1. Kinetic Parameters of Metal-Substituted bLeuAP[a]

enzyme form		L-Leu-*p*-NA			L-Leu-*p*-anisidine			L-Leu-amide		
site 1	site 2	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})
Zn^{2+}	Zn^{2+}	7	5900	0.1	12	6000	0.2	2322	51000	4.6
Co^{2+}	Zn^{2+}	11	1200	0.9	59	2200	2.7	2108	20000	10.5
Mg^{2+}	Zn^{2+}	151	2600	5.8	362	3500	10.3	2160	14000	154.3
Zn^{2+}	Co^{2+}	10	1100	0.9	19	1800	1.1	1243	3100	40.1
Co^{2+}	Co^{2+}	14	260	5.4	103	940	10.9	3186	350	91.0
Mg^{2+}	Co^{2+}	146	900	16.2	319	1400	22.8	2268	2000	113.4

[a] Data from Allen et al.[23] Assay conditions: 0.01 M $NaHCO_3$, 0.2 M NEM pH 7.5, 30 °C. To facilitate comparisons to other enzymes, the units for k_{cat} have been converted from μmol min^{-1} mg^{-1} to min^{-1} assuming a molecular mass of 324 000 Da and six active sites. The standard errors for each measurement have also been omitted.

Table 2. Kinetic Parameters of Metal-Substituted pkLeuAP

enzyme form		L-Leu−*p*-NA[a]			Leu−Gly−NHNH−Dns[b]		
site 1	site 2	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})
	Zn^{2+}	4.2	1000	0.4	190	80	240
Zn^{2+}	Zn^{2+}	2.0	1000	0.2	630	180	360
Mn^{2+}	Zn^{2+}	55	1000	5.5	1800	370	490
Mg^{2+}	Zn^{2+}	42	1000	4.2	1200	330	360
Ni^{2+}	Zn^{2+}	3.0	900	0.3	750	230	330
Cu^{2+}	Zn^{2+}	2.3	1000	0.2	660	180	360

[a] Data from Van Wart and Lin.[25] Assay conditions: 0.01 M Tris pH 8.0, 22 °C. [b] Data from Lin et al.[27] Assay conditions: 0.01 M Tris pH 9.0, 0.1 M KCl, 23 °C.

be obtained when the apoenzyme is first treated with one equivalent of Zn^{2+} or Co^{2+} to fill the "tight" binding site (site 2) followed by the addition of a second equivalent of Zn^{2+}, Co^{2+}, Mn^{2+}, or Mg^{2+} ions to fill the "readily exchangeable" site (site 1).[31,41,42] Scatchard analysis confirmed the presence of two binding sites and that the high affinity site, site 2, is filled first.[42]

It should be noted that the initial assignment as to which metal site, 1 or 2, corresponds to the "tight" or "weak" site was arbitrary. Unfortunately, the original designation, i.e., site 1 being the tight site, needed to be reversed in light of further experimentation and crystallographic work[43] described later in the review. This realization has been taken into account above and when presenting the results from kinetic and structural analyses.

The differences in the binding affinities of the metal sites allow the sequential addition of metal ions (Zn^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Co^{2+}) and the creation of metallohybrids. These mixed metal centers have been analyzed in an effort to attribute a functional role to each metal ion in catalysis. For example, metal ion analyses showed that two ions were bound to each bLeuAP monomer, even after removal of excess metal ions.[23] The desired ratios for substitution were achieved except for the [ZnCo-(bLeuAP)] and [CoZn(bLeuAP)] hybrids. The ratios for the latter hybrids were most likely skewed as a result of the Zn^{2+} and Co^{2+} ions exchanging from site to site during the long dialysis period. In these instances, the level of correct substitution was estimated to be greater than 90%.

The representative kinetic data for the bLeuAP and the porcine kidney LeuAP (pkLeuAP) metallohybrids shown in Tables 1 and 2 illustrate the complex effects of metal substitution on activity toward different substrates.[23−25] These studies have built upon earlier kinetic work and have taken into account some of the pitfalls of previous methods, assay conditions, and assumptions.[31,41,42] As seen previously, the addition of Mg^{2+} to bLeuAP containing either Zn^{2+} or Co^{2+} in site 2 results in an ~10-fold increase in the turnover number (k_{cat}) and also in the specificity or apparent second-order rate constant (k_{cat}/K_m) for all three substrates. The presence of Co^{2+} in site 2 leads to a consistent decrease in the apparent binding constant (K_m). Moreover, the substitution of metal ions in site 1 results in a decrease in K_m of the relative order Zn^{2+} > Mg^{2+} > Co^{2+}. This study clearly indicates that there are complex interactions between the metal sites and the substrate that affect all three catalytic constants.

In contrast to bLeuAP, pkLeuAP (Table 2) has weaker affinity for metal ions in site 1 and therefore retains only a single metal ion when excess metal ions have been removed by ion-exchange resin or gel-filtration.[24−27] The mono-Zn^{2+} enzyme is active. The activity (k_{cat}) is increased, however, when site 1 is filled by adding excess metal (0.1−5 mM) to the assay buffer, particularly for the Leu−Gly−NHNH−Dns substrate. The substitution of Mn^{2+} and Mg^{2+} also leads to an increase in k_{cat} for both substrates. The typical 10-fold increase in k_{cat}/K_m seen for bLeuAP, however, appears to be masked by a reduction in K_m when the more physiological, dipeptide substrate is used. Lin et al. suggest that this phenomenon points to the need for caution when drawing conclusions on the importance of activity modulation by metal ions based on substrates that do not contain a P_1' residue.[24] Assuming that the dipeptide substrate more accurately reflects the situation in vivo, the addition of different metals to site 1 activates the enzyme (increases k_{cat}) when under zero-order conditions ([S_0]

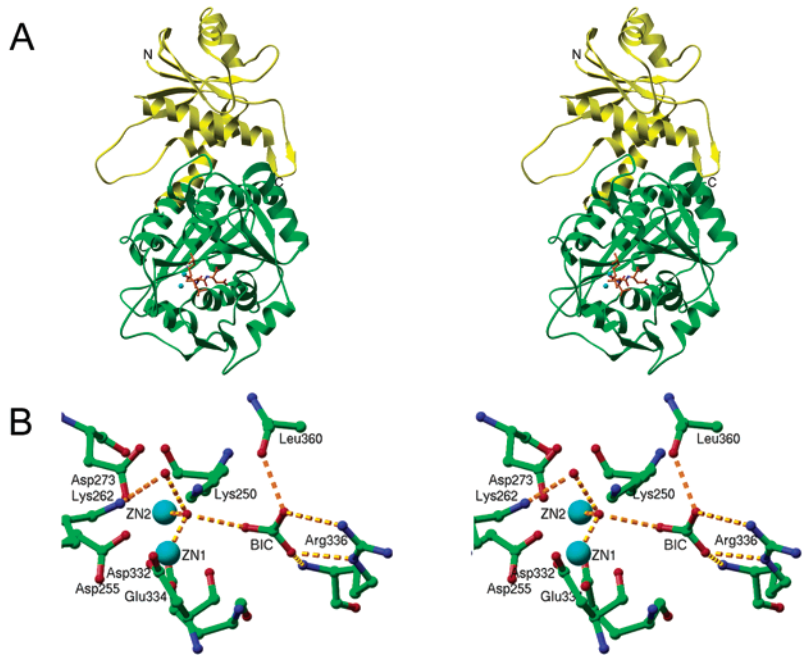


Figure 1. Stereoviews of bLeuAP. (A) General structure of the N-terminal domain (yellow) and the catalytic, C-terminal domain (green). The transition state analogue amastatin is shown in ball-and-stick representation (PDB: 1BLL).[51] The Zn^{2+} ions are shown as spheres. (B) Dinuclear metal center containing Zn^{2+} ions and a bicarbonate molecule (PDB: 1LAM).[56] Atom coloring scheme used throughout unless indicated otherwise: red, oxygen; blue, nitrogen; pink, phosphorus; boron, white; yellow, iodine; Zn^{2+}, cyan; Co^{2+}, magenta; Mn^{2+}, gold. Carbon atoms of the enzyme and bound ligands are colored green and orange, respectively. Dashed gold bonds indicate putative hydrogen bonding and metal interactions. Solvent molecules in the vicinity of the metal center are shown as individual, red spheres. Only the backbone carbonyl group of Leu360 is shown for clarity.

$\gg K_m$). If, on the other hand, the in vivo conditions are first order ($[S_0] \ll K_m$), the relative activities are reflected by k_{cat}/K_m. In this situation, the effects of metal substitution are very small and may not be biologically significant.

C. Macromolecular Structure and Metal Center

The architecture of the native, zinc-containing bLeuAP hexamer has been determined by X-ray crystallography.[44,45] Each monomer (Figure 1a) consists of two domains: the N-terminal (residues 1−150) and C-terminal (residues 151−482). The interface between them is made up of one α-helix from the N-terminal domain packing against two α-helices of the C-terminal domain. The monomers within the hexamer are arranged in two layers of trimers. Each trimer has the C-terminal domains of the monomer pointing toward the center with the N-terminal domains radiating outward. It appears that the role of the N-terminal domains is to provide the majority of the interactions necessary for the two trimers to stack on top of one another (see section V.C.).

Concomitant with the structural work, the C-terminal domain of bLeuAP was shown to contain the active site by photoaffininty labeling with azidobestatin, an analogue of the potent, natural product inhibitor bestatin.[46,47] Further studies also showed that the mode of binding of bestatin was slow and competitive with one binding site per monomer as seen for pkLeuAP.[48,49] The K_i and K_i* values for the initial and final complexes are approximately 1.1×10^{-7} and 1.3×10^{-9} M, respectively.

The C-terminal domain contains the cocatalytic metal center (Figure 1b).[44] The differences in the electron density observed for the metal ions within the [MgZn(bLeuAP)] crystal structure definitively showed that site 2 is the high affinity site.[43] The two metal ions, Zn1 and Zn2 (numbered Zn488 and Zn489 within the structure, respectively), are liganded by the side chains of conserved amino acid residues. Asp255, Asp332, and Glu334 bind Zn1. Lys250, Asp255, Asp273, and Glu334 bind Zn2. These interactions result in a distorted, trigonal−pyramidal coordination sphere for both metal ions with a metal separation distance of ∼3.0 Å. The differences in the nature of the ligands at each site, however, support their nonequivalence.

Throughout the course of the structural work on bLeuAP, there has been some ambiguity as to whether a solvent molecule bridges between the metal ions.[44,50−54] The source of electron density adjacent both to the metal center and to the conserved residue Arg336 was also unclear. The use of cryo-cooling to collect data on crystals of native bLeuAP and the homologous PepA (52% sequence identity within the catalytic domain[55]) have improved the quality and resolution of the structures and as a result clarified the situation.[53,56,57] A water or hydroxide molecule was found to bridge between the Zn^{2+} ions in both structures. In bLeuAP, hydrogen bonding interactions are made from this bridging molecule to Lys262 via an intervening solvent molecule, and to Arg336

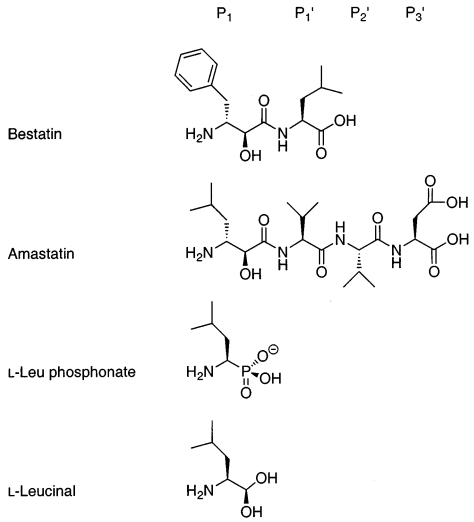


Figure 2. Ligands used to study the metal center of bLeuAP by X-ray crystallography. The nomenclature of Schechter and Berger[2] is used to indicate the location of the amino acid side chain within a peptide substrate relative to the site of cleavage (P_1*P_1'). The N-terminal side chains of bestatin and amastatin are in the D-configuration. All other side chains are in the L-configuration.

through an intervening bicarbonate molecule (Figure 1b).

D. Comparison of Ligand Complexes

The structures of several ligand complexes have been determined for bLeuAP (Figure 2). Each compound is thought to mimic the tetrahedral, *gem*-diolate intermediate or transition state of the hydrolytic reaction. The P_1 hydroxyl group of bestatin and amastatin ($K_i^* \sim 10^{-9}$ M)[48,49] can be considered to represent the nucleophile that attacks the scissile peptide bond and has been shown to be essential for potent inhibition.[58] The P_1 carbonyl group is also important for potency. A 50000-fold decrease in affinity toward pkLeuAP is observed if the carbonyl group is reduced.[59] Because an additional functional group has been inserted, there is a frame-shift of one atom (Figure 2). The P_1 side chain is also in the D-configuration. Therefore, care must be taken when extrapolating the observed interactions to those that may occur with substrates. The amino acid phosphonates are potent inhibitors of the mono-zinc peptidases carboxypeptidase A and thermolysin.[60−62] Likewise, L-Leu phosphonate (LeuP) inhibits pkLeuAP with a K_i of 230 nM.[63] The oxygen atoms of the phosphate group represent the substrate carbonyl oxygen and amide nitrogen atoms and the oxygen atom of the putative hydroxide nucleophile. The aminoaldehyde, L-Leucinal, potently inhibits pkLeuAP (K_i = 60 nM) and binds as the hydrated *gem*-diol adduct.[53,64,65]

Bestatin and amastatin have the same binding mode to the metal center.[44,51,52] Amastatin, for example, binds as an extended β-strand with alternating hydrogen bonding and metal interactions (Figure 3a). The inhibitor chelates the metal center as originally proposed,[66] displacing solvent molecules bound in the native enzyme (Figure 1b). The observation that the N-terminus binds to Zn2 is consistent with the requirement for a free N-terminus.[19] The P_1 hydroxyl group bridges between Zn1 and Zn2, while the carbonyl group interacts with Lys262. These new metal interactions expand the coordination sphere of each metal ion to a distorted octahedral geometry. The amide nitrogen of Gly362 interacts with the carbonyl oxygen of the P_1' residue.

The P_1 side chain protrudes into a primarily hydrophobic crevice generated by Met270, Thr359, Gly362, Ala451, and Met454 (not shown). Since the S_1 subsite is not well defined or enclosed, the promiscuity of the enzyme toward a wide variety of substrates is understandable. In a similar manner, the S_1' subsite is fully solvent accessible with enzyme residues Asn330, Ala333, Arg336, and Ile421 further than 5 Å away. The side chain of Arg336 in the amastatin complex was modeled in two conformations, one of which places the side chain in the location of the bicarbonate molecule seen in the native structure (Figure 3a).

LeuP binds to the metal center (Figure 3b) in an analogous manner.[54] The N-terminus and one of the oxygen atoms of the phosphate group bind to Zn2 and to both metal ions, respectively. In contrast to the P_1 carbonyl group of amastatin, one of the phosphate oxygen atoms interacts directly with Zn1 and Lys262. Hydrogen-bonding interactions from the third phosphate oxygen atom are also found to the backbone carbonyl group of Leu360 and to the amide group of Gly362 via an intervening solvent molecule. A triad of solvent molecules was reported to form a hydrogen-bonding network between LeuP and Arg366 (Figure 3b). In light of the subsequent higher-resolution native structure,[56] however, these putative solvent molecules could represent a bicarbonate molecule.

The interactions observed for L-Leucinal, a true transition state analogue, are similar to those for LeuP (Figure 3c).[53] Differences in solvent interactions were found, however, in the region near Arg336. Two of these solvent molecules were reported to be extremely close together (2.3 Å), and it was suggested that they might represent a bihydroxide ($H_3O_2^-$) anion. Following the above discussion, however, it would seem that this cluster of putative solvent molecules might actually be a bicarbonate ion.

E. Proposed Reaction Mechanism

Recent kinetic and structural data for the LeuAPs have permitted mechanistic schemes to be refined. The results from isotope studies ($H_2{}^{18}O$) and the lack of nucleophilic residues in the active site have ruled out the possibility that catalysis occurs through an anhydride mechanism involving a covalent intermediate.[67] One of the earliest schemes, based on the bestatin and amastatin complexes (Figure 3a), suggested that a water molecule bridging between the metal ions was activated with the help of Asp255.[51,68] On the basis of the "alternative" conformation, Arg336 was also postulated to stabilize the resulting oxyanion intermediate. This hypothesis was modified after the determination of the LeuP complex (Figure 3b) and is presented in Figure 4 (Intermediate I).[54] In this proposal, the P_1' amide nitrogen atom also binds to Zn1 and is protonated as the leaving group by Lys262.

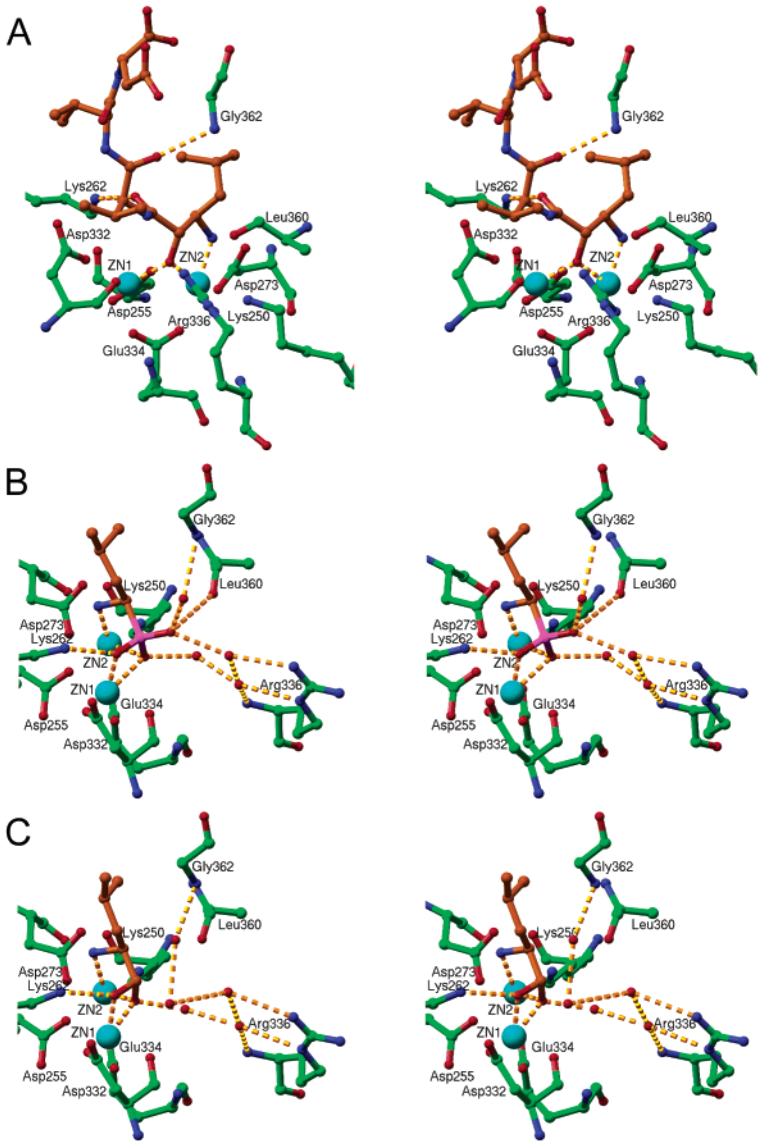


Figure 3. Stereoviews of representative ligand complexes of bLeuAP. (A) The substrate-like transition state analogue amastatin (PDB: 1BLL).[51] (B) L-Leu phosphonic acid (PDB: 1LCP).[54] (C) L-leucinal (PDB: 1LAN).[53] Only the backbone carbonyl group of Leu360 is shown for clarity.

The location of the metal-bridging solvent molecule and the bicarbonate molecule bound to Arg336 (Figure 1b) has ruled out these proposed roles for Asp255 and Arg336. The binding mode of LeuP (Figure 3b) also clearly suggests that the nitrogen of the scissile bond does not bind to Zn1, but is in the position of the phosphate oxygen atom that interacts with Leu360, the putative bicarbonate anion and Arg336. The interaction of the oxygen atoms of intermediate I, either directly or indirectly with Arg336, also seems unlikely. On the basis of these observations, it is also not likely that a solvent molecule in the vicinity of Arg336 could be activated strongly enough to act as the nucleophile of the reaction.[54]

The putative transition state intermediates II and III (Figure 4) were formulated based on the binding modes of LeuP and L-leucinal. The proposed interactions are reminiscent, however, of those seen for bestatin and amastatin in that two oxygen atoms interact with both metal ions and Lys262. The main difference between these intermediates is the origin of the attacking nucleophile, O_N. If O_N originates from the vicinity of the two metal ions (Scheme 2), the carbonyl group is attacked from the *re* enantiotopic face and the negatively charged oxygen atom of the carbonyl group, O_C, is stabilized by interactions with Zn1 and Lys262. In the converse arrangement (Scheme 3), the nucleophile O_N originates from the vicnity of Zn1 and the attack occurs on the *si* side of the carbonyl group with the developing negative charge stabilized by both metal ions.

The structures of the unliganded forms of bLeuAP and PepA (not shown) did not show a solvent molecule bound exclusively to Zn1. Moreover, the NMR analysis of [MnZn(bLeuAP)] in complex with the inhibitor *N*-(leucyl)-*O*-aminobenzoate and the crystallographic analyses of ligands supports the binding of the substrate carbonyl group to the site 1 metal.[69] Together these observations support mechanism II where the solvent molecule bridging between the metal ions functions as the nucleophile. Since water molecules bound to metals can exhibit pK_a values as

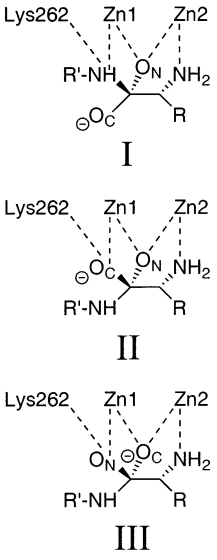


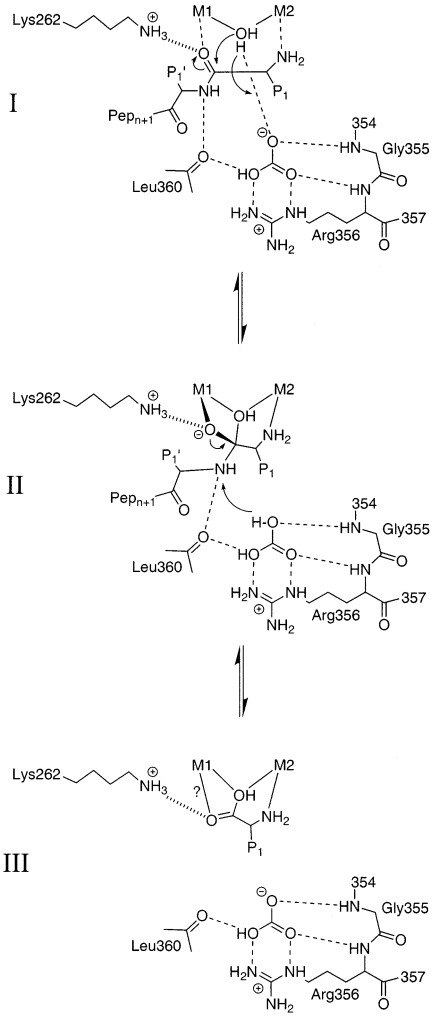
Figure 4. Three possible binding modes of the transition state to bLeuAP. Figure adapted from the diagrams presented by Sträter and Lipscomb.[53,54] The oxygen atoms are labeled according to their proposed origin: O_N, attacking nucleophile; O_C, the substrate carbonyl oxygen atom. In intermediate I the oxyanion, O_C, is potentially stabilized directly by Arg336 or indirectly by intervening water molecules bound to Arg336. Intermediates I and II are both stabilized by the metal center, but differ in the location O_N.

low as pH 7, this molecule most likely exists as a hydroxide ion.[70] The activation of the water molecule may also be facilitated by its interaction with a bicarbonate or bihydroxide ion, which may function as a general base.

The importance of bicarbonate, Arg336, and Lys262 to catalysis has been evaluated through the mutational analysis of the corresponding residues in PepA.[56] The presence of bicarbonate in the assay buffer increases the turnover number (k_{cat}) for the wild-type enzyme 8-fold. The R336A and R336M mutants exhibit activity levels that are similar to the wild-type enzyme in bicarbonate-free buffer. Thus, Arg336 can enhance catalysis, but is not essential. The similar activity levels in the bicarbonate-free buffer also suggest that a bihydroxide ion does not contribute to enzyme activity. In contrast, the Lys262Ala mutant shows a 100- to 600-fold decrease in k_{cat} and a ~20-fold increase in K_m. These observation support the direct interaction of Lys262 with the substrate and the subsequent stabilization of the transition state. Similar results have been observed for the corresponding Lys and Arg mutants of the tomato LeuAP.[71]

On the basis of the compilation of structural, kinetic, and mutational data, the currently favored reaction mechanism is presented in Scheme 1. The slow-binding characteristics of bestatin analogues and the observation of at least two pre-steady-state intermediates for pkLeuAP suggest that the initial binding process may be sequential.[26,48,49] As the substrate enters the active site it may briefly bind to Zn1 before it slides into the correct register with its N-terminus bound to Zn2 and carbonyl group bound to Zn1 (intermediate I). The resulting alteration of the metal center electronics coupled with

Scheme 1



proton abstraction by the bicarbonate ion activates the metal-bridging water molecule as the nucleophile. The tetrahedral intermediate (intermediate II) is stabilized by further interactions with Zn1 and Lys262. Collapse of the intermediate is facilitated by proton transfer to the amide leaving group via the bicarbonate molecule. As shown by the residual activity of the Arg336 mutants, water molecules in the vicinity may supplant the role of the biocarbonate molecule. The resulting product complex (intermediate III) most likely maintains the N-terminal amine and bridging oxygen interactions. It is unclear whether the interactions with Zn1 and Lys262 are also maintained.

The only observation that appears to be at variance with the proposed mechanism is the reported activity of pkLeuAP with a metal ion only in site 2 (Table 2). Mechanistically, the site 1 metal is thought to provide critical binding and stabilizing interactions to the substrate and the transition state. It may be noted, however, that the apparent dependence on activity of metal in site 1 varies substantially for different substrates. Also it is difficult to ensure that the metals tested do not migrate from one site to the other. Unfortunately, the sequence of pkLeuAP is not in the protein database and an identification of possible amino acid substitutions that might help explain the behavior of this enzyme is not possible.

Table 3. Kinetics Parameters of Metal-Substituted ApAP[a]

enzyme form		L-Ala-*p*-NA			L-Val-*p*-NA			L-Leu-*p*-NA		
site 1	site 2	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	$(k_{cat}/K_m) \times 10^{-4}$ (M^{-1} min^{-1})
Zn^{2+}	Zn^{2+}	17.3	1000	1.7	10.6	25	42.4	4720	23	20520
Zn^{2+}	Co^{2+}	21.2	1250	1.7	10.5	22	47.7	2166	14	15470
Zn^{2+}	Ni^{2+}	27.0	286	9.4	14.6	20	73	4200	23	18260
Zn^{2+}	Cu^{2+}	14.2	740	1.9	12.4	29	42.8	3270	13	25150
Co^{2+}	Co^{2+}	44.8	435	10.3	7.3	11	66.4	1360	4	34000
Co^{2+}	Zn^{2+}	52.2	870	6.0	9.5	7	135	1990	8	24880
Co^{+2}	Ni^{2+}	85.6	333	25.7	9.8	11	89.1	942	5	18840
Co^{2+}	Cu^{2+}	41.3	80	51.6	5.9	1.0	590	706	4	17650
Ni^{2+}	Ni^{2+}	53.6	137	39.1	5.3	0.9	589	784	10	7840
Ni^{2+}	Zn^{2+}	312.0	213	146.0	14.8	0.4	3700	248	16	1550
Ni^{2+}	Co^{2+}	93.3	122	76.5	8.1	0.9	900	713	6	11880
Ni^{2+}	Cu^{2+}	14.4	114	12.6	4.1	0.8	513	271	13	2090
Cu^{2+}	Cu^{2+}	5.4	36	15.0	2.6	1	260	240	13	1850
Cu^{2+}	Zn^{2+}	61.5	25	246.0	10.5	3	350	557	15	3710
Cu^{2+}	Co^{2+}	27.6	39	70.8	3.9	1.0	390	447	6	7450
Cu^{2+}	Ni^{2+}	15.3	64	23.9	3.9	0.6	650	523	21	2490

[a] Data from Bayliss and Prescott.[81] Assay conditions: 0.01 M Hepes pH 7.5.

As discussed below, consideration of the other classes of metalloaminopeptidases shows that there are cases in which side chains in the active site region are able to assume the function of a metal ion.

III. Aeromonas proteolytica (ApAP) and Streptomyces griseus (SgAP) Aminopeptidases

The metalloaminopeptidases from the marine organism *Aeromonas proteolytica* (E.C. 3.4.11.10) and the soil microbe *Streptomyces griseus* have been the focus of a variety of studies as a consequence of their small size, ease of purification, heat stability, and activity as a monomer.[72,73] Early studies were also motivated by the need for effective additives for the two-stage assays of other peptidases[74,75] and the search for an enzyme that could be used for sequencing of proteins.[76] Both enzymes show remarkable similarities in their action against substrates, tertiary structures, metal centers, and putative reaction mechanisms even though they share only 30% sequence identity.[77]

A. Substrate Specificity

The substrate preferences for ApAP and SgAP have been determined with dipeptides and oligopeptide substrates derived from the degradation of other proteins.[76,78,79] Both enzymes prefer large hydrophobic amino acids in the P_1 position of the substrate with Leu being the most efficiently cleaved. The enzymes can accommodate all residues in the P_1' position except for Pro and Glu. On the basis of these findings, the substrate specificity of both enzymes is similar to that of LeuAP.

B. Metal Dependence and Modulation of Activity

Metal analyses of the native ApAP and SgAP enzymes indicate the presence of two Zn^{2+} ions per ~30 kDa monomer.[72,73] Removal of the metal ions by chelating agents inactivates the enzymes.[72,80] The type of metal ion and the order of substitution can drastically affect the enzyme activity as seen for the LeuAPs. The activity of SgAP is also affected by the addition of Ca^{2+}.

Near wild-type levels of activity (~80%) can be restored to the metal-free form of ApAP by the addition of one equivalent of either Zn^{2+} or Co^{2+} ions.[80] Zn^{2+} in site 1 has an approximate dissociation constant of 10^{-10} M. Enzymatic activity is, however, enhanced or modulated by filling the lower affinity site (site 2) to form a dinuclear metal center.[72,73,80,81] Representative data for ApAP reconstituted with Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} to form metallohybrids are given in Table 3. The most important factors governing the activity are the choice of substrate and the metal present in site 1 (ref 81). No combination of metal ions alters the overall substrate preference as expressed by k_{cat}/K_m, i.e., L-Leu-*p*-NA > L-Val-*p*-NA > L-Ala-*p*-NA. The highest k_{cat} values are observed for the Leu-based substrate. Zn^{2+} in site 1 decreases the affinity (K_m) for all substrates. The enhancement of activity over the di-zinc enzyme, previously observed[80] with Ni^{2+} or Cu^{2+} in site 1, only occurs with the poorer Ala- and Val-based substrates. Similar substrate and metal-dependent effects on activity have been observed for SgAP.[72,79,82] Thus, it is apparent that ApAP and SgAP can function with a variety of metals and that the effects can vary somewhat from substrate to substrate.

C. Unique Metal Center Despite Structural Similarity to Leucine Aminopeptidase

The high-resolution crystal structures[83−89] of the unliganded forms of both ApAP and SgAP have revealed that the cocatalytic metal center is somewhat symmetric in nature (Figure 5a) with an interion distance of ~3.5 Å. Both metal ions have distorted tetrahedral coordination with ligands from two histidines, a bifurcated interaction from a bridging Asp side chain, and terminal Asp and Glu residues. The terminal acidic residues may provide a fifth ligand to the metal ions via an asymmetric, bidentate interaction with the second oxygen atom of the side chain. Glu131 and Glu151 in SgAP and ApAP,

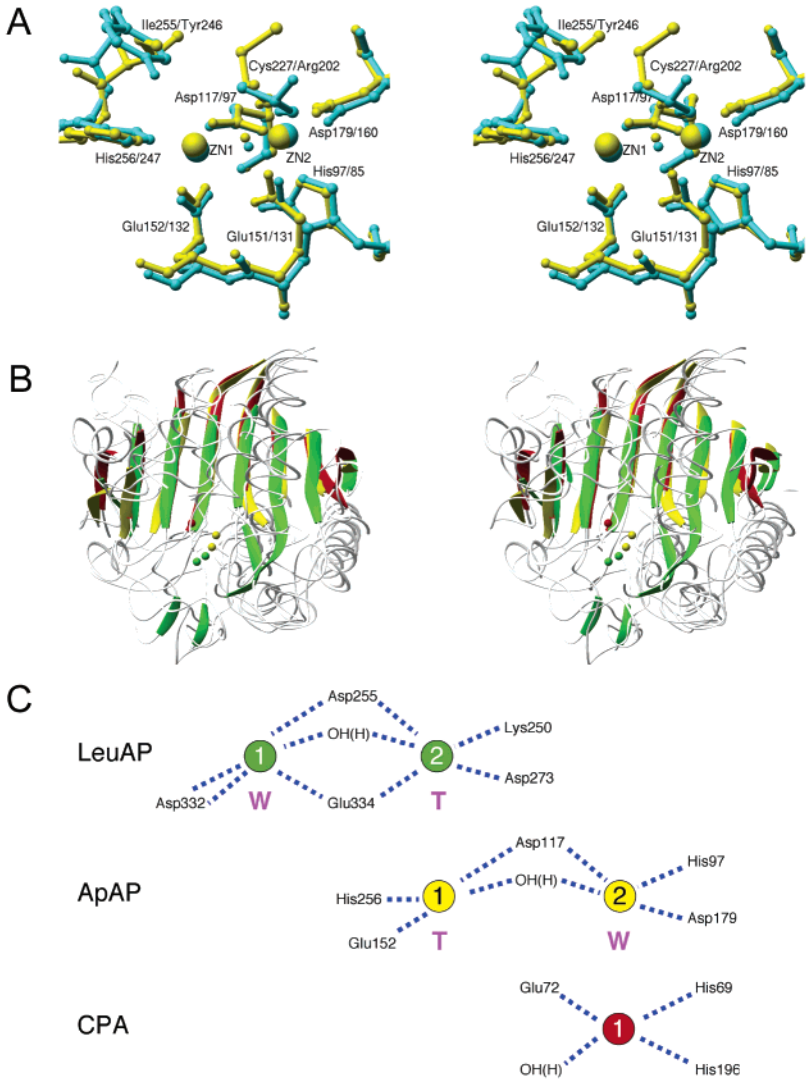


Figure 5. Structural and metal center relationships between some members of the zinc hydrolase superfamily. (A) Overlay of the metal centers of ApAP (yellow, PDB: 1CP6)[86] and SgAP (cyan, PDB: 1CP7).[87] The labels indicate the ApAP/SgAP residues. (b) Comparison of bLeuAP, ApAP, and CPA. The coordinates for LeuAP (green, PDB: 1LAM),[56] ApAP (yellow) and CPA (red, PDB: 1CPX)[205] were superimposed based on the hydrogen-bonding patterns for the β-sheet and α-helices using the program EDPDB.[206] The central β-sheet is spatially more conserved than the α-helical and loop regions (white). The metal ions are shown as spheres. The nonconserved regions have been colored white and partially sliced out of view to enhance the clarity of the metal ions and the central β-sheet. (C) Diagrammatic representation of the metal centers showing their approximate positional relationship as shown in (B) and their relative affinity for metals. The weak (W) and tight (T) binding sites exhibit significant differences in affinity for divalent metal ions. The catalytic metal ion of CPA lies in the same plane as the site 2 metal ion of ApAP and SgAP. The metal ion, however, has apparently been translocated slightly in order to facilitate carboxypeptidase rather than aminopeptidase activity (see text for details).

respectively, form a hydrogen bond to a bridging water molecule or hydroxide ion.

The protein folds of ApAP and SgAP are, as expected, very similar. Although the enzymes have little sequence homology, they have similarities to the structures of bLeuAP and carboxypeptidase A (CPA).[90,91] All have a common, central, eight-stranded β-sheet which can be used to superimpose these members of the zinc hydrolase superfamily (Figure 5b). This superposition suggests that the dinuclear enzymes (bLeuAP and ApAP) have "translocated" metal centers (Figure 5c) with one metal site being conserved but having differences in metal ligands. The conserved metal site, site 2 for bLeuAP and site 1 for ApAP, exhibits higher affinity for metal ions. The single metal site of CPA is located in close proximity to the site 2 metal of ApAP. As a consequence of the apparent translocation of the metal center within the protein fold, mutations in active site residues and changes in the substrate binding subsites have occurred.[90] A comparison of ligand complexes suggests that these changes have resulted in the re-registration and reorientation of the substrate necessary for enzymes that function as N- or C-terminal exopeptidases.

D. The Effect of Calcium on the Stability and Catalytic Properties of SgAP

The addition of calcium to SgAP results in increased stability at 75 °C.[72,82] In contrast, ApAP is destabilized under similar conditions. Calcium binding also alters the activity of SgAP and increases its affinity for inhibitors and substrates.[79,82,92] The inhi-

Table 4. Inhibition of [ZnZn(SgAP)] in the Absence and Presence of Ca^{2+}

	$CaCl_2$ 1 mM	K_i (μM)
Leu-CH_2−Cl	−[a]	5.5
	+	0.5
Leu-CH_2−Cl	−	5.3
	+	1.9
amastatin	−	0.55
	+	0.02
bestatin	−	5.4
	+	2.6

[a] Data from Papir et al.[82] Assay conditions: 50 mM Tris pH 8.0, 100 mM NaCl, 30 °C.

bition constants for the Leu- and Phe-based chloromethyl ketones, amastatin and bestatin (Table 4) are decreased by 2- to 34-fold. Moreover, the K_m for Leu-*p*-NA decreases from 3 to 0.6 mM with a concomitant 2-fold increase in k_{cat}. The modulation of activity, however, is not always positive and depends on the substrate used. L-Lys-*p*-NA, for example, is cleaved more poorly in the presence of calcium.[79] The analysis of the Cd^{2+}-substituted enzyme also suggests that calcium is an ideal modulator of activity by enabling the enzyme to be active at much lower metal concentrations.[92]

X-ray crystallography revealed that the calcium binding site is generated by three acidic residues (Asp3, Asp262, Asp266) and the carbonyl oxygen atom of Ile4 (Figure 6).[89] When calcium is removed, the side chain of Asp3 side chain rotates away from the ion site (not shown). The lack of the corresponding residues in ApAP supports the exclusivity of the calcium dependence to SgAP.[82]

Surprisingly, the Ca^{2+} site is 22−25 Å away from the two metal ions in the active site (Figure 6). A hydrogen-bonding network involving Asn96, Asp97 (one of the Zn^{2+} ligands), Gly163, Asn257, Asp258, and Asp262 (one of the Ca^{2+} ligands) has been postulated as the molecular mechanism whereby calcium binding is propagated or transmitted to the active site.[82] According to this proposal, calcium binding may move the secondary structural elements of the enzyme that underpin the hydrogen-bonding network. This small movement may then alter the bifurcated interaction of Asp97 in a way as to affect the electronics of the metal center and thus catalysis. The analysis of the temperature-dependence of hydrolysis shows, however, that the binding of calcium leads to the tighter binding of substrates and, as a result, decreases the entropy of activation ($\Delta S^{\ddagger}$) and the free energy of activation ($\Delta G^{\ddagger}$).[92] The magnitude of the free energy change ($\Delta\Delta G^{\ddagger} \sim 2.6$ kJ mol^{-1}) suggests that the proposed "relay network" and electrostatic effects do not fully account for the observed calcium effects. We are not aware of a case in which long-distance effects in a protein are transmitted by such a chain of interactions involving individual side chains. Rather, long-distance effects tend to result from domain shifts or other changes in the global character of the protein.

E. Comparison of Ligand Complexes

The structures of several ligand complexes have been determined for ApAP and SgAP (Figure 7). These complexes illustrate how a metal chelator, two transition state analogues, and several N-terminal products bind to the dinuclear metal centers. Amino acid hydroxamates are potent inhibitors of metalloaminopeptidases by their ability to form bidentate, chelate complexes to transition metals.[93] The D-configuration for the side chain is preferred.[94] The metal chelate, *p*-iodo-D-Phe hydroxamate (D-IPH), inhibits ApAP with a K_i of 0.4 μM.[85] The transition state analogues LeuP and butane boronic acid (BBA) inhibit ApAP with K_i values of 6.6 and 10 μM, respectively.[83,95] Several product complexes of SgAP have also been determined. Met, Leu, and Phe weakly inhibit SgAP with K_i values of about 10 mM.[82] Amastatin and bestatin also potently inhibit this group of aminopeptidases ($\sim 10^{-8}$ to 10^{-10} M) but await structure solution and analysis.[49]

D-IPH binds to the active site of ApAP (Figure 8a) replacing the metal-bridging solvent molecule of the native enzyme with the hydroxamate hydroxyl group (P_1').[85] The carbonyl group contacts Zn1. The aromatic ring of the iodobenzyl moiety stacks against Phe244 while the N-terminus interacts with the hydroxyl group of Tyr225. Two interactions are also observed to Glu151. Since the N-terminal, α-amino group is not required for potency,[94] it is apparent that

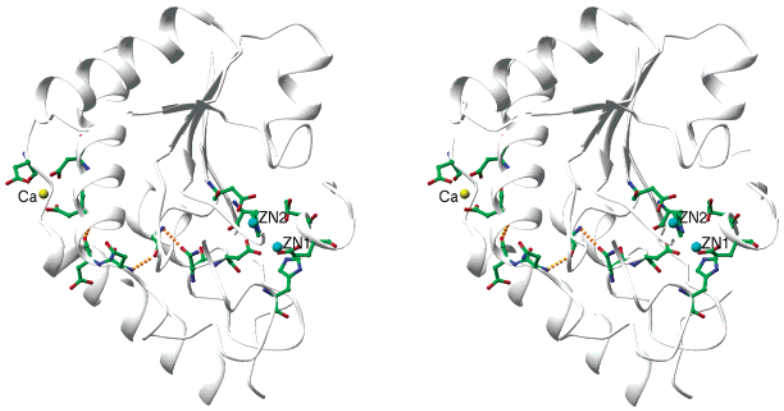


Figure 6. Spatial relation between the metal center and the calcium-binding site within SgAP. Several residues (green) generate a hydrogen-bonding network (dashed gold bonds) between the active site Zn(II) ions (cyan) and the Ca^{+} ion (yellow) (PDB: 1CP7).[87] Asp3, Asp262, Asp266, and the carbonyl oxygen atom of Ile4 on the surface of the protein facilitate Ca^{+} binding. Parts of the molecule have been sliced out of view and colored white for clarity.

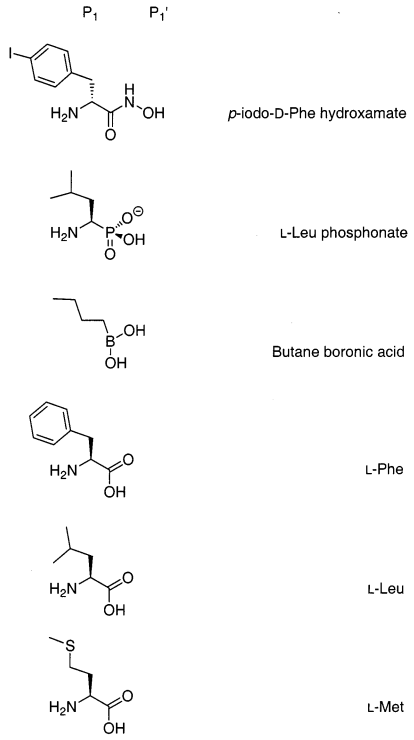


Figure 7. Ligands used to study the metal centers of ApAP and SgAP by X-ray crystallography.

the hydroxamate interactions dominate binding to the metal center. Given the D-configuration of the side chain, the binding mode of D-IPH cannot fully represent a true substrate. The mode of binding is consistent, however, with the bLeuAP complexes in that the carbonyl oxygen atom does bind to Zn1.

BBA interacts only with Zn1 of ApAP (Figure 8b).[86] The oxygen and boron atoms of BBA normally exhibit trigonal planar (sp^2) geometry although a trigonal pyramidal (sp^3) configuration is observed in the complex. Binding is associated with the release of the metal-bridging solvent molecule of the native enzyme, and the location of a new water molecule 2.4 Å from the boron atom. This complex is thought to represent an intermediate between the Michaelis complex and the transition state.

The binding of LeuP to the metal center of ApAP results in the expansion of the coordination sphere of both metal ions (Figure 8c).[83] Zn1 exhibits a distorted five-coordinate geometry if the bidentate interaction from Glu152 is assumed. Zn2 also exhibits a distorted five-coordinate geometry with Asp179 providing a bidentate interaction. As seen in the LeuP complex of bLeuAP, the N-terminus binds to Zn2 and the oxygen atom of the phosphate moiety that represents the substrate carbonyl group binds to Zn1. The binding mode of LeuP to ApAP is different, however, in that the oxygen atom bound to Zn2 does not bind to Zn1 and a metal-bridging interaction is not maintained.

The product complexes of SgAP illustrate the potential for a different binding mode.[87,88] Instead of the N-terminus binding to Zn2 as seen for all other metalloaminopeptidases, the N-terminus of Leu, for example, binds to Asp160, Glu131, and the backbone carbonyl group of Arg202 (Figure 8d). Despite the apparent rotation of the ligand within the active site relative to ApAP, the carboxyl oxygen atoms maintain the typical metal-bridging and Zn1 interactions. A hydrogen-bonding interaction is also seen from the Zn1-bound oxygen atom to Tyr246 in a manner reminiscent to that of Lys242 of bLeuAP.

The apparent anomalous location of the N-terminus may be explained by the differences in the composition and structure of the loop in this vicinity of the S_1 subsite between SgAP and ApAP. The loop of SgAP is composed of Glu196−Thr197−Glu198−Gly199−Asp200−Gly201−Arg202. The fact that this loop is only observed in the presence of a ligand suggests that it is relatively mobile when compared to the loop found in ApAP. The corresponding loop in ApAP is composed of Asp221−Thr222−Cys223−Gly224−Tyr225−Ala226−Cys227 with a disulfide bond between the Cys residues. Despite these differences, the backbone carbonyl atoms of Arg202 and Cys227 are in roughly the same location in space. These observations suggest that this carbonyl group may bind to the scissile peptide bond nitrogen atom as in ApAP or to the N-terminal nitrogen atom as in SgAP. The resolution of this issue awaits the structural analysis of ligand complexes for both enzymes.

The side chains of LeuP and Leu fit into the active sites of ApAP and SgAP in a similar manner. The side chain in each case interacts with an expansive, primarily hydrophobic S_1 subsite. Within ApAP, only Met180, Met242, Phe244, and the disulfide bridge of Cys223 and Cys227 are within 5 Å of the ligand side chain. For SgAP, only Met161, Phe219, and the backbone atoms of Gly199 and Arg202 are within 5 Å of the ligand side chain. The open nature of both active sites supports their ability to cleave a wide variety of substrates.

F. Proposed Reaction Mechanism for ApAP

The combination of spectroscopic and structural data for ApAP has led to a detailed description of the hydrolytic reaction. Many elegant studies measuring the electronic absorption, CD, MCD, NMR, and EPR spectra of homonuclear and heteronuclear ApAPs have clearly indicated that the metal sites are electronically distinct even though they are essentially equivalent structurally.[83,92,96−107] These studies have exploited the metal site differences to analyze ligand binding events and the change in the location of the metal-bridging solvent molecule. For example, the differences between the EPR spectra of [CoCo(ApAP)], [CoZn(ApAP)], and [ZnCo(ApAP] seen in the absence and presence of LeuP indicate that the metal ions are spin-coupled and that ligands bind directly to both.[83,98,106] Previously proposed reaction schemes[98,102] based on the metal chelator D-IPH have been revised following insights gained from the native enzyme structure and the BBA and LeuP complexes (Figures 5a and 8a−c).[83]

Scheme 2 presents the reaction mechanism assembled from the available data and adapted from the schemes presented by the Holz and Petsko laboratories.[83,100,101] The kinetic and spectroscopic analysis of a series of aliphatic alcohols that bind

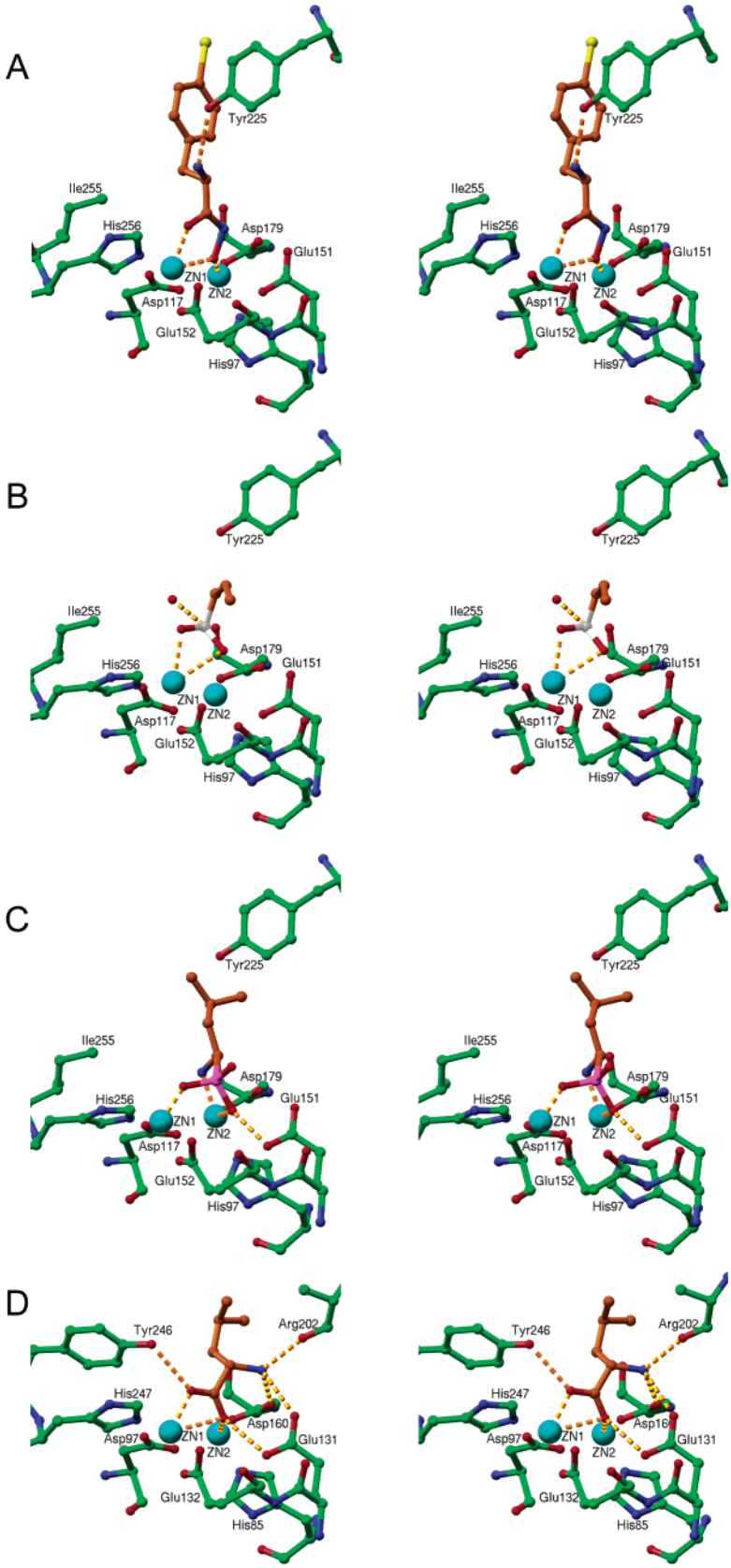
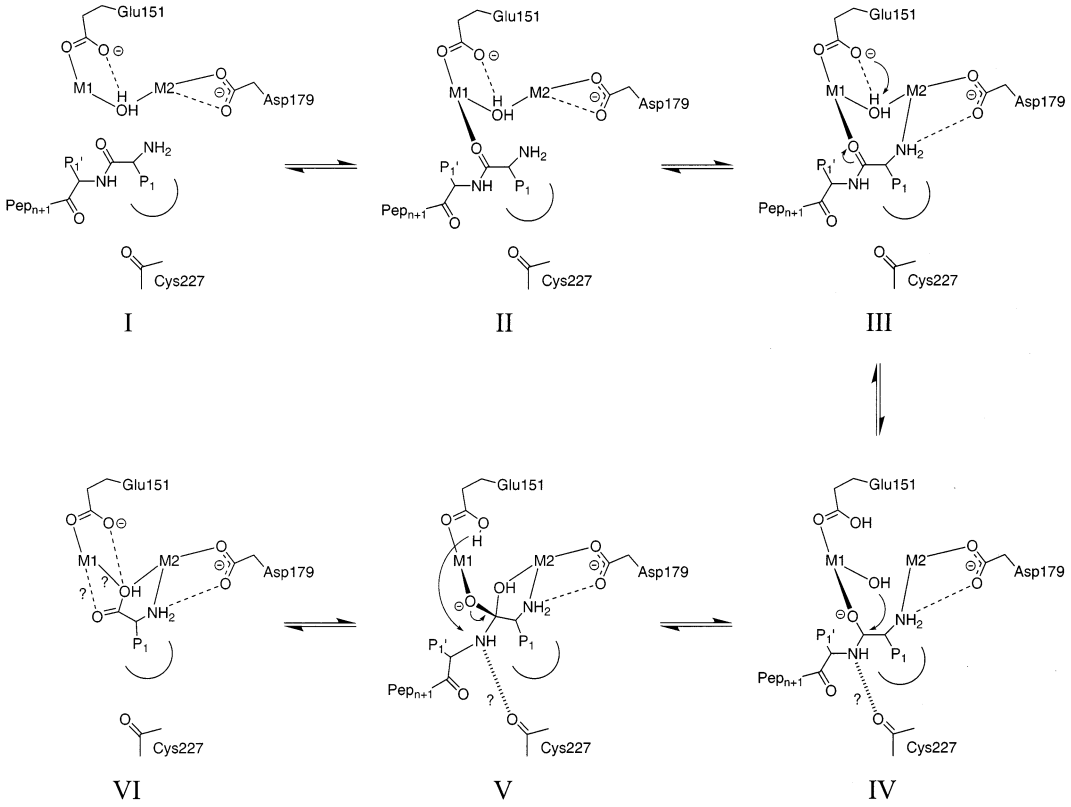


Figure 8. Stereoviews of selected ligand complexes of ApAP and SgAP. (A) *p*-iodo-D-Phe hydroxamate bound to ApAP (PDB: 1IGB).[85] (B) Butane boronic acid bound to ApAP (PDB: 1CP6).[86] Only three of the four carbons atoms were observed in the electron density. (C) L-Leu phosphonic acid bound to ApAP (PDB: 1FT7).[83] (D) L-Leu bound to SgAP (PDB: 1F2O).[88] The side chain of Arg202 has been truncated for clarity.

competitively to ApAP suggests that the Leu side chain first binds to the hydrophobic S_1 pocket (intermediate I).[103] The carbonyl group of the scissile peptide bond then binds to Zn1 (intermediate II). This step is supported by the EPR and crystallographic analysis of BBA showing that BBA binds only to the

Scheme 2



site 1 metal.[86,98,105] The analysis of peptides with the scissile peptide bond modified to contain a thiocarbonyl group, or with thiol groups appended to the N-terminus further support this notion.[99,100] Use of the thiocarbonyl substrate also indicates that the third step (intermediate III) in the reaction is the binding of the N-terminal amine group to Zn2, which is consistent with the interaction seen in the LeuP complex. On the basis of geometrical considerations, the proposed N-terminal interaction with Asp179 is more speculative. This second binding event is thought to alter the electronics of the metal center such that the metal-bridging water/hydroxide molecule loses its interaction with Zn2 and becomes associated with Zn1 (intermediate IV). Glu151 is thought to facilitate the formation of the nucleophile by acting as a general base. Hydrogen bonding networks involving metal ligands and adjacent enzyme residues may also help break the Zn2−OH(H) interaction.[83,86]

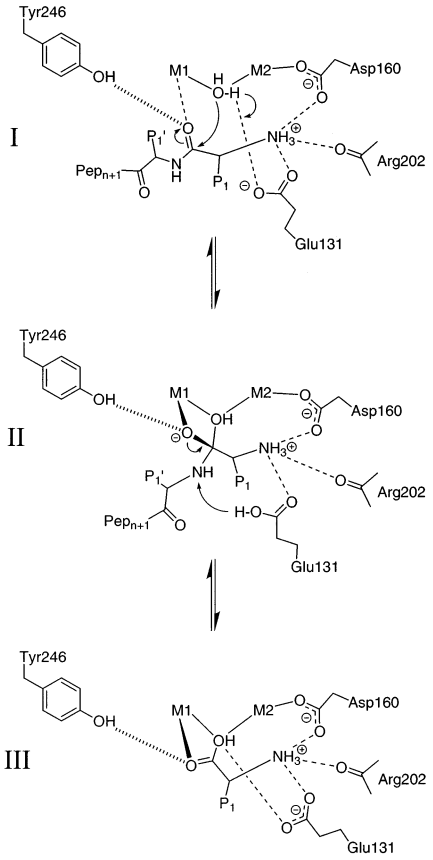
The transfer of the nucleophile to Zn1 is also supported by the fact that ApAP is ~80% active with one metal ion and uncompetitvely inhibited by fluoride, indicating that inhibition occurs only after substrate binding.[80,102] The Zn1-bound nucleophile attacks the carbonyl group leading to the formation of a tetrahedral, *gem*-diolate intermediate (intermediate V). The breakdown of the intermediate has been shown to be the rate-limiting step of the reaction and to involve the transfer of one proton.[101,102] A proton is most likely transferred from Glu151 to the amide nitrogen atom of the leaving group. The binding mode of the resulting free amino acid (intermediate VI) is uncertain. If the product binds in the manner observed for the product complexes of SgAP (Figure 8d) and methionine aminopeptidase, the carboxyl group interactions with both metal ions may be maintained.[87,88,108]

The high activity of the mononuclear form of ApAP also strongly suggests that the role of the site 2 metal ion in binding the N-terminus of the substrate can also be performed by either Asp179 or possibly Asp117 (ref 83). In this scenario, the N-terminal amine is most likely in the protonated state. This possibility of an alternative interaction for the N-terminus of the substrate while maintaining the necessary interactions with the catalytic site 1 metal ion is supported, in principle, by the product complexes of SgAP. Additional support for this proposal has been put forward from the study of the mononuclear form of methionine aminopeptidases described in more detail below.

G. Proposed Reaction Mechanism for SgAP

A reaction mechanism for SgAP (Scheme 3) has been formulated based on the native structure and the binding mode of several product complexes (Figures 5a and 8d).[87−89] Similar to the mechanisms proposed for ApAP and bLeuAP, the carbonyl group of the scissile peptide bond is thought to bind to Zn1 (intermediate I). Tyr246 stabilizes this interaction in a manner similar to Lys242 of bLeuAP. The N-terminal amine group, in contrast, is proposed to interact with the side chains of Glu131, Asp160, and the backbone carbonyl group of Arg202. This alternative mode of binding, however, still enables the oxygen atoms of the tetrahedral intermediate (intermediate II) to be stabilized by interactions to both metals and Tyr246. Glu131 functions as a general base similar to Glu151 and bicarbonate ion of ApAP

Scheme 3



and bLeuAP, respectively. The resulting product complex (intermediate III) maintains all metal contacts and the interaction with Tyr246.

IV. The "Pita-Bread" Enzymes: Methionine Aminopeptidase (MetAP), Aminopeptidase P (AAP), Prolidase

This family of enzymes is able to cleave amido-, imido-, and amidino-containing bonds (Table 5). MetAP (E.C. 3.4.11.18), APP (X-Pro aminopeptidase; E.C. 3.4.11.9), and prolidase (X-Pro dipeptidase; E.C. 3.4.13.9) exhibit relatively narrow substrate specificities when compared to the other metalloaminopeptidases[109,110] The restrictive nature of these enzymes is associated with a common "pita-bread" fold that contains a metal center flanked by well-defined S_1 and S_1' substrate binding pockets. The ability of this group of enzymes to cleave a restricted subset of N-terminal peptide bonds suggests that they play roles in the regulation of biological processes rather than general protein degradation.

Table 5. Substrates of the Pita-Bread Enzymes[a]

enzyme	substrate
methionine aminopeptidase	H-Met*Xaa-Yaa
aminopeptidase P	H-Xaa*Pro-Yaa
prolidase	H-Xaa*Pro-OH
creatinase	$H_2N-C(=NH)*N(CH_3)-CH_2-COOH$

[a] The locations of the amido-, imido, and the amidino-scissile bonds of the different substrates are indicated by the asterisks.

A. Physiological Roles and Substrate Specificity of MetAPs

The removal of the N-terminal Met from proteins by MetAPs appears to primarily occur cotranslationally.[111,112] Mutational analysis of the N-terminal zinc-finger domains of the yeast MetAP-1 has shown that it associates with the 60S ribosomal subunit and the 80S translational complex.[113] Trimming at the amino-terminus is required for biological activity, subcellular localization, and the eventual degradation by other enzymes.[114−118] The importance of MetAP activity is underscored by the lethality of the gene knockouts of *E. coli* and *Salmonella typhimurium*.[119,120] Yeast with both MetAP genes deleted are also nonviable.[121−123] Moreover, this family of enzymes is involved in the regulation of protein synthesis[124−127] and the processing of key proteins necessary for the formation of new blood vessels in normal development, tumor growth, and metastasis.[128−134]

The N-terminal sequence analysis of endogenous and systematically substituted recombinant proteins showed that MetAPs remove the N-terminal Met (P_1) only when the second residue (P_1') is small and uncharged (i.e., Gly, Ala, Ser, Thr, Val, Pro, Cys).[135−137] The in vitro analysis of peptides cleaved by the *E. coli* enzyme confirmed these observations, showing that the N-terminus cannot be blocked and that the substrate must be at least three residues in length.[138−144] This restrictive substrate specificity is opposite of the "N-end rule" for protein lifetime and degradation. MetAP will not remove the N-terminal Met if a "destabilizing" amino acid is revealed.[114]

Eukaryotic organisms express more than one form of MetAP, each with the same general substrate specificity.[109,145] These different forms, however, show subtle differences in the efficiency of substrate cleavage and marked differences in inhibition by the natural product fumagillin.[121,122,144,146−150] For example, fumagillin, a potent anti-angiogenesis agent, selectively inhibits the type 2 yeast and human enzymes, yMetAP-2 and hMetAP-2, respectively. Moreover, yMetAP-2 is unable to process in vivo some substrates readily cleaved by the type 1 enzyme.[149] Human MetAP-2 also appears to be different from *E. coli* MetAP, a type 1 enzyme (see structural discussion), by being able to cleave dipeptides and small chromogenic and fluorogenic substrates, albeit with significantly poorer rates than typical tripeptide and larger substrates.[144]

B. Physiological Roles and Substrate Specificities of APP and Prolidase

APP and prolidase also appear to process specific substrates including bioactive peptides involved in the cardiovascular and pulmonary systems and degradation products of collagen.[110,151−153] Both enzymes cleave the N-terminal residue from substrates of the form Xaa−Pro with proline in the penultimate (P_1') position.[110,154]

Prokaryotic, eukaryotic, and plant APPs have the same general substrate specificity despite showing a variety of oligomerization states ranging from dimer to tetramer.[110,154−160] The eukaryotic enzymes

can be further delineated as being either cytoplasmic or membrane bound via a glycosylphosphatidylinositol lipid anchor. The preferred substrates for APPs contain a free N-terminus, a hydrophobic or basic residue at P_1 and a trans proline in the P_1' position. Dipeptides are cleaved slowly while extended substrates are readily cleaved. The increased activity is thought to be a function of the extended S_2' and S_3' subsites that are generated across monomers within the oligomer. The mammalian membrane-bound enzymes appear to be more restrictive than the *E. coli* enzyme by not being able to accommodate bulky amino acid side chains in the P_2' position.

As a consequence of only being able to cleave dipeptides, prolidase is also known as X-Pro dipeptidase. This dimeric enzyme favors nonpolar residues in the P_1 position (i.e., Met, Leu, Val, Phe, Ala) and requires the P_1' Pro residue to be in the trans configuration.[161−164] The study of picolinylproline substrate analogues has also been informative for this group of enzymes.[165]

C. Metal Dependence of MetAPs

Historically, the observed Co^{2+}-dependent activation of metal-free eMetAP suggested that all MetAPs utilized Co^{2+} in vivo.[138] In other aerobic studies, activity has been observed in the presence of Co^{2+} and Zn^{2+} for eMetAP; Co^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+} for yMetAP-1; Co^{2+} for pfMetAP; and Co^{2+} and Mn^{2+} for hMetAP-2 (refs 123, 138, 140, 141, 144, 150, 166, and 180). Upon the basis of the findings that the Zn^{2+}-substituted yMetAP-1 enzyme was as active as the Co^{2+}-substituted enzyme and that only the Zn^{2+}-mediated activity was retained in the presence of reduced glutathionine, Walker et al. proposed that the Zn^{2+} was the in vivo metal ion.[140,141] The subsequent anaerobic analysis of eMetAP where extreme precautions were taken to remove contaminating metal ions from buffers and glassware have, however, suggested that the addition of Zn^{2+} is inhibitory.[167−169] Zn^{2+} has also been shown to inhibit hMetAP-2 (ref 144). At this time, further data on the yeast enzyme are needed to reconcile this discrepancy.

In an effort to experimentally determine the metal ion used in vivo, the metal content of bacterial extracts with the plasmid-based overexpression of eMetAP was determined. These studies showed an increase in Fe^{2+}, Mn^{2+}, and Zn^{2+} levels, with the concentration of Fe^{2+} being 4−6 times greater than the other metal ions.[168] A negligible change in the Co^{2+} level was observed. Anaerobic experiments with the recombinant enzyme indicated that Co^{2+} and Fe^{2+} gave the highest activity even in the presence of reduced glutathione. The levels of activity with Mn^{2+} and Zn^{2+} were 5- to 11-fold lower than the Co^{2+}-substituted enzyme, respectively. These differences in observed activity highlight the necessity of performing the experiments anaerobically due to the rapid oxidation of the metal center, particularly in the case of Fe^{2+}. The lower abundance of cobalt compared to iron within cells was taken as evidence that Fe^{2+} is used by MetAPs in vivo.

Further metal titration experiments indicate that eMetAP is maximally stimulated with the addition of one equivalent of Co^{2+} or Fe^{2+} and that the first metal ion binds with a binding constant of ∼0.2−0.3 μM.[167] The binding constant for the second metal site was estimated to be ∼2.5 mM based on the titration of the electronic absorption spectrum. The addition of excess metal ions (>50 equivalents) resulted in a loss in activity as previously seen with lower concentrations of Zn^{2+}. The ^1H NMR spectrum of eMetAP containing one equivalent of Co^{2+} suggested that tight metal binding occurs at site 1 which contains a histidine ligand. The EPR spectra of eMetAP with two equivalents of metal added indicated that no significant spin−spin interactions between the metal ions were occurring as suggested by the crystal structure, described below. Moreover, an EXAFS study of the Co^{2+}- and Fe^{2+}-substituted enzyme confirmed the presence of a mononuclear metal center with a histidine ligand.[169] These results clearly indicate that eMetAP needs only one metal ion for catalysis. When two equivalents are added only metal site 1 is filled as a consequence of the low affinity of site 2 for metal ions. In contrast, the addition of more than one equivalent of Co^{2+} to hMetAP leads to optimal activity.[144] Therefore, in vivo the number and identity of the metals bound presumably depend in part on the concentrations of ions that are present in the cell. Possible ancillary proteins that import, store, or deliver metal ions may also contribute. This needs to be kept in mind in interpreting the in vitro experiments. For example, as described for APP above and will be described in more detail below, the amino acid residues that are ligands to the site 2 metal ion in the X-ray crystal structures may, in the cell, bind the N-terminus of the substrate.

D. Metal Dependence of APP and Prolidase

APP was originally identified in *E. coli* and was shown to be inhibited by EDTA and activated by Mn^{2+} ions.[110,170,171] The sequence similarities to the MetAPs suggested that APP also contained a dinuclear metal center.[172] The X-ray crystal structure of the Mn^{2+}-substituted *E. coli* enzyme confirmed this notion.[173] Early studies identified 0.2 to 1 equivalents of Zn^{2+} bound to the porcine membrane-bound APP and the *E. coli* APP.[171,174,175] The significant activation of the enzymes by Mn^{2+}, however, suggests that the Zn^{2+} ion may be binding to a metal site distinct from the active site. Moreover, recent experiments have shown that the overexpression of human cytosolic APP in *E. coli* results in an enzyme that is activated by glutathione and contains only one Mn^{2+} ion.[157] These results confirm the previously unexplained EPR data showing the presence of only a single metal ion.[176] The only metal other than Mn^{2+} to cause a slight activation of the mononuclear enzyme was Co^{2+}. The addition of Ca^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+} lead to inactivation. Once the single metal ion was removed from the protein by chelation, only Mn^{2+} restored full activity. The addition of Fe^{2+} leads to only partial activity. A mutational analysis of the metal ligands and two other conserved histidines that flank the metal center (see below) has confirmed the importance of these residues for catalysis by the porcine membrane-bound APP.[156]

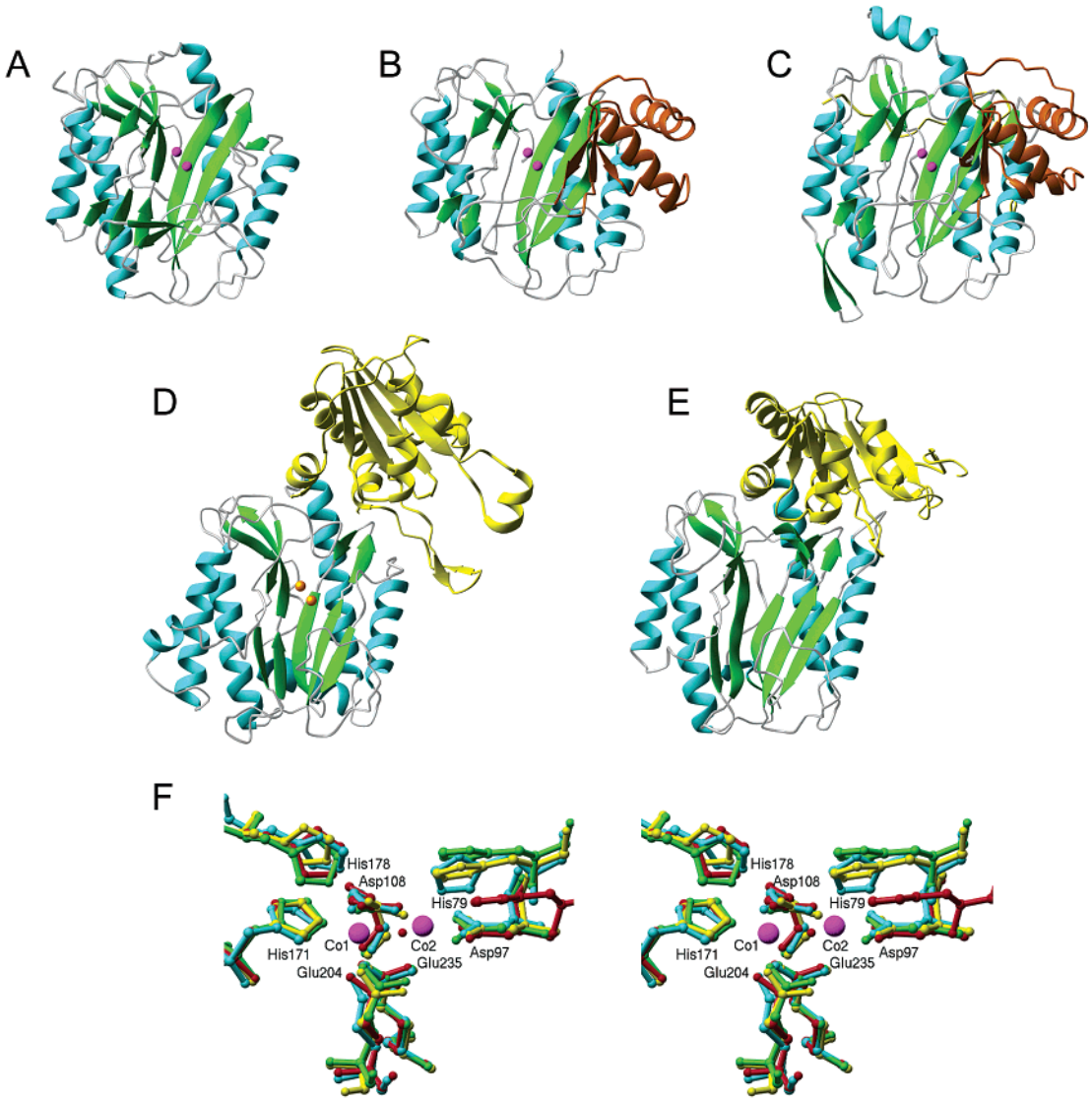


Figure 9. Type I and type II MetAPs and other "pita-bread" enzymes: (A) eMetAP-1 (PDB: 2MAT),[166] (B) PfMetAP-2 (PDB: 1XGS),[180] (C) hMetAP-2 (PDB: 1BN5),[179] (D) APP (PDB: 1A16),[173] (E) Creatinase (PDB: 1CHN).[183] In contrast to the type 1 enzymes, the type 2 MetAPs contain an α-helical subdomain insert (orange) within the catalytic domain (cyan and green α-helices and *β*-strands, respectively). The N-terminal domain extensions (yellow) of APP and creatinase share the same fold, but are in a slightly different orientation in relation to the rest of the protein. (F) Comparison of the dinuclear metal centers and flanking His residues of eMetAP (red) and its relatives. Residues are numbered according to eMetAP. The crystal structures do show additional solvent molecules bound to the metal center of eMetAP, PfMetAP (cyan), hMetAP (yellow), and APP, but these differ slightly in their locations and are not shown for clarity.

Prolidase from a variety of organisms also appears to be activated by Mn^{2+} ions.[110] A comparison of the native and recombinant enzyme from *Pyrococcus furiosus* showed, however, one equivalent of Co^{2+} and two equivalents of Zn^{2+} ions.[161,162] The removal of the Zn^{2+} ions with EDTA did not affect activity. Co^{2+} and to a lesser extent Mn^{2+} could restore activity. These studies also showed that more than one equivalent of Co^{2+} was required to reach full activity and that the enzyme was inhibited by Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, and Ni^{2+}. At this time, it is unclear why APP and prolidase utilize Mn^{2+} or Co^{2+} in the active site whereas the MetAPs may use Fe^{2+}, since they contain the same metal ligands. It is tempting to speculate that Mn^{2+} is preferred in some manner due to the requirement to hydrolyze the unique imido-scissile bond of a Xaa−Pro substrate. The pending crystal structure of prolidase and other metal-dependence studies will hopefully shed light on this question.[177]

E. Macromolecular Structure

The initial crystallographic analysis of the MetAP from *E. coli* (eMetAP) revealed the "pita-bread" fold (Figure 9a).[178] The subsequent structural analyses of the MetAPs from *P. furiosis* and human and the APP from *E. coli*[172,178,179] (Figure 9b−d) confirmed this pseudo 2-fold symmetric fold in which a dinuclear metal center is generated by five conserved residues (Figure 9f): Asp97, Asp108, His171, Glu204, and Glu235 (eMetAP numbering).[172] These studies and a higher-resolution structure of eMetAP[166] revealed several solvent molecules in the vicinity of the metal center including a metal-bridging water or hydroxide

ion. Despite the overall pseudo symmetry of the molecule, the S_1 subsite is asymmetrically located with residues contributed primarily from the N-terminal portion of the molecule.[108,166]

The MetAPs were initially divided into the type 1 and type 2 classes based on sequence comparisons.[145,172] The type 2 enzymes, in contrast to the type 1 class, have an α-helical domain of approximately 60 residues in length inserted within a surface loop of the C-terminal half of the molecule (Figure 9b,c). This insert shows no sequence or structural homology to any other protein. The proximity of this insert to the active site has been suggested as one potential source for the observed differences in specificity of the two classes. The absence (subclass a) or presence (subclass b) of N-terminal extensions further differentiate the enzymes.[181] For example, eMetAP (type 1a) is the prototypical enzyme containing only the catalytic domain. The yeast enzyme, yMetAP-1b, has an N-terminal extension that contains two zinc-finger domains. The type-2 human and yeast enzymes, yMetAP-2b and hMetAP-2b, contain polybasic and polyacidic residues blocks within an N-terminal domain extension involved in interactions with the translation initiation factor eIF-2α.[124−126,150,182]

The crystal structure of APP confirmed the tetrameric oligomerization state of the enzyme and the "pita-bread" fold of the C-terminal domain (Figure 9d). The N-terminal domain appears to be primarily responsible for higher order oligomerization and exhibits the same structure as the N-terminal domain of the dimeric protein creatinine amidinohydrolase (creatinase) (Figure 9e).[183,184] The C-terminal catalytic domain of creatinase also exhibits the "pita-bread" fold. Remarkably, this enzyme cleaves the amidino-scissile bond of the substrate (Table 5) without the use of metal ions. As will be discussed in more detail below, the mechanistic similarities between creatinase and the other members of the family that contain metal ions is informative.

As presented above, it has recently been argued that the MetAPs and APP exhibit mononuclear rather than dinuclear metal binding in vivo. Notwithstanding the outcome of this discussion, the analysis of the dinuclear forms of MetAPs and APP has been instrumental to the understanding of substrate specificity and catalysis.

F. Comparison of Noncovalent Ligand Complexes

The structures of several noncovalent ligand complexes of eMetAP and APP have been determined (Figure 10).[108,166,173] In an effort to specifically inhibit eMetAP, transition state analogues were designed based on amastatin and bestatin and the known substrate specificity of the enzyme.[185,186] The N-terminal (P_1) Leu residue of amastatin was changed to Met or norleucine (Nle), a Met analogue with the sulfur atom of the side chain replaced with carbon. The stable Nle side chain is also useful since the oxidation of Met to Met sulfoxide results in a poor or noncleavable substrate.[138] The P_1' Val of amastatin was replaced with Ala. The resulting compound, SL648, contains the (3*R*)-amino-(2*S*)-hydroxyheptanoic acid metal chelator[58,187] and inhibits the enzyme with an IC_{50} of 5 μM.[186]

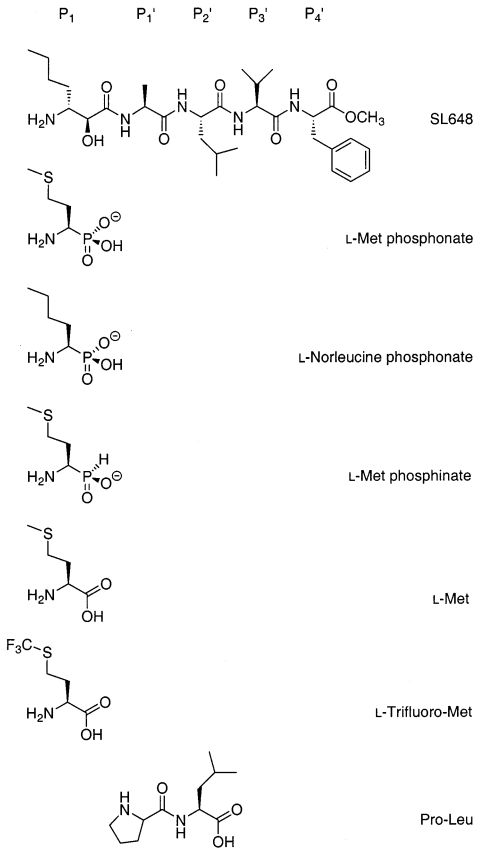


Figure 10. Noncovalent ligands used to study the metal centers of MetAPs and APP by X-ray crystallography. The N-terminal side chain of SL648 is in the D-configuration. All other side chains are in the L-configuration.

SL648 binds to the active site of eMetAP in a manner (Figure 11a) reminiscent of the complexes of amastatin and bestatin with bLeuAP.[166] The inhibitor coordinates the metal ions through three interactions: N-terminal ligation to Co2, bridging coordination between the metal ions, and ligation to Co1 via the inserted carbonyl oxygen atom. Hydrogen bonds were also observed to His79 and His178, two conserved residues on either side of the metal center.

The SL648 complex allowed for the first time a rationalization of the substrate specificity of the MetAPs. The side chain of the Met mimic, Nle, bound into the hydrophobic S_1 subsite (Figure 12a) generated by residues Cys59, Tyr62, Tyr65, Cys70, His79, Phe177, and Trp221. This binding mode supports the observation that only Met, Nle, and other Met analogues can fit into this subsite. The binding of β-branched amino acids such as Leu is most likely excluded due to potential collisions with Phe177, Tyr62, and Cys59. The side chain of the P_1' Ala residue interacts with the shallow S_1' subsite (Figure 12b) lined with Met206, Gln233, and Tyr168. The ability of these residues to dictate the selection of substrates has been confirmed by the mutational analyses of the corresponding Met and Gln residues in yMetAP-1. Truncation of these residues to the shorter Ala side chain resulted in variants that were capable of cleaving substrates with larger P_1' side chains.[141] It is also interesting to note that only the first three residues of the pentapeptide analogue were visible in the electron density. This observation

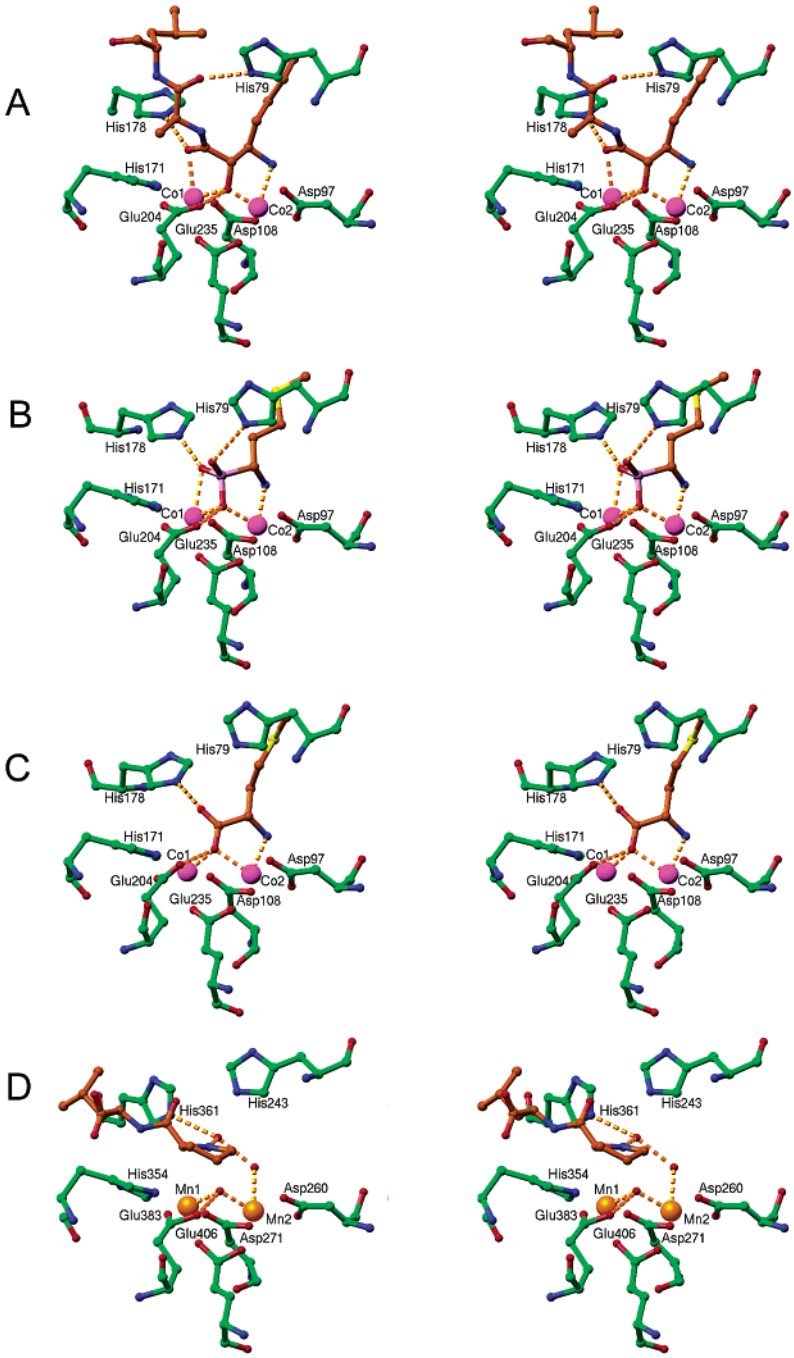


Figure 11. Stereoviews of selected noncovalent ligand complexes of eMetAP and APP. (A) SL648 bound to eMetAP (PDB: 3MAT).[166] (B) L-Met phosphonate bound to eMetAP (PDB: 1C23).[108] (C) L-Met bound to eMetAP (PDB: 1C21).[108] (D) Pro−Leu bound to APP (PDB: 1A16).[173]

is consistent the minimal requirement for a tripeptide substrate even though no direct enzyme contacts are made to the P_2' residue.[138]

Several phosphonate and phosphinate analogues of the reaction product Met (Figure 10) were synthesized to mimic the putative tetrahedral intermediate of the reaction.[108] Two phosphonates and one phosphinate bound to the metal center (Figure 11b) with similar N-terminal, metal bridging, and bifurcated interactions with Co1 and His178. As a consequence of the movement of the entire loop containing His79 toward the metal center, His79 is also able to make a hydrogen-bonding interaction (3.0 Å). In contrast, the Met and trifluoro-Met complexes (Figure 11c) showed a loss of interactions to Co1 and to His79 while maintaining interaction with His178 and the metal ions. These observations suggest that the coordination sphere of Co1 changes during the course of the reaction.

The Pro−Leu complex of Mn^{2+}-substituted APP (Figure 11d) illustrates the putative binding mode of the C-terminal product of the hydrolysis reaction.[173] Pro−Leu binds directly above the water/hydroxide molecule that bridges between the metal ions. The N-terminal amine interacts through a solvent molecule to His361 (His178 in eMetAP) and an axially associated solvent molecule to Mn2. A weak interaction is also found to His243 (His79 in

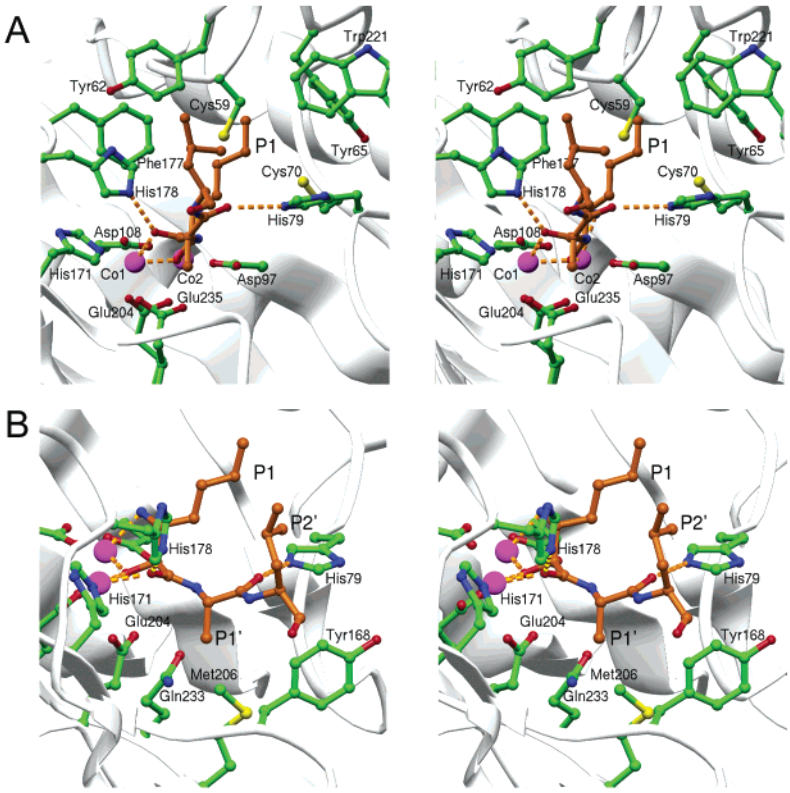


Figure 12. Subsite interactions that dictate the substrate specificity of eMetAP. (A) Residues of the S_1 subsite that interact with the N-terminal Met residue. (B) Residues of the S_1' subsite that interact with the penultimate (P_1') residue.

eMetAP). His350 and Arg404 pack against the Pro ring (not shown). His361 and His354 of the S_2' subsite interact with the Leu side chain. This complex also illustrates how residues from the adjacent monomers in the tetramer can play a role in substrate binding. For example, the C-terminal carboxyl group interacts with Arg370 of monomer A and Arg404 of monomer B (not shown). The extension of the active site to the S_3' subsite most likely involves residues Asp38, Asp88, and Phe89 of monomer D. Even with these substantial insights, a rationale for needing a P_1' proline residue is not readily apparent.

G. Covalent Modification of MetAPs by Fumagillin and Its Congeners

The epoxide-containing natural products fumagillin and ovalicin (Figure 13) have been under intense investigation as a result of their ability to function as anti-angiogenesis agents thus preventing the vascularization and metastasis of tumors.[188,189] A variety of biochemical and structural experiments have shown that these unique compounds inhibit MetAPs by covalent attachment.[146−148,179,188−190] They contain at their core a cyclohexane ring decorated with an expoxide at C2−C3, an epoxide-containing, hydrophobic side chain at C4, and a methoxy group at C5. The C4 and C6 positions show some variability. The C6 position of fumagillin is substituted with a monoester of decatetraenedioic acid while ovalicin contains only a keto group. The C6 position of TNP-470, a semi-synthetic analogue, contains a chloro-acetylcarbonyl substitution at C6 and has been used to treat a variety of cancer types.[191−194]

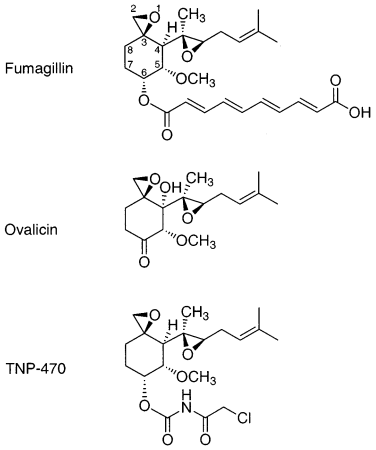


Figure 13. Anti-angiogenesis compounds that covalently inhibit MetAPs. Numbering scheme from Griffith et al.[146]

The functional groups necessary for inhibition of MetAPs have been identified.[146,147] An intact epoxide at C2−C3 is required while the epoxide on the C4 substituent is dispensable. Changes in the C6 moiety can lead to differences in the ability to decrease enzyme activity and endothelial cell proliferation. For example, the deletion of the C6 substituents of TNP-470 and fumagillin appears not to affect potency, but the replacement of the keto group of ovalicin by a hydroxyl group substantially decreases efficacy against cell proliferation. The therapeutic promise of these compounds has intensified the search for other natural and synthetic fumagillin analogues that may be useful as anticancer, antimicrobial, and antimalarial agents.[195−199]

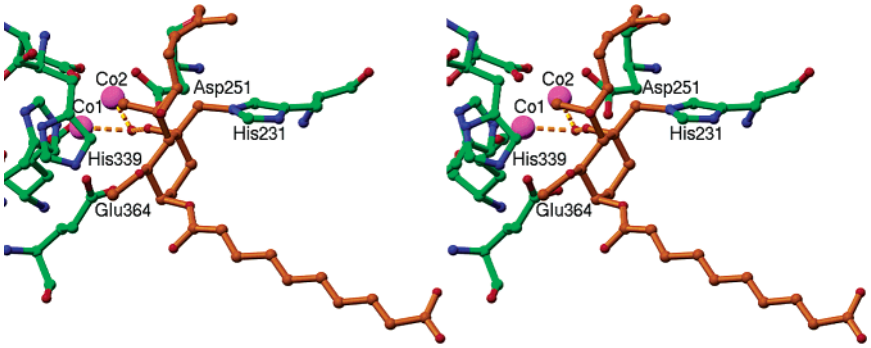


Figure 14. Binding-mode of fumagillin to the active site of hMetAP-2. Fumagillin is covalently cross-linked to His231 (His79 in eMetAP) via the C2-epoxide moiety (Figure 12) (PDB: 1BOA).[179] Leu328, Leu447, and Asp376 interact with the conjugated, C6 substituent, but are not shown for clarity.

The modification of MetAP-2 with fumagillin was initially identified within bovine brain and human umbilical vein endothelial cells.[148] Experiments testing isogenic yeast strains containing either yMetAP-1 or yMetAP-2 also showed that only the type-2 enzyme was modified and inhibited.[146] The site of covalent attachment was first identified in eMetAP despite the observed preference for the type-2 enzymes.[190] Similar N-terminal sequence and mass spectroscopic analyses of proteolytic fragments of the fumagillin−hMetAP-2 complex confirmed the attachment to His231 (His79 in eMetAP).[147]

The molecular details of the binding mode of fumagillin were revealed in the crystal structure of the fumagillin−hMetAP-2 complex (Figure 14).[179] Fumagillin spans the active site from His231 to the metal center and His339 (His178 in eMetAP), thus preventing the binding of substrates. The hydrophobic C4 substituent docks into the S_1 subsite generated by Phe219, His331, Ile338, His339, and Tyr444. The conjugated C6 moiety extends out of the active site and interacts with Leu328 and Leu447 of the S_1' subsite and Asp376 (not shown). The oxygen atom of the opened C2−C3 epoxide is involved in a hydrogen-bonding network with the metal bridging water/hydroxide through an intervening water molecule. An overlay of the eMetAP structure onto the fumagillin complex suggests that several residues, His79, Tyr62, Cys78, Tyr168, and Phe177, would need to move to allow fumagillin binding and covalent attachment. These movements could explain why formation of the fumagillin−eMetAP complex requires a higher concentration of the ligand than for the type-2 enzymes.[190] EXAFS spectra of the fumagillin−eMetAP complex supports the lack of a direct interaction between fumagillin and the metal center.[169]

H. Proposed Reaction Mechanism

The biochemical and structural analyses of MetAP, APP, and prolidase suggest that these enzymes have a common reaction mechanism. The binding mode of the bestatin analogue SL648 (Figure 11a) to eMetAP suggested two potential reaction intermediates.[166] The main difference between them, similar to alternatives put forward for LeuAP (Figure 4), is the difference in the location of carbonyl oxygen atom of the scissile peptide bond upon binding. The subsequent determination of product and product-based transition state analogue complexes (Figure 11b,c) support the view that the scissile-bond carbonyl group binds to Co1 in eMetAP.[108]

The crystallographic data also suggested that the N-terminus of the substrate binds to the dinuclear metal center in a manner similar to that observed for LeuAP and APP. In this scenario, the N-terminus of the substrate interacts with the Co2 or the Mn2 atom. The careful analysis of the metal content and activity of several enzymes, however, indicates that this family of enzymes may function with a mononuclear metal center in vivo. The residual activity of the Asp219Asn variant of yMetAP-1 (Asp97 in eMetAP) supports this notion.[200] As a result of these findings, Asp97 and potentially Asp108 and Glu235 in eMetAP are thought to meditate substrate binding by interacting with the positively charged N-terminus. A corollary to this proposition is that the binding of a second metal ion may be used in vivo to regulate enzymatic activity.

An overlay of the LeuP−eMetAP complex onto the Pro−Leu−APP complex (Figure 15a) suggests how the tetrahedral *gem*-diolate intermediate binds to the site 1 metal ion. In particular, one of the oxygen atoms of LeuP superimposes on the N-terminal amine nitrogen of Pro−Leu. In addition, it appears that His79 (His243 in APP) is poised to interact with the nitrogen atom of the scissile peptide bond. The oxygen atoms corresponding, respectively, to the nucleophile and the carbonyl oxygen of the substrate are stabilized by interactions with M1 and with His178 (His361 in APP).

The His residue that corresponds to His79 of eMetAP is conserved in all "pita-bread" enzymes including creatinase.[172] Mutation of this residue in eMetAP, hMetAP-2, and APP leads to variants with negligible activity.[147,156,166] These observations coupled with the modification of this residue by fumagillin and related analogues (Figures 13 and 14) clearly suggest that it is important for catalysis and may also be essential for productive substrate binding. The mutation of His178 leads to variants that have reduced but still significant levels of activity. Therefore, His178 clearly affects catalysis but is not essential.

His178 is conserved in all MetAPs and APP, but is replaced in creatinase by Arg335, which forms a key salt bridge with carboxylate group of the inhibitor carbamoyl sarcosine (Figure 15b).[183] Creatinase

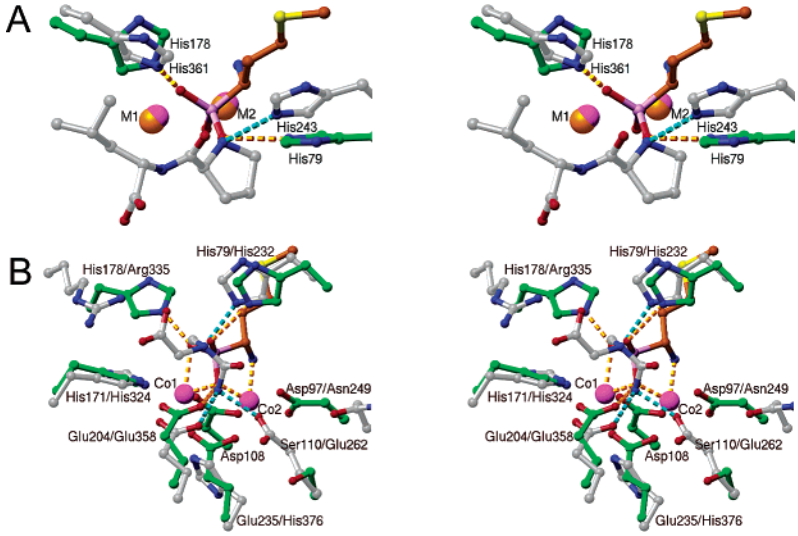
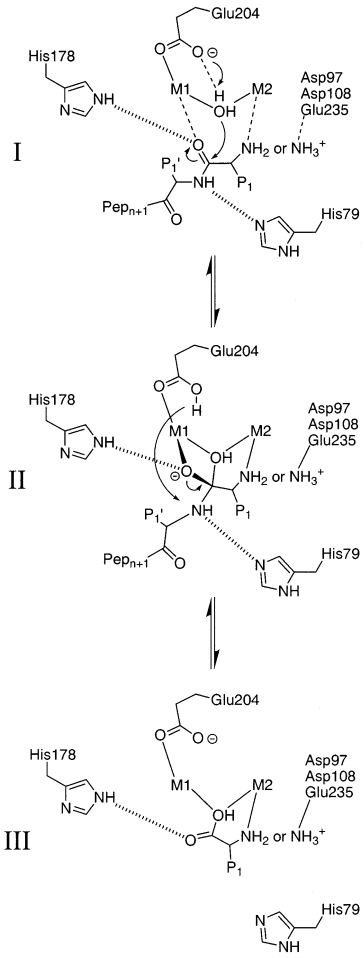


Figure 15. Comparison of ligand complexes between members of the "pita-bread" enzyme family. (A) Superposition of the L-Met phosphonate complex of eMetAP and the Pro−Leu complex of APP (Figure 5b,c). The coordinates of the metal ions and the metal-bridging oxygen atom were used to superimpose the eMetAP (green and orange) and APP (gray) structures. (B) Superposition of the MetP-eMetAP complex with the carbamyl sarcosine complex of creatinase (PDB: 1CHM).[183] The superposition is based on the C^{α} atoms of eMetAP and the C-terminal domain of creatinase (gray), which have a RMS discrepancy of 1.5 Å. The labels indicate the eMetAP/creatinase residues.

cleaves the guanidinium group of creatinine in a metal-independent reaction. The superposition of the creatinase complex with the Met phophonate complex of eMetAP illustrates that the carbon atoms attacked during the reaction (or at least their counterparts in the inhibitors) are within 1.0 Å of each other. Both enzymes utilize His79/His232 to contribute a direct ligand interaction. In creatinase, His232 is thought to be essential for activating a water molecule for nucleophilic attack, binding of substrate, and the shuttling of a proton. This role would be somewhat analogous to His231 of hMetAP-2 attacking the epoxide group of fumagillin (Figure 14). Glu358 of creatinase (Glu204 of eMetAP) is proposed to help bind the guanidinium group of the substrate. Although Glu358 is apparently close to the nitrogen atom of the scissile bond, it has not been proposed to act as a general base or as a proton donor to the leaving group.[183] Since such a role has, however, been ascribed to Glu204 of eMetAP and Glu383 of APP[108,166,173] further investigation of Glu358 in creatinase would seem justified.

Glu204 of eMetAP (Glu383 in APP) interacts with M1 through one of the oxygen atoms of its side chain. The other oxygen atom is within hydrogen bonding distance of the water/hydroxide molecule that bridges between the two metal ions in the crystal structures (Figure 9f). This interaction suggests that Glu204 helps activate the nucleophilic water molecule. This role can be envisaged whether there is a dinuclear or a mononuclear metal center. Moreover, a study of synthetic carboxylate-bridged dimetallic complexes suggests that deprotonation of a metal-bound water molecule is facilitated by the strong hydrogen-bonding interaction with the dangling carboxylate oxygen atom.[201] This observation is somewhat surprising since one would not normally think that a carboxylate group interacting with a metal ion would be particularly basic in nature.

Scheme 4



On the basis of the available data, a mechanistic scheme for the metal-dependent "pita-bread" enzymes is presented in Scheme 4 with eMetAP residue numbering. The substrate binds with the N-terminus interacting with three potential acidic residues in a

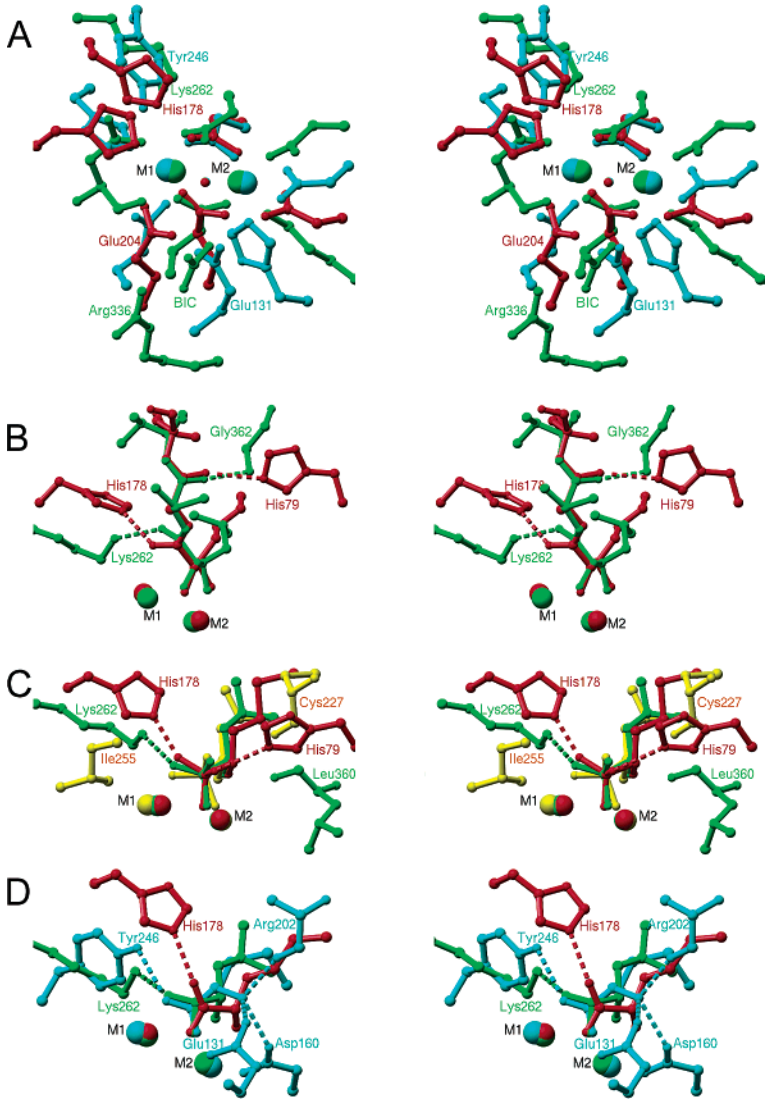


Figure 16. Comparison of the metal centers and ligand complexes from selected broad-class and substrate-specific metalloaminopeptidases. (A) The ligand-free metal centers of eMetAP (red), bLeuAP (green), and SgAP (cyan, PDB: 1CP7).[87] (B) Overlay of the amastatin and SL648 complexes of bLeuAP and eMetAP, respectively. (C) Superposition of the L-Leu phosphonate complexes of bLeuAP and APP (orange) with the L-Met phosphonate complex of eMetAP. (D) Comparison of the L-Met complexes of eMetAP and SgAP to the leucinal complex of bLeuAP. The metal ions are denoted M1 and M2. See the individual structures for the metal composition (Figures 1, 3, 5, 8, 9, and 11). In each case, the coordinates of the metal ions and the metal-bridging oxygen atom were used to superimpose the structures.

manner that remains to be determined (intermediate I). The carbonyl and amide groups of the scissile peptide bond interact with M1 plus His178 and His79, respectively. M1 and Glu204 of eMetAP facilitate the activation of a water molecule to become a nucleophilic hydroxide ion. Two interactions to M1 and one to His178 stabilize the tetrahedral intermediate (intermediate II). Breakdown of the intermediate is facilitated by donation of a proton from Glu204 to the amine of the leaving group. The resulting product complex (intermediate III) maintains interactions with M1 and His178.

Following the relatively recent suggestion that several MetAPs may use only one metal ion, together with the proximity of His79 and His178 to M1 in eMetAP, other scenarios are not required but may be possible or contribute in some manner to the scheme described above. For example, His79 could function as a proton donor to the leaving amino group as proposed for His141 of arginase.[202] His178 could also, in principle, expedite the formation of the nucleophile through an intervening water molecule observed in the crystal structure of the native eMetAP.[166] This possibility would be similar to that observed for His64 of carbonic anhydrase II.[203,204]

V. Common Structure–Function Motifs

The biochemical and structural analyses of several metalloaminopeptidases suggest that they utilize fundamentally similar reaction mechanisms despite differences in overall structure and metal centers. The differences in the ligand binding pockets generate the observed preferences for substrates. This selection process can either be relatively broad in nature as in LeuAP, ApAP, and SgAP, or restrictive as observed in the “pita-bread” enzymes. In each enzyme system, the metal center plays an essential

role in the activation of the nucleophile and the stabilization of the putative tetrahedral intermediate. Conserved amino acid side chains and backbone atoms that are adjacent to the metal center also provide key interactions. On the basis of the apparent similarities, a generalized mechanistic scheme can be proposed. Some of the metalloaminopeptidases also appear to exploit their oligomeric state as part of the selection criterion for substrates, thus preventing unwanted or processive proteolysis.

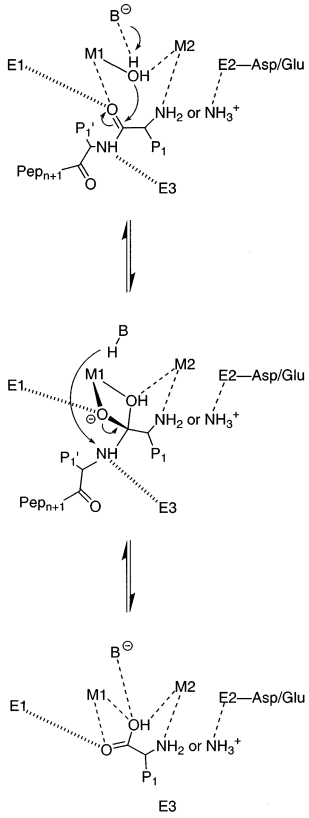
A. Role of the Metal Center and Neighboring Residues

The superposition of the metals centers of eMetAP, bLeuAP, and SgAP (Figure 16a) reveals one of the hallmarks of metalloexopeptidases, i.e., the use of acidic residues to mediate a bifurcated interaction between metal ions. The variability seen in the chemical composition of the metal ligands supports the observed differences in metal affinities and preferences. For example, the tighter binding site in LeuAP is M2 (Figure 5c). In contrast, M1 is the more tightly bound metal ion in ApAP and eMetAP. These differences offer a rationale for why bLeuAP requires two metal ions for activity while ApAP and, as more recently suggested, eMetAP can function with only M1.

The site 1 metal ion is thought to be essential for the activation of a water molecule to a nucleophilic hydroxide ion. Glu204 of eMetAP, Glu131/Glu151 of SgAP/ApAP, and the bicarbonate-arginine pair of LeuAPs facilitate this process by functioning as a general base to abstract a proton. The analyses of natural product, transition state analogue and product complexes suggest that the oxygen atoms of the putative, *gem*-diolate transition state intermediate are stabilized by additional interactions with M1 and His178 for eMetAP, Lys262 for bLeuAP, and potentially Tyr246 of SgAP (Figure 16b−d). Therefore, when only a single equivalent of metal ion is available, bLeuAP fills M2 first and cannot initiate hydrolysis. In the presence of additional metal ions, site 1 is filled and enzymatic activity is restored. Further interactions between the substrate and enzyme side chains and backbone atoms may also be essential for docking the substrate in a productive manner, e.g., with His 79 and the Leu360 carbonyl group of eMetAP and bLeuAP, respectively.

The majority of the crystal structures indicate that the N-terminus of the substrate interacts with M2. The ability of ApAP and eMetAP to function with only the site 1 metal ion, however, suggests that the M2-substrate interaction can be replaced by the acidic M2 ligands and other residues in the vicinity. In these cases, the charged form of the N-terminus is most likely bound. Support for such a scenario comes from the product complexes of SgAP where the N-terminus binds to Asp160, Glu131, and the backbone carbonyl group of Arg202 (Figure 16d). This concept points to that possibility that the addition of the second metal ion may be inhibitory or utilized in the in vivo regulation of activity.

Scheme 5



B. Generalized Reaction Mechanism

On the basis of the similarities of the metalloaminopeptidases, a generalized mechanistic scheme can be formulated (Scheme 5). The substrate binds to the active site with the carbonyl group of the scissile peptide bond interacting with M1 and a conserved enzyme residue, E1. The lack of an E1 interaction within ApAP appears to be the only exception (Figure 16c). The mutational analysis of the E1 residues of eMetAP and PepA, however, shows that the interaction with M1 is sufficient for catalysis. The N-terminus either interacts with M2 or with one or more acidic enzyme residues in the vicinity, E2. Additional enzymic histidine or backbone carbonyl group interactions, E3, facilitate substrate binding in the correct register. The scissile peptide bond is attacked by a solvent molecule that has been activated by its interaction with the metal ion(s) and an enzyme residue that functions as a general base, B. Whether or not the subsequent tetrahedral intermediate is stabilized by interactions to both metal ions and E2 side chains depends on the particular enzyme system. Breakdown of the intermediate is most likely promoted by the addition of a proton to the leaving amine group from the former general base, B−H, as first suggested for thermolysin.[62] In a manner unique to the MetAPs, proton donation to the leaving group may possibly occur through an E3 histidine side chain, though definitive evidence is needed. The product complexes of several of the enzymes suggest that interactions with the metal ion(s), E1 or B are quite variable.

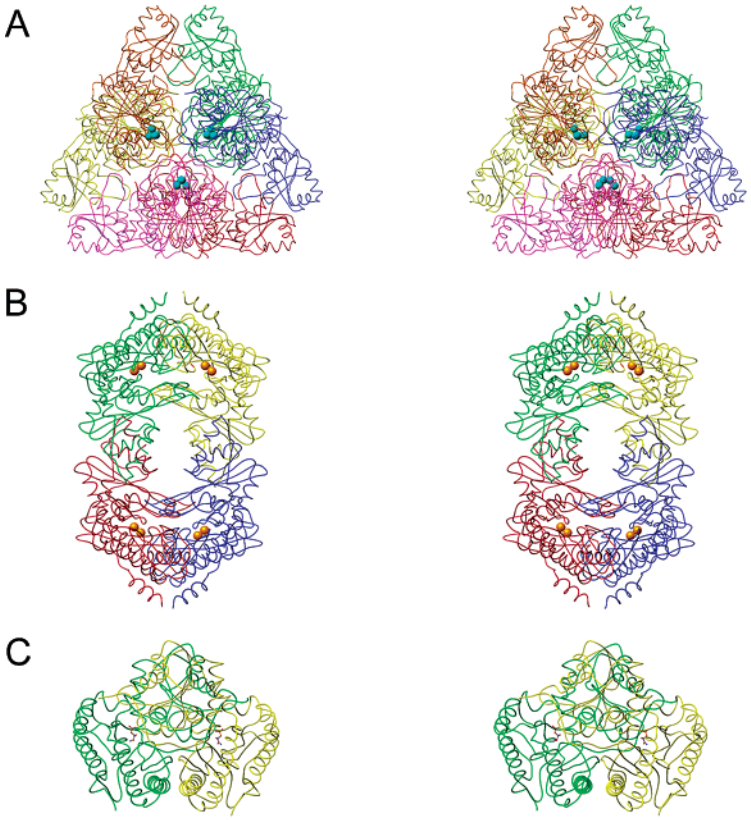


Figure 17. Quaternary structural relationships of LeuAP, APP, and creatinase. (A) Each monomer of the LeuAP hexamer is colored uniquely. Each active site contains two Zn^{2+} ions (cyan) and is located at the center of the oligomeric structure.[44] (B) The APP tetramer with two Mn^{2+} ions (gold) in each active site.[173] Surface loops of the adjacent monomer directly contribute to the generation of extended subsites. (C) The dimer structure of creatinase.[183] The creatine molecule is shown as ball-and-stick representation docked into an active site that does not contain metal ions. In this case, residues from the adjacent monomer are also essential for substrate recognition.

C. Does the Oligomerization State Influence Substrate Selection?

Many of the metalloaminopeptidases function in an oligomeric state. LeuAPs, for example, function as hexamers. The monomers are arranged as two layers of trimers with the active sites in the interior of the oligomer (Figure 17a).[44] This arrangement appears to restrict access to only di- and tripeptide substrates. Without this limitation the LeuAPs, having broad specificity, would most likely function in a processive manner and continue to move down the protein chain resulting in complete hydrolysis. Because of this potential for unregulated proteolysis, it is unclear why the structurally related enzymes ApAP and SgAP function as monomers.

APP is active as a tetramer (Figure 17b).[173] In contrast to the LeuAPs, the active site of APP is not sequestered from bulk solvent. The contribution of residues to the active site from the adjacent monomer results in the extension of the substrate binding site and the ability to cleave larger substrates. It is tempting to speculate that the oligomeric nature of APP in relation to the monomeric MetAPs is necessary to facilitate the hydrolysis of the relatively cleavage-resistant imido scissile bond. Support for this notion comes from the observed activity of creatinase (Figure 17c), a metal-independent, "pita-bread" enzyme that hydrolyzes the amidino scissile bond of creatine.[183] In this example, the enzyme functions as a dimer with side chains of the adjacent monomer making direct contact to the substrate (Figure 15b).

VI. Summary and Future Directions

The structure−function relationships of the metalloaminopeptidases have been investigated through a myriad of experimental techniques. Biochemical, elemental, and spectroscopic analyses have shown that the enzymes require metal ions for catalysis. Some enzymes require two metal ions for activity. For other enzymes, one metal ion appears to be sufficient and conserved amino acid residues that were originally thought to be metal ligands may function to bind the N-terminus of the substrate. The analysis of a variety of ligand complexes has been instrumental in understanding substrate preferences and in testing mechanistic proposals.

Many questions remain to be investigated. Chief among these relate to the metal center itself. Most of the crystal structures of these enzymes clearly show two metals bound, yet it has also been established that in some cases a single metal will suffice for activity. What is the nature of metal binding in the cell? Do the number and identity of metal ligands change under different conditions? If so, are such changes in metal binding physiologically relevant and are they used to regulate activity? This review has clearly established that although there are many

structural and biochemical differences, similar functional themes are utilized for catalysis. The stage is now set to more fully understand how the differences between the individual enzymes relate to their biological activities within the cell.

VII. Abbreviations

amastatin	(2*S*,3*R*)-3-amino-2-hydroxy-5-hexanoyl-L-valyl-L-valyl-L-aspartic acid
ApAP	*Aeromonas proteolytic* aminopeptidase
APP	aminopeptidase P
BBA	butane boronic acid
bestatin	(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanol-L-leucine
CD	circular dichroism
CPA	carboxypeptidase A
creatinase	creatine amidinohydrolase
D-IPH	*p*-iodo-D-Phe hydroxyanate
Dns	5-(dimethylamino)-naphthalene-1-sulfonyl
EPR	electron paramagnetic resonance
EXAFS	extended X-ray absorption fine structure spectroscopy
Hepes	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
LeuAP	leucine aminopeptidase
LeuP	L-Leu phosphonate
MCD	magnetic circular dichroism
MetAP	methionine aminopeptidase
NA	nitroanilide
NEM	*N*-ethylmorpholine
NMR	nuclear magnetic resonance
PepA	*E. coli* leucine aminopeptidase
SgAP	*Streptomyces griseus* aminopeptidase
Tris	Tris(hydroxylmethyl)aminomethane

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IX. Note Added in Proof

Several reports related to the area of discussion have appeared since the submittal of this review. The reader is referred to the following references: MetAP (refs 207−211) and ApAP (refs 212, 213).

X. References

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