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Crystal Structure of the Leucine Aminopeptidase from *Pseudomonas putida* Reveals the Molecular Basis for its Enantioselectivity and Broad Substrate Specificity

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The zinc-dependent leucine aminopeptidase from *Pseudomonas putida* (ppLAP) is an important enzyme for the industrial production of enantiomerically pure amino acids. To provide a better understanding of its structure–function relationships, the enzyme was studied by X-ray crystallography. Crystal structures of native ppLAP at pH 9.5 and pH 5.2, and in complex with the inhibitor bestatin, show that the overall folding and hexameric organization of ppLAP are very similar to those of the closely related di-zinc leucine aminopeptidases (LAPs) from bovine lens and *Escherichia coli*. At pH 9.5, the active site contains two metal ions, one identified as Mn²⁺ or Zn²⁺ (site 1), and the other as Zn²⁺ (site 2). By using a metal-dependent activity assay it was shown that site 1 in heterologously expressed ppLAP is occupied mainly by Mn²⁺. Moreover, it was shown that Mn²⁺ has a significant activation effect when bound to site 1 of ppLAP. At pH 5.2, the active site of ppLAP is highly disordered and the two metal ions are absent, most probably due to full protonation of one of the metal-interacting residues, Lys267, explaining why ppLAP is inactive at low pH. A structural comparison of the ppLAP-bestatin complex with inhibitor-bound complexes of bovine lens LAP, along with substrate modelling, gave clear and new insights into its substrate specificity and high level of enantioselectivity.

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**Keywords:** leucine aminopeptidase; X-ray crystallography; di-zinc proteases; substrate specificity; enantioselectivity

**Introduction**

Aminopeptidases are metalloproteinas that cleave N-terminal residues from proteins and small oligopeptides. These enzymes are widely distributed in nature and play crucial roles in several important physiological processes, including protein degradation and turnover, protein maturation, the metabolism of biologically active peptides and antigen presentation. Aminopeptidases have attracted additional interest due to their applicability for the production of peptides and amino acids used in the food, agrochemical and pharmaceutical industries. An example of such an industrial enzyme is the leucine aminopeptidase from *Pseudomonas putida* ATCC 12633 (ppLAP), which has a longstanding use as a whole-cell biocatalyst for the enantioselective hydrolysis and enzymatic resolution of a broad range of DL-amino acid amide racemates. *Pp* LAP is a member of the M17 family of di-zinc-dependent leucine aminopeptidases (LAPs; EC 3.4.11.1 and EC 3.4.11.10), which also includes the well studied LAPs from bovine lens (bLAP) and *Escherichia coli* (eLAP, also known as PepA). X-ray crystallographic analysis of bLAP and eLAP, which share a level of sequence identity with ppLAP of 31% and 53%, respectively, has provided important insights into the structure and
catalytic mechanism of M17 LAPs.\textsuperscript{11-16} In particular, on the basis of the crystal structures of bILAP bound with inhibitors and transition-state analogues like bestatin and L-leucinephosphonic acid, the LAP residues with proposed roles in catalysis, in coordinating the zinc ions, and/or binding substrate were identified.

Knowledge of the biochemical, catalytic, and structural properties of \textit{ppLAP} is important to improve its effectiveness as an industrial enzyme. Like its homologues, \textit{ppLAP} requires the presence of divalent metal ions for its activity, in particular Zn\textsuperscript{2+} and/or Mn\textsuperscript{2+}. It displays clear amide-hydrolysing activity between pH 7 and pH 11, but is inactive at pH 6 or lower.\textsuperscript{6,7} Dipeptides are hydrolysed as well as single amino acid amides, with a clear preference for substrates with a hydrophobic side chain at their N-terminus. Substrates with an N-terminal leucine residue are most readily hydrolysed, but significant activity is found with substrates with an N-terminal methionine, phenylalanine, or isoleucine residue. In addition, a variety of non-proteinogenic amino acid amides with different hydrophobic side chains, such as phenylglycine amide and various allylglycine amides, form good \textit{ppLAP} substrates.\textsuperscript{6} In contrast, peptides and amides with small or negatively charged N-terminal amino acid residues, such as glycine, alanine, serine, valine, aspartic acid and glutamic acid, are poor substrates. Because its activity requires that the chiral C\textsubscript{α} atom has one proton substituent, α,α-disubstituted amino acid amides like D,L-α-methyl-valine amide, are not hydrolysed. Finally, the enzyme is highly enantiopreference towards substrates that have an S-configuration at their N-terminal chiral C\textsubscript{α} atom (i.e., L-amino acid amides).\textsuperscript{6,7}

To provide an accurate structural model for explaining the biochemical and catalytic properties of \textit{ppLAP}, we have analysed this enzyme by X-ray crystallography. Here, we report a high-pH and a low-pH crystal structure of unliganded \textit{ppLAP} determined at 2.2 Å resolution and at 2.75 Å resolution, respectively. In addition, we describe the high-resolution crystal structure of a \textit{ppLAP}-bestatin complex determined at 1.5 Å resolution. Analysis of these structures, along with substrate modelling studies, allowed us to provide new insights into the structural and functional features of \textit{ppLAP}.

### Results

#### Overall structure

Crystal structures of \textit{ppLAP} were elucidated to a resolution of 2.75 Å (pH 5.2, unliganded), 2.2 Å (pH 9.5, unliganded), and 1.5 Å (pH 7.5, with bound bestatin inhibitor) (see Table 1 for the crystallographic statistics). The overall features of the \textit{ppLAP} structure are identical in all three crystal forms and structural differences are restricted mainly to the active site region. The crystals reveal the presence of a \textit{ppLAP} hexamer that is highly similar to the cLAP and bILAP hexamers (Fig. 1a). In solution, \textit{ppLAP} exists also as hexamers, which was evident from gel-filtration and dynamic light-scattering analysis (data not shown). The subunits that form the hexamer contain two domains with mixed α/β structure that are linked by a long α-helix (Fig. 1b).

#### Table 1. Data collection and refinement statistics of \textit{ppLAP}

<table>
<thead>
<tr>
<th></th>
<th>Bestatin-bound</th>
<th>High pH (pH 9.5)</th>
<th>Low pH (pH 5.2)</th>
</tr>
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<tbody>
<tr>
<td><strong>A. Data collection</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Beam line (ESRF)</td>
<td>ID14-1</td>
<td>ID23-2</td>
<td>ID14-3</td>
</tr>
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<td>Wavelength (Å)</td>
<td>0.9340</td>
<td>0.8726</td>
<td>0.9300</td>
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<td>Space group</td>
<td>P1</td>
<td>P1</td>
<td>P6\textsubscript{3}</td>
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<td>Unit cell parameters</td>
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<tr>
<td>a (Å)</td>
<td>95.9</td>
<td>95.8</td>
<td>116.9</td>
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<tr>
<td>b (Å)</td>
<td>95.9</td>
<td>95.9</td>
<td>116.9</td>
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<tr>
<td>c (Å)</td>
<td>96.0</td>
<td>96.3</td>
<td>137.9</td>
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<tr>
<td>α (°)</td>
<td>108.8</td>
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<tr>
<td>β (°)</td>
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<tr>
<td>γ (°)</td>
<td>93.2</td>
<td>94.9</td>
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<tr>
<td>Highest R-factor (Å)</td>
<td>1.50</td>
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<td>2.75</td>
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<tr>
<td>Resolution (Å)</td>
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<td>2.75</td>
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<td>Completeness (%)</td>
<td>93.0 (92.5)</td>
<td>99.1 (96.1)</td>
<td>100 (99.5)</td>
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<tr>
<td>Rmerge</td>
<td>0.054 (0.194)</td>
<td>0.044 (0.372)</td>
<td>0.033 (0.353)</td>
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<tr>
<td>Mean 1/αR</td>
<td>15.0 (3.6)</td>
<td>20.2 (2.4)</td>
<td>39.7 (3.8)</td>
</tr>
<tr>
<td><strong>B. Refinement</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>94 – 1.50</td>
<td>91 – 2.20</td>
<td>58 – 2.75</td>
</tr>
<tr>
<td>Rwork</td>
<td>0.149</td>
<td>0.192</td>
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<tr>
<td>Rfree</td>
<td>0.173</td>
<td>0.251</td>
<td>0.267</td>
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<tr>
<td>Overall B-factor (Å)</td>
<td>12.9</td>
<td>13.7</td>
<td>32.1</td>
</tr>
<tr>
<td>Composition of asymmetric unit</td>
<td>Six polypeptide chains (residues 1–497), 6 Zn\textsuperscript{2+}, 6 Mn\textsuperscript{2+}, 6 K\textsuperscript{+}, 6 bicarbonate ions 6 bestatin inhibitors, 2974 water molecules</td>
<td>Six polypeptide chains (residues 1–497), 6 Zn\textsuperscript{2+}, 6 Mn\textsuperscript{2+}, 6 K\textsuperscript{+}, 6 bicarbonate ions 702 water molecules</td>
<td>Two polypeptide chains (residues 1–146, 150–269, 291–497)</td>
</tr>
<tr>
<td>rmsd from ideal Bond lengths (Å)</td>
<td>0.015</td>
<td>0.015</td>
<td>0.016</td>
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<tr>
<td>Bond angles (°)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.7</td>
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<tr>
<td>Ramachandran plot Most favoured (%)</td>
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<td>97.2</td>
<td>98.0</td>
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<tr>
<td>Additionally allowed (%)</td>
<td>1.8</td>
<td>2.8</td>
<td>2.0</td>
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<tr>
<td>Molprobity Score</td>
<td>1.51</td>
<td>2.0</td>
<td>1.95</td>
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</tbody>
</table>

Data in parentheses are for the highest resolution shell. $R_{work} = \Sigma |F_{obs}|-|F_{calc}|/\Sigma |F_{obs}|$, where the crystallographic R-factor was calculated with 95% of the data used in the refinement. $R_{free}$ is the crystallographic R-factor based on 5% of the data selected at random and withheld from the refinement for cross-validation.

...
smaller of the two domains, the N-terminal domain (residues 1–164), is composed of a six-stranded mixed parallel/anti-parallel β-sheet flanked by several α-helices on both sides. The C-terminal domain (residues 193–497) contains a central eight-stranded mixed parallel/anti-parallel β-sheet, surrounded by α-helices on both sides and a small three-stranded β-sheet involved in oligomerization. The long α-helix that connects the N- and C-terminal domains comprises residues 165–192. The C-terminal domain contains the active site and shows the highest degree of similarity with the other LAP structures, both in sequence and in three-dimensional structure. Domain superpositions of the high-pH structure of ppLAP with ecLAP and blLAP reveal a root-mean-square deviation (rmsd) in Cα positions of 0.6 Å (304 residues, 63% sequence identity) and 1.3 Å (302 residues, 42% sequence identity), respectively. The N-terminal domain of ppLAP is highly conserved, but less than the C-terminal domain, with rmsd values of 1.5 Å (ecLAP, 161 residues, 35% sequence identity) and 2.5 Å (blLAP, 132 residues, 18% sequence identity), respectively. Like the other LAPs, the six protomers in the ppLAP hexamer form a dimer of trimers with 32 symmetry. The C-terminal domains are at the core of the hexamer, where they pack around the central 3-fold axis and stabilise the trimer-to-trimer packing. The N-terminal domains form the corners of the triangular-shaped hexamer and further stabilise the trimer-to-trimer packing by making dimeric interactions with each other.

Structure of unliganded ppLAP at pH 9.5

In the high-pH crystal structure three metal ions are bound to each protomer in the hexamer (Fig. 1b). Two metal-binding sites are located in the active site (Fig 2a; Supplementary Data Fig. S1A), similar to blLAP and ecLAP. Previously, it was shown for blLAP that one of these metal-binding sites (site 1) allows exchangeable binding of various divalent metal cations (e.g., Zn²⁺, Mn²⁺, Mg²⁺ and Co²⁺), whereas the other metal-binding site (site 2) is specific for either Zn²⁺ or Co²⁺ and its bound ion cannot be readily exchanged.¹²,¹⁷ X-ray fluorescence analysis of ppLAP at beamline ID29 of the ESRF, Grenoble, revealed the presence of zinc and manganese in the high-pH ppLAP crystals (Fig. 2b). On the basis of that analysis and the high degree of structural similarity of ppLAP with blLAP, we expect the non-exchangeable metal site 2 in ppLAP to be fully occupied by a Zn²⁺ and the exchangeable metal site 1 by Mn²⁺ or a mixture of Mn²⁺ and Zn²⁺. Figure 2a shows the geometry of metal-binding sites 1 and 2 of ppLAP. All residues that coordinate the two metal ions are identical with those found in the active sites of ecLAP and blLAP, and the coordinating bond distances and metal-to-metal distances are very similar to those reported for the homologous LAP structures. The site-1 Mn²⁺/Zn²⁺ and site-2 Zn²⁺ are both pentacoordinated in a distorted pyramidal coordination geometry. The metal-coordinating atoms are mostly carboxylate oxygens from the side chains of three aspartate and one glutamate residue (Asp272, Asp290, Asp291, Glu271, and Glu274).
Asp349 and Glu351). In addition, the site-1 Mn\(^{2+}\)/Zn\(^{2+}\) is coordinated by the main chain carbonyl oxygen of Asp349, and the site-2 Zn\(^{2+}\) makes a bond with the \(\varepsilon\)-amino group of a lysine residue (Lys267). A water molecule or hydroxide ion is observed at a bridging position, binding to both metal ions simultaneously, as was observed in the unliganded structures of blLAP and ecLAP.\(^{13,15}\) The high-pH structure of ppLAP also shows the presence of a bicarbonate ion bound to the active site, at a position identical with that observed in blLAP and ecLAP. The bicarbonate ion is bound to Arg353 and makes a hydrogen bond to the metal-bridging water molecule/hydroxide ion.

The third metal ion bound in ppLAP is located at the C-terminal end of the inter-domain linker helix (Fig. 1b). This metal-binding site 3 has so far been identified only in blLAP.\(^{13,14}\) The coordination geometry and relatively long metal–ligand bond distances are most suited for a monovalent sodium or potassium ion. Because the crystallization procedure of ppLAP involved the presence of potassium, it appears likely that a K\(^{+}\) is bound to metal-binding site 3 of ppLAP, which was confirmed by a B-factor analysis (not shown). As discussed for blLAP,\(^{14}\) the role of the metal ion in site 3 is unclear. Most likely it has a structural role stabilizing the interface between the linker helix and the C-terminal domain.

**Active site metal composition and metal-dependent activity**

To better define the metal composition of site 1 in the ppLAP structure and analyse the effect on catalysis when either Zn\(^{2+}\) or Mn\(^{2+}\) occupies this site, the activity of purified ppLAP used for crystallization was compared to the activities of EDTA-treated ppLAP for which the metal in site 1 was fully replaced by either Zn\(^{2+}\) or Mn\(^{2+}\) (Fig. 3a). The results indicate that ppLAP is significantly less active when site 1 is occupied with Zn\(^{2+}\) than when it is occupied with Mn\(^{2+}\). This was
confirmed by competitive activation/inhibition experiments in which the activity of (Zn^{2+}-Zn^{2+})-bound and (Mn^{2+}-Zn^{2+})-bound ppLAP (referring to the metals occupying sites 1-2) was analysed after the addition of a 17-fold excess of either Zn^{2+} or Mn^{2+} (Fig. 3b and c). Addition of Zn^{2+} to (Zn^{2+}-Zn^{2+})-bound ppLAP or Mn^{2+} to (Mn^{2+}-Zn^{2+})-bound ppLAP did not significantly affect the enzyme activity (the small decrease in activity can be attributed to measurement errors and/or instability of the enzyme). However, addition of Mn^{2+} to (Zn^{2+}-Zn^{2+})-bound ppLAP resulted in a ∼2.5-fold increase of activity, and addition of Zn^{2+} to (Mn^{2+}-Zn^{2+})-bound ppLAP caused a ∼70% decrease in activity. Purified protein isolated from the E. coli cytoplasm, which was used for the crystallizations, has an activity that is comparable to that of treated ppLAP with Mn^{2+} in site 1, indicating that in the high-pH ppLAP structure site 1 is predominantly occupied by Mn^{2+}.

Structure of unliganded ppLAP at pH 5.2

In contrast to the high-pH ppLAP structure, the active site region is highly disordered and the active site metals are absent from the low-pH structure of ppLAP (Figs. 1b and 2b). The loss of the two metals from the active site at pH 5.2 is most likely the result of Lys267 being in a fully protonated state, and therefore unsuitable to serve as coordinating ligand for the site-2 metal ion. This would explain also why ppLAP is inactive at pH 6 or below. Partial protonation of some of the metal-coordinating carboxylate groups might further destabilize metal binding. A large segment of the active site in the low-pH ppLAP structure, residues 270–290, is not visible in the 2Fo–Fc and Fo–Fc electron density maps (Fig. 1b). This segment contains two of the metal-coordinating ligands and its disorder in the low-pH ppLAP structure signifies the importance of the metals for maintaining the integrity of the active site.

Structure of bestatin-bound ppLAP

Highly ordered and well diffracting crystals of bestatin-bound ppLAP were obtained from protein subsequently treated with EDTA and Mn^{2+} to ensure site 1 was fully occupied by Mn^{2+}. The ppLAP-bestatin crystal structure showed excellent density for the bestatin inhibitor in the active site (Supplementary Data Fig. S1B). Structural representations of the binding mode of bestatin are provided in Supplementary Data Fig. S2. No significant difference was observed in the positions of residues and metal ions in the active site when comparing the bestatin-bound ppLAP structure with the unliganded, high-pH structure. The binding interactions of bestatin in the active site of ppLAP are very similar to those reported for the bILAP complexes.
with bestatin, and the bestatin derivative microginin FR1. A schematic overview of the polar interactions of bestatin with **ppLAP** is given in Fig. 4. The most conspicuous interaction is the replacement of the bridging water/hydroxide ion between the two active site metal ions by the hydroxyl group of bestatin. Two additional metal-coordinating bonds are formed by the inhibitor, between the terminal amino group and site-2 Zn$^{2+}$ and between the peptide carbonyl group and site-1 Mn$^{2+}$, such that both metals are 6-fold coordinated in an octahedral geometry. The D-phenylalanine side chain binds in the hydrophobic S1 pocket (following the nomenclature of protease sub-sites in Ref. 20) and is stabilized by van der Waals interactions with Met287, Thr376, Ile382, Ala466 and Trp470. The L-leucyl side chain binds in the S1’ subsite making van der Waals contacts with Ala350, Asn347 and Leu377, while the terminal carboxylate group is more solvent-exposed, forming one hydrogen bond with the main chain amide of Gly379.

**Comparison of bestatin-bound ppLAP with L-leucinephosphonic acid-bound bILAP**

Bestatin is not a true transition state analogue of ppLAP, and therefore one may expect differences between its binding mode and that of ppLAP substrates. In particular, in bestatin the chiral C3 carbon atom to which the terminal amino group and phenylalanine side chain are attached (the P1 residue of the inhibitor) has an R-configuration, but the equivalent Cα atom of the ppLAP peptide substrates has an S-configuration (Supplementary Data Fig. S3). In addition, bestatin contains a methyl hydroxyl group, inserted between the chiral C3 carbon atom and the peptide bond, which is not present in the normal ppLAP substrates. To investigate the interaction of ppLAP with natural substrates, the ppLAP-bestatin structure was superimposed on the structure of bILAP complexed with l-leucinephosphonic acid (LPA) (Fig. 5). This latter complex is considered to closely resemble the presumed tetrahedral gem-diolate transition state of the LAP reaction, based in particular on the configuration and interactions of the phosphonate group of LPA in the active site of bILAP. From the superposition it is evident that the interactions of bestatin in the active site of ppLAP are remarkably similar to the interactions of LPA in the active site of bILAP, notwithstanding the significant differences between both inhibitors. In particular, the terminal amino groups of LPA and bestatin are bound at equivalent positions and make identical interactions in the active site of bILAP and ppLAP, respectively, while the C2 hydroxyl group of bestatin binds at the same metal-bridging position as one of the three phosphoryl oxygens of LPA (O1 in Fig. 5). The P–O bond associated with this metal-bridging oxygen atom is thought to represent the carbon–oxygen bond that is formed in the transition state

![Fig. 4. A diagram of the binding mode of bestatin in the active site of ppLAP. Hydrogen bonds with bestatin and metal-coordinating bonds are indicated with broken lines.](image-url)
upon attack of the water or hydroxide ion nucleophile on the carbonyl carbon atom of the scissile peptide bond. One of the other two phosphoryl oxygens of LPA (O2 in Fig. 5) is proposed to represent the oxyanion of the transition state (the former carbonyl oxygen of the scissile peptide bond). In the LPA-bound bLAP structure it is coordinated to the site-1 metal ion and hydrogen bonded to Lys262 (equivalent of Lys279 in ppLAP). The third phosphoryl oxygen of LPA (O3 in Fig. 5) is thought to represent the former peptide nitrogen atom of the substrate. This oxygen is within hydrogen bonding distance from the backbone carbonyl oxygen of Leu360 of bLAP (equivalent to Leu377 of ppLAP). In the ppLAP–bestatin complex the peptide bond is shifted away from the dimetal centre due to the extra C–C backbone bond present in the P1 residue of the inhibitor. Nevertheless, the carbonyl oxygen and amide nitrogen of bestatin are close (within 1 Å) to the positions of the O2 and O3 phosphoryl oxygens of LPA, making similar, albeit weaker, interactions with ppLAP and the site-1 metal ion. This is possible due to the inverted configuration at the C3 carbon of bestatin (R instead of S) that allows a change in overall binding orientation of the inhibitor such that the terminal amino group, the metal-bridging hydroxyl group and the carbonyl oxygen all bind close to the dimetal centre, while the phenylalanine side chain occupies the S1 pocket. The high degree of similarity between the bestatin-binding interactions in ppLAP and the LPA-binding interactions in bLAP provides a clear framework for modelling substrates in both the ground state and transition state configuration in the active site of ppLAP (see below).

Molecular modelling of the substrate-binding modes in ppLAP

To examine the structural basis for the substrate preferences and high enantioselectivity of ppLAP, the bestatin-bound ppLAP structure was used as a template to model the binding modes of the amide forms of the L-amino acids leucine, phenylglycine, valine, isoleucine, glutamic acid and arginine (Fig. 6). Earlier it was shown that among these compounds, the leucine and phenylglycine amides are the best ppLAP substrates. The valine and isoleucine amides, which have an extra methyl group connected to their Cβ atom, are poor substrates, while almost no amidase activity is measured with glutamic acid amide as a substrate. No ppLAP activity data are available for the amide forms of L-lysine and L-arginine, but the L-arginine amide has been reported to form a good substrate for the highly similar ecLAP. The amino acid amides were modelled in the active site of ppLAP in energetically favourable conformations, under consideration of the crucial binding interactions implied by the comparison of the bestatin-bound ppLAP structure with the LPA-bound structure of bLAP and the probable mechanism described below. The modelling included the placement of a nucleophilic water molecule at the position of the hydroxyl oxygen of bestatin in the bestatin-bound ppLAP structure. The results clearly show how the L-amino acid amides of leucine and phenylglycine (with the S-configuration at their chiral Cα atom) might bind to the active site in a productive mode allowing formation of the metal-coordinating bonds by their α-amino and carbonyl groups, while their Cβ side chains fit snugly in the hydrophobic S1 pocket (Fig. 6a and b).
mode, the Cα-H proton of the amino acid amide substrates is in close proximity (<3 Å) to the backbone carbonyl oxygen of residue 377, which leaves no space for any larger substituent at that position, explaining why ppLAP is inactive with α-methyl-substituted amino acid amides. It explains also the high enantioselectivity of ppLAP, as substrates with an R-configuration at their chiral Cα...
atom will either be excluded from the active site due to steric hindrance of the C\(^{\alpha}\) side chain, or due to unfavourable interactions with their C\(^{\alpha}\)-linked amino and carbonyl groups. The valine and isoleucine amides are poor substrates because their C\(^{\gamma}\)2 methyl groups are positioned unfavourably between the NH amide of Gly379 and the amino group of Lys279 (distances of 3.5–4 Å; Fig. 6c and d). In addition, the small hydrophobic side chain of the \(\alpha\)-valine amide does not fully occupy the S1 pocket, thus further weakening the binding interactions. The side chains of aspartate, asparagine, glutamate or glutamine amides could optimally fill the S1 pocket (Fig. 6e), but their polar or charged head groups are not tolerated by the hydrophobic protein environment, explaining why these amides do not form substrates of \(pp\)LAP. On the other hand, we predict that the \(L\)-arginine amide is indeed a putative substrate of \(pp\)LAP, as its side chain is long enough to traverse the S1 pocket with its charged head group extending away from the protein surface (Fig. 6f).

Discussion

The structural similarities of \(pp\)LAP with bILAP and \(ec\)LAP, in particular with respect to its di-metal coordination geometry and binding mode of bestatin, confirm that these enzymes share a common catalytic mechanism. In this mechanism, which has been analysed extensively for bILAP,\(^{11,13,14,16}\) the metal-bridging water molecule or hydroxide ion observed in the active site of the unliganded structure is believed to represent the nucleophile that will attack the scissile amide bond of the substrate. Besides having a role in positioning and activating the nucleophile, the two active site metals are important for substrate binding and transition state stabilisation. The site-2 metal ion is crucial for binding the N-terminal amino group of the substrate, while the site-1 metal ion binds the carbonyl oxygen of the scissile amide bond and stabilizes the negative charge that develops on this atom (the oxyanion) in the presumed tetrahedral gem-diolate transition state. The oxyanion is further stabilized by an interaction with the nearby lysine residue (Lys297 in \(pp\)LAP). The bicarbonate ion is believed to act as a general base in this mechanism, abstracting a proton from the nucleophilic water molecule and transferring it to the amino-terminal group of the P1’ product after cleavage of the peptide bond.

Our results indicate that while metal-binding site 2 in the unliganded high-pH structure of \(pp\)LAP contains a Zn\(^{2+}\), metal-binding site 1 is occupied mainly by Mn\(^{2+}\). Since no manganese was present in the solutions used for protein purification and crystallization, it must have been picked up by \(pp\)LAP from the cytoplasm of \(E. coli\) during protein expression. Assuming that the intracellular concentration of free Mn\(^{2+}\) in \(E. coli\) is similar to that of Zn\(^{2+}\), these findings indicate that metal-binding site 1 of \(pp\)LAP has a higher specificity for Mn\(^{2+}\) than for Zn\(^{2+}\). Alternatively, during expression in \(E. coli\) the intracellular levels of Mn\(^{2+}\) were significantly higher than those of Zn\(^{2+}\). Whether Mn\(^{2+}\) is the preferred metal ion in binding site 1 of \(pp\)LAP under physiological conditions remains to be investigated. In addition, it is unclear whether the observed increase in activity of (Mn\(^{2+}\)-Zn\(^{2+}\))-bound versus (Zn\(^{2+}\)-Zn\(^{2+}\))-bound \(pp\)LAP has biological relevance. A similar activation effect of Mn\(^{2+}\) has been observed for other members of the M17 LAPs, including bILAP,\(^{2,22}\) and in some of these enzymes the metal-activation effect is substrate specific.\(^{6,5}\) Although the precise mechanistic basis for these effects is unclear, it must result from subtle differences in the active site, depending on which metal ion is present at the low-affinity site. Such a metal-dependent modulation of activity could serve as a regulatory mechanism to alter the hydrolytic activity of LAPs towards certain substrates in response to changes in the environment. However, in the case of \(pp\)LAP it can be argued that the Mn\(^{2+}\) activation is only a secondary effect, and that the metal exchangeability of site 1 serves merely to make the enzyme more robust and less vulnerable to large fluctuations in the environment.

The present crystal structures of \(pp\)LAP and their analysis clearly explain the pH-dependence of this enzyme and its high enantioselectivity, and provide a structural basis for its observed substrate specificity. They also suggest target residues for mutagenesis (e.g. Met287, Ile382, Ala466) in order to change the substrate specificity and thus may serve as a platform for future protein engineering to enhance the applicability of this enzyme in the stereoselective synthesis of proteinogenic and non-proteinogenic \(L\)-amino acids.

Materials and Methods

Purification and crystallization

\(pp\)LAP was produced by heterologous expression in \(E. coli\), using the expression vector pTrpLAP as described.\(^{14}\) All enzyme purification steps were done at 7 °C, following a procedure based on previously established protocols.\(^{14}\) In brief, 10 g of bacterial pellet was suspended in buffer A (20 mM Hepes–KOH, pH 8.0, 1 mM DTT) containing 200 mM MgSO\(_4\), which was followed by sonication and high-speed centrifugation to obtain a cell-free extract. The resulting supernatant was filtered, diluted with buffer A to a final concentration of 25 mM MgSO\(_4\) and subsequently loaded onto a 6 ml Resource-S cation-exchange column (GE Healthcare), previously equilibrated with buffer A containing 25 mM MgSO\(_4\). Protein was eluted by a linear gradient of 25 mM to 500 mM MgSO\(_4\). The \(pp\)LAP-containing fractions were pooled and then further purified on a Superdex 200 10 × 300 mm gel-filtration column (GE Healthcare), using buffer A containing 100 mM MgSO\(_4\) as a running buffer. \(pp\)LAP eluted from the column as a single peak (apparent molecular mass 270 kDa) corresponding to the expected molecular mass of a hexameric species. Purified \(pp\)LAP was concentrated to 8 mg/ml in buffer A, and subsequently used for crystallization. Initial screening for crystallization conditions was done in hanging drops using different commercial screens. Subsequent optimization resulted in the growth of X-ray
diffraction analysis of averaged intensities. A search model was obtained by molecular replacement with Phaser, using the high-pH ppLAP structure as a template, which was subsequently optimized by refinement and model building using Refmac5 and Coot, respectively.

Activity assays

Metal-dependent activity assays were carried out as described using L-phenylglycine amide as a substrate. To prepare (Zn\(^{2+}\)-Zn\(^{2+}\))-bound and (Mn\(^{2+}\)-Zn\(^{2+}\))-bound forms of the enzyme, EDTA-treated ppLAP was incubated overnight at room temperature with a 13-fold molar excess of ZnSO\(_4\) and MnSO\(_4\), respectively. After incubation the enzyme was washed and concentrated to 3.7 mg/ml in 20 mM Hepes-KOH, pH 8.0, 0.1 M K\(_2\)SO\(_4\) in the presence of a 1.4-fold molar excess of ZnSO\(_4\) or MnSO\(_4\). Competitive activation/inhibition was analysed by incubating these ppLAP preparations for 2 h with a 17-fold molar excess of ZnSO\(_4\) or MnSO\(_4\) before measuring the activity.

Modelling

Amide forms of the L-amino acids were modelled manually in the active site of ppLAP using the program Coot. Coordinates and topology files of the L-amino acid amides were generated using the PRODRG2 server. The bestatin-bound structure of ppLAP was used as a protein model. Manual docking was guided by the superposition of bestatin-bound ppLAP to L-leucinephosphonic acid-bound biLAP, restraining the positions of the N-terminal amino nitrogen, the carbonyl oxygen and the amide nitrogen to the equivalent atoms in L-leucinephosphonic acid. A water molecule was placed at the position of the hydroxyl oxygen in bestatin, and bad contacts were removed by several cycles of energy minimization using CNS.

Figures

Figures 1, 2, 5 and 6 were prepared using the program PyMOL.*

Protein Data Bank accession number

Coordinates and structure factors have been deposited in the Protein Data Bank under accession numbers 3H8E (unliganded, low pH), 3H8F (unliganded, high pH) and 3H8G (bestatin-bound complex).

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† http://www.pymol.org
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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.03.042

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