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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 matur-

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Abbreviations used: ppLAP, *Pseudomonas putida* leucine aminopeptidase; LAP, leucine aminopeptidase; ecLAP, *Escherichia coli* leucine aminopeptidase, biLAP, bovine lens leucine aminopeptidase; rmsd, root-mean-square deviation; LPA, L-leucinephosphonic acid.
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{p}pLAP is important to improve its effectiveness as an industrial enzyme. Like its homologues, \textit{p}pLAP 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 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{p}pLAP 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\textsuperscript{α} atom has one proton substituent, α,α-disubstituted amino acid amides like DL-α-methyl-valine amide, are not hydrolysed. Finally, the enzyme is highly enantiomeric, selective towards substrates that have an S-configuration at their N-terminal chiral C\textsuperscript{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{p}pLAP, we have analysed this enzyme by X-ray crystallography. Here, we report a high-pH and a low-pH crystal structure of unliganded \textit{p}pLAP 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{p}pLAP-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{p}pLAP.

\section*{Results}

\subsection*{Overall structure}

Crystal structures of \textit{p}pLAP 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{p}pLAP 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{p}pLAP hexamer that is highly similar to the \textit{c}LAP and biLAP hexamers (Fig. 1a). In solution, \textit{p}pLAP 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). The

\begin{table}[h]
\centering
\caption{Data collection and refinement statistics of \textit{p}pLAP}
\begin{tabular}{|c|c|c|c|}
\hline
 & Bestatin-bound & High pH (pH 9.5) & Low pH (pH 5.2) \\
\hline A. Data collection & & & \\
Beam line & ID14-1 & ID23-2 & ID14-3 \\
Wavelength (Å) & 0.9340 & 0.8726 & 0.9300 \\
Space group & P1 & P1 & P6\_3 \\
Unit cell parameters & & & \\
a (Å) & 95.9 & 95.8 & 116.9 \\
b (Å) & 95.9 & 95.9 & 116.9 \\
c (Å) & 96.0 & 96.3 & 137.9 \\
α (°) & 100.8 & 68.4 & 90 \\
β (°) & 107.8 & 76.3 & 90 \\
γ (°) & 93.2 & 94.9 & 120 \\
Highest resolution (Å) & 1.50 & 2.20 & 2.75 \\
No measured reflections & 941,319 & 205,478 & 111,116 \\
No unique reflections & 484,976 & 166,778 & 27,831 \\
Completeness (%) & 93.0 (92.5) & 99.1 (96.1) & 100 (99.5) \\
R_{merge} & 0.054 (0.194) & 0.044 (0.372) & 0.033 (0.353) \\
Mean 1/σ & 15.0 (3.6) & 20.2 (2.4) & 39.7 (3.8) \\
B. Refinement & & & \\
Resolution range (Å) & 94 – 1.50 & 91 – 2.20 & 58 – 2.75 \\
R_{work} & 0.149 & 0.192 & 0.212 \\
R_{free} & 0.173 & 0.251 & 0.267 \\
Overall & 12.9 & 13.7 & 32.1 \\
B-factor (Å\textsuperscript{2}) & & & \\
Composition of asymmetric unit & 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 & Six polypeptide chains (residues 1–497), 6 Zn\textsuperscript{2+}, 6 Mn\textsuperscript{2+}, 6 K\textsuperscript{+}, 6 bicarbonate ions 702 water molecules & Two polypeptide chains (residues 1–146, 150–269, 291–497) \\
rmsd from ideal bond lengths (Å) & 0.015 & 0.015 & 0.016 \\
Bond angles (°) & 1.5 & 1.5 & 1.7 \\
Ramachandran plot & Most favoured (%) & 98.2 & 97.2 & 98.0 \\
Additionally allowed (%) & 1.8 & 2.8 & 2.0 \\
Molprobity score & 1.51 & 2.0 & 1.95 \\
\hline
\end{tabular}
\end{table}
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,
Asp349 and Glu351). In addition, the site-1 Mn\textsuperscript{2+}/Zn\textsuperscript{2+} is coordinated by the main chain carboxyl oxygen of Asp349, and the site-2 Zn\textsuperscript{2+} makes a bond with the ε-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 bILAP and ecLAP\textsuperscript{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 bILAP 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 bILAP\textsuperscript{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\textsuperscript{+} is bound to metal-binding site 3 of ppLAP, which was confirmed by a B-factor analysis (not shown). As discussed for bILAP\textsuperscript{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\textsuperscript{2+} or Mn\textsuperscript{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\textsuperscript{2+} or Mn\textsuperscript{2+} (Fig. 3a). The results indicate that ppLAP is significantly less active when site 1 is occupied with Zn\textsuperscript{2+} than when it is occupied with Mn\textsuperscript{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 $\sim$2.5-fold increase of activity, and addition of Zn$^{2+}$ to (Mn$^{2+}$-Zn$^{2+}$)-bound ppLAP caused a $\sim$70% decrease in activity. Purified protein isolated from the E. coli cytoplasm, which was used for the crystallizations, has an activity that is comparable to that of treated ppLAP with Mn$^{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.$^6$ 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 $2F_o$ – $F_c$ 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 blLAP complexes.
with bestatin\textsuperscript{11,18} and the bestatin derivative microginin FR1.\textsuperscript{19} A schematic overview of the polar interactions of bestatin with \textit{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\textsuperscript{2+} and between the peptide carbonyl group and site-1 Mn\textsuperscript{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\textsuperscript{′} 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 \textit{ppLAP} with \textit{L-leucinephosphonic acid-bound blLAP**}

Bestatin is not a true transition state analogue of \textit{ppLAP}, and therefore one may expect differences between its binding mode and that of \textit{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 C\textsubscript{α} atom of the \textit{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 \textit{ppLAP} substrates. To investigate the interaction of \textit{ppLAP} with natural substrates, the \textit{ppLAP}-bestatin structure was superimposed on the structure of blLAP complexed with \textit{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 blLAP.\textsuperscript{14} From the superposition it is evident that the interactions of bestatin in the active site of \textit{ppLAP} are remarkably similar to the interactions of LPA in the active site of blLAP, 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 blLAP and \textit{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 \textit{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 pLAP). 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 pLAP). In the pLAP–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 pLAP 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 pLAP 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 pLAP (see below).

**Molecular modelling of the substrate-binding modes in pLAP**

To examine the structural basis for the substrate preferences and high enantioselectivity of pLAP, the bestatin-bound pLAP 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 pLAP 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 pLAP 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.10,21 The amino acid amides were modelled in the active site of pLAP in energetically favourable conformations, under consideration of the crucial binding interactions implied by the comparison of the bestatin-bound pLAP 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 pLAP 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 Cα 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 Cα side chain, or due to unfavourable interactions with their Cα-linked amino and carbonyl groups. The valine and isoleucine amides are poor substrates because their Cα2 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 additional hydrophobic side chain of the L-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 ppLAP. On the other hand, we predict that the L-arginine amide is indeed a putative substrate of ppLAP, 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 ppLAP with biLAP and ecLAP, 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 ppLAP). The bicarbonate ion is believed to act as a general base in this mechanism, abstracting a proton in the product after ‘‘ product after

Materials and Methods

Purification and crystallization

PpLAP was produced by heterologous expression in E. coli, using the expression vector pTrpLAP as described.6 All enzyme purification steps were done at 7 °C, following a procedure based on previously established protocols.25 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 MgSO4, 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 MgSO4 and subsequently loaded onto a 6 ml Resource-S cation-exchange column (GE Healthcare), previously equilibrated with buffer A containing 25 mM MgSO4. Protein was eluted by a linear gradient of 25 mM to 500 mM MgSO4. The ppLAP-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 MgSO4 as a running buffer. ppLAP eluted from the column as a single peak (apparent molecular mass 270 kDa) corresponding to the expected molecular mass of a hexameric species. Purified ppLAP 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
differing crystals at two different conditions, at low pH (8 mg/ml protein, 11% (w/v) PEG 8000, 0.2 M sodium formate, 0.1 M Mes–NaOH, pH 5.2, 1 mM NaN₃ at 5 °C) and at high pH (4 mg/ml protein, 15% (w/v) PEG 1500, 0.1 M propionic acid, cacodylate, bis-Tris propane (PCB) cocktail buffer, pH 9.5 at 23 °C). The low-pH ppLAP crystals (apo form) were hexagonal and reached an average size of 0.3 mm × 0.3 mm × 1.0 mm within three days, whereas the high-pH ppLAP crystals (active form) were triclinic and grew overnight to an average size of 0.1 mm × 0.1 mm × 0.1 mm. To obtain well diffracting crystals of a bestatin-bound ppLAP–inhibitor complex it was necessary to first dialyse purified ppLAP against 20 mM Hepes–KOH, pH 8.0, 0.1 M K₂SO₄, 100 mM EDTA, followed by extensive dialysis against EDTA- and metal-free buffer (treated with Chelex 100 Resin from Bio-Rad). The protein was then incubated with a sixfold molar excess of MnSO₄ (relative to the concentration of the ppLAP hexamers) for 2 h at 23 °C, before adding a 30-fold molar excess of bestatin. Hexagonal crystals of the ppLAP–bestatin complex (0.12 mm × 0.12 mm × 0.10 mm) were obtained from 0.2 M DL-malic acid, pH 7.0, and 22.5% (w/v) PEG 3350 at 23 °C.

Data collection and structure determination

Diffraction data for the ppLAP crystals were measured with synchrotron radiation at the ESRF in Grenoble, France. Before data collection crystals were transferred to a cryoprotecting solution and flash-frozen in liquid nitrogen. The high-pH crystals were transferred in two steps, first to 35% PEG 1500, then to 45% PEG 1500 in 0.1 M PCB cocktail buffer, pH 9.5, and the low-pH crystals were transferred in a single step to mother liquor containing 35% PEG 8000. The ppLAP–bestatin crystals were transferred to mother liquor containing 20% (v/v) glycerol. The data were processed with Mosflm and were transferred to mother liquor containing 35% PEG 8000. The ppLAP–bestatin crystals were transferred in a single step to mother liquor containing 20% (v/v) glycerol. The data were processed with Mosflm and merged using Scala as implemented in the CCP4 software suite. The relevant data statistics are given in Table 1.

The high-pH ppLAP crystals diffracted to 2.20 Å resolution and belonged to space group P1. Analysis of the Matthews coefficient and inspection of self-rotation Patterson maps indicated the presence of one hexamer per unit cell, with a solvent content of 49% (v/v). An initial set of phases was obtained by the molecular replacement method using the program MOLREP. Using the FFAS of phases was obtained by the molecular replacement suite. The relevant data statistics are given in Table 1.

Analysis of $\text{Zn}^{2+}$-bound ppLAP and (Mn$^{2+}$–Zn$^{2+}$)-bound forms of the enzyme, EDTA-treated ppLAP was incubated overnight at room temperature with a 15-fold molar excess of ZnSO₄ and MnSO₄, 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 K₂SO₄ in the presence of a 1.4-fold molar excess (96 μM) of either ZnSO₄ or MnSO₄. Competitive activation/inhibition was analysed by incubating these ppLAP preparates for 2 h with a 17-fold molar excess of ZnSO₄ or MnSO₄, 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. Using the FFAS and SCRWL servers a search model was constructed based on the structure of $\text{L}$-amino acid (PDB identifier 1GYT), which included all conserved side chains with the remaining non-alanine/glycine residues truncated at the C⁰ atom (a so-called mixed model). Several iterations of manual building using the program Coot were alternated with maximum-likelihood refinement using the program Refmac5. Water molecules were added using Coot during the last refinement cycles.

The low-pH ppLAP crystal used for data collection diffracted to 2.75 Å resolution and belonged to space group P6₃, with two subunits per asymmetric unit (solvent content 50% (v/v)). The crystal was twinned with a twinning fraction of 0.47 as determined in CNS. The twin-related reflection intensities were averaged to simulate the case of perfect twinning, following the suggestion made by Yeates. A starting model for refinement was obtained by molecular replacement with the program Phaser with a single subunit of the high-pH ppLAP hexameric structure as a search model. Model refinement was carried out using CNS, with protocols designed for twinned data. Model building, and the placement of water molecules was done with Coot.

The ppLAP crystal complexed with bestatin diffracted to 1.5 Å and belonged to space group P1 with one hexamer per unit cell. An initial structure 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.

The model quality was validated for all structures using Coot and MolProbity. The statistics of the refined structures are given in Table 1.

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 15-fold molar excess of ZnSO₄ and MnSO₄, respectively. After incubation the enzyme was washed and concentrated to 3.7 mg/ml in water containing 0.1 M K₂SO₄ in the presence of a 1.4-fold molar excess (96 μM) of either ZnSO₄ or MnSO₄. Competitive activation/inhibition was analysed by incubating these ppLAP preps for 2 h with a 20-fold molar excess of ZnSO₄ or MnSO₄, before measuring the activity.

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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**Supplementary Data**

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

**References**


