Structure of a Zn$^{2+}$-containing D-alanyl-D-alanine-cleaving carboxypeptidase at 2.5 Å resolution

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Bacteria possess proteases that are specific for the peptide bonds between D-alanine residues, one of which has a free α-carboxyl group. These D-alanyl-D-alanine peptidases catalyse carboxypeptidation and transpeptidation reactions involved in bacterial cell wall metabolism, and are inactivated by β-lactam antibiotics. We have now elucidated the structure, at 2.5 Å resolution, of the penicillin-resistant Zn$^{2+}$-containing D-alanyl-D-alanine peptidase of Streptomyces albus (Zn$^{2+}$ G peptidase). The enzyme is shown to consist of two globular domains, connected by a single link. The N-terminal domain has three α-helices, and the C-terminal domain has three α-helices and five β-strands. The Zn$^{2+}$ ion is ligated by three histidine residues, and located in a cleft in the C-terminal domain. The mechanism of action of the enzyme may be related to that of other carboxypeptidases, which also contain functional Zn$^{2+}$ ions.

Crystals of the Zn$^{2+}$ G peptidase were grown, using the vapour diffusion technique, from a solution containing 50 mM Tris-HCl pH 8.0, 5 mM MgCl$_2$, 10 mM Na$_2$SO$_4$, and a protein concentration of 2%. The crystals were prismatic and belonged to the space group P2$_1$ (a = 51.1 Å, b = 49.7 Å, c = 38.7 Å and β = 100.6°). The asymmetric unit contained one protein molecule.

The heavy-atom derivatives were prepared by soaking native crystals in appropriate solutions: 5.6 mM K$_2$PtCl$_6$, 3 mM NaAuCl$_4$, and 30 mM K$_2$CO$_3$. X-ray diffraction data were collected on a four-circle diffractometer (Hilger-Watts) with a Ni-filtered CuKα radiation and an θ-scan procedure. Two equivalent reflections for the native and four for the derivatives, including Bijvoet pairs, were measured in shells of increasing θ values. The heavy-atom sites were located using F$_{calc}$ Patterson functions. Heavy-atom parameters were refined by the F$_{calc}$ procedure, followed by three cycles of phase refinement. The mean figure of merit was 0.66 for 6,700 reflections. Despite this rather low value, the high quality of the electron density map can be judged from Fig. 1.

The interpretation of the 2.5 Å electron density map of the Zn$^{2+}$ G peptidase was carried out more or less simultaneously with the establishment of its primary structure. Initially, the N-terminal 55 residues and the C-terminal 45 residues could be located directly on the mini-map and six fragments (totaling 84 residues) could be correctly positioned using the Evans-Sutherland graphic system (at the University of London). However, examination of all possible tracings of the polypeptide chain and re-evaluation of the C$\alpha$—C$\alpha$ distances led to the conclusion that a 28-residue fragment was missing. This fragment was subsequently isolated and sequenced so that, at this time, the techniques are in agreement except that at a few residues located outside the catalytic cavity. The complete sequence will be published elsewhere, but Table 1 gives the...
positions and proposed functions of some critical amino acid residues.

Besides the Zn\(^{2+}\) ion, cystines 92–140 and 168–210 were the most prominent features of the electron density map. Electron density at the methionine residues was rather weak and the weakest Met 153 was located near an Au-binding site. Tracing the polypeptide chain was easy except for two regions, C\(^{\text{N}}\)C\(^{\text{C}}\) and C\(^{\text{C}}\)C\(^{\text{N}}\), respectively. The enzyme molecule has an overall dimension of 48 Å × 34 Å × 28 Å and consists of two distinct globular domains of different sizes (Figs 2, 3). The small N-terminal domain (76 residues) contains three \(\alpha\)-helices (43%) and is connected to the large C-terminal domain (136 residues) by a single link that exhibits a rather unusual folding. The C-terminal domain belongs to the \(\alpha/\beta\)-type secondary structure\(^6\), containing three \(\alpha\)-helices (34%) and five \(\beta\)-strands (17%). About 38% and 11% of the total number of amino acid residues are comprised of \(\alpha\)-helix (80 residues) and \(\beta\)-structure (23 residues), respectively. To our knowledge, such a secondary structure has not been found previously in any other protein.

The five \(\beta\)-strands of the C-terminal domain form a mixed sheet with the usual left-handed twist. The cross-over connection is right-handed, a widely observed rule in protein folding\(^8\). This sheet constitutes the lining of one side of the catalytic cavity. Other remarkable features of the C-terminal domain are the long, 24-residue-containing helix and, especially, the two loops (C\(^{\text{C}}\)C\(^{\text{C}}\)C\(^{\text{C}}\)C\(^{\text{C}}\) and C\(^{\text{C}}\)C\(^{\text{C}}\)C\(^{\text{C}}\)C\(^{\text{C}}\)) which, in turn, form the edges of the catalytic cavity. This cavity cuts the C-terminal domain into two parts, with the Zn\(^{2+}\) cofactor bound inside coordinated with the three protein ligands His 152, His 193 and His 196. Previous studies at 4.5 Å resolution\(^10\) have shown this cleft to be the binding site of the two enzyme competitive inhibitors, the dipeptide acetyl-d-Ala-d-Val and the \(\beta\)-lactam compound p-iodo-7-\(\beta\)-phenylacetylaminocephalosporanic acid. The cavity of the native enzyme was not free of electron density; this might be due either to the presence of ordered solvent molecules or to spurious peaks originating from the heavy-atom binding sites. (In fact, each of the three heavy-atom derivatives studied had at least one binding site inside the cavity.)

A full characterization of the catalytic cavity of the Zn\(^{2+}\) G peptidase must await the final fitting of the protein molecule in the Richard’s box and crystallographic refinements. Similarly, a full identification of all the residues involved in the binding and catalytic processes must await study at high resolution of various inhibitor (activator)—enzym complexes. Nevertheless, at the present stage of the analysis, one may make interesting hypotheses concerning the mechanistic properties of this peptidase. Most likely, Arg 136 is concerned with binding, by charge pairing, of the carboxylate substrate. In addition, two Asp residues (159 and 192) and His 190 are located near the Zn\(^{2+}\) cofactor and their side chains have the right orientation to interact with the bound peptide substrate molecule. Contrary to all the other \(\beta\)-lactam compounds tested, \(\beta\)-iodopenicillanate bound irreversibly to the catalytic cavity of the Zn\(^{2+}\) G peptidase in rather mild conditions (unpublished results). The difference Fourier synthesis at 2.8 Å resolution of the complex thus formed showed two peaks in the vicinity of Arg 136 and His 190, respectively. Permanent inactivation of the peptidase may be due to alkylation of this His residue.

In the proposed catalytic mechanism of carboxypeptidase A and thermolysin\(^11\), the Zn\(^{2+}\) ion functions as a negative-charge stabilizer, the side-chain oxygen of a Glu residue as a proton abstractor (with or without a water molecule as the nucleophile), and a His or Tyr residue as a proton donor. By analogy, one may propose that in the Zn\(^{2+}\) G peptidase, Asp 159 or Asp 192 also acts as proton abstractor and His 190 as proton donor. Following this view, the Zn\(^{2+}\) G peptidase would be mechanistically similar to the usual metalloendopeptidases and its specificity would depend entirely on the shape and structural features of its substrate binding cavity.

Two further comments deserve attention. Contrary to observations on carboxypeptidase A, whose active centre is a closed cavity, that of the Zn\(^{2+}\) G peptidase is an open cleft. This feature, which is reminiscent of that of thermolysin, should permit accommodation of extended structures. It might be related to the ability of the Zn\(^{2+}\) G peptidase to perform endopeptidase activities effectively on complex peptide substrates (possessing a free carboxyl group in \(\alpha\) position to the scissile linkage)\(^12\). Finally, the role of the small N-terminal domain remains unknown.

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**Table 1** Positions and proposed functions of some critical amino acid residues

<table>
<thead>
<tr>
<th>Residue</th>
<th>Assigned Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn-Gly 1-2</td>
<td>N-terminal(^*)</td>
</tr>
<tr>
<td>Cystine 3-80</td>
<td>S-S bridge</td>
</tr>
<tr>
<td>Cystine 92-140</td>
<td>S-S bridge</td>
</tr>
<tr>
<td>Cystine 168-210</td>
<td>S-S bridge</td>
</tr>
<tr>
<td>His 152, His 193, His 196</td>
<td>Zn(^{2+}) ligands</td>
</tr>
<tr>
<td>Arg 136</td>
<td>Charge pairing with substrate</td>
</tr>
<tr>
<td>Asp 159 or 192</td>
<td>Proton abstractor</td>
</tr>
<tr>
<td>His 190</td>
<td>Proton donor</td>
</tr>
<tr>
<td>Ile 212</td>
<td>C-terminal</td>
</tr>
</tbody>
</table>

\(^*\) Partly in the form of a cyclic imide.