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Inhibition of carboxypeptidase A by excess zinc: analysis of the structural determinants by X-ray crystallography

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Abstract Pancreatic metallocarboxypeptidases are inhibited by a millimolar excess of zinc together with other exo- and endometalloproteases. We have analyzed the structure of bovine carboxypeptidase A inhibited by an excess of zinc ions using X-ray crystallography at 1.7 Å overall resolution. Under these conditions, a second zinc is observed to bind to the enzyme active site, establishing a distorted tetrahedrally coordinated complex which involves Glu-270 (the general base for catalysis), a water molecule, a chloride ion, and a hydroxide ion. This hydroxide ion forms a 114° angular bridge between the inhibitory and the catalytic zinc ions, which are at a distance of 3.3 Å from one another. The inhibitory zinc holds the hydroxide at nearly the same location as a previously observed active site water molecule (W571) and probably perturbs the substrate positioning and stereochemical rearrangements required for substrate cleavage during catalysis.

Key words: Metalloenzyme; Carboxypeptidase A; Enzyme inhibition; Zinc inhibition; X-ray crystallography

1. Introduction

Carboxypeptidase A (CPA, EC 3.4.17.1) is a zinc metalloenzyme that catalyses the hydrolysis of C-terminal residues from peptide or ester substrates by cleavage of the peptide or ester bond [1,2]. One Zn^{2+} ion is strongly coordinated at the active site and is involved in catalysis [2]. CPA has been the subject of a large number of biochemical and crystallographic studies both in its free state and bound to peptidic and peptidomimetic inhibitors [2,3]. Additionally, the interactions of the enzyme with its 95-residue activation segment in the catalytically inactive procarboxypeptidase A [4], and with the specific protein inhibitor isolated from potato [5], have been analysed in depth [6].

Within a few years of finding that CPA is a zinc-dependent enzyme [7], it was reported that millimolar concentrations of $ZnCl_2$ inhibit its hydrolytic activity in a competitive way for both ester and peptide substrates [8]. These results were later corroborated by equilibrium dialysis experiments, indicating that CPA loosely binds a second equivalent of zinc in the pH range from 7 to 10 [9,10]. Additional evidence was obtained by stopped-flow and spectrophotometric methods using the derivatized azo-carboxypeptidase A [11]. From these experiments, it was not possible to locate precisely the binding site of the excess zinc ions but the results suggested that the zinc binding site for inhibition was not far from the enzyme active site but did not involve the substrates.

Later, using kinetic approaches, Larsen and Auld studied the state(s) of hydroxylation of the inhibitory zinc and its possible site(s) of interaction with CPA [12]. The authors showed that the pH dependence of the pK_i follows a pattern which indicates that the enzyme is selectively inhibited by zinc monohydroxide, $ZnOH^+$ ($K_i = 7.1 \times 10^{-7}$ M). Furthermore, they postulated that the ionization of a ligand in the enzyme's inhibitory site should be obligatory for binding of $ZnOH^+$ to the complex and, after kinetic studies of the pH dependence of substrate hydrolysis, they proposed that the inhibitory zinc ion binds to the carboxylate of Glu-270. Also, they suggested that the inhibition process is specific for zinc monohydroxide because it allows the formation of a stabilizing hydroxide bridge between the inhibitory and catalytic zinc ions. The latter was corroborated in further studies [13] where the mode of interaction for several inhibitory metal ions (Zn^{2+}, Pb^{2+}, and Cd^{2+}) with Glu-270 was investigated by the specific chemical modification of this residue. Finally, a novel type of metal binding site based on a bridging interaction between the Glu-270-coordinating metal hydroxide and the catalytic metal ion was proposed.

Besides CPA, several zinc proteases have been shown to be inhibited by excess zinc, e.g. thermolysin [14], the neutral metallo-endopeptidase from rabbit kidney brush border [15], human neutrophil collagenase [16], and angiotensin converting enzyme [17], among others. However, except for the endoproteinase thermolysin [18], none of these enzymes have been studied by X-ray crystallography to confirm the biochemical data. The present work reports the structural features of the complex of bovine CPA with two zinc ions as derived by X-ray crystallography. These features clarify the inhibitory effect of excess zinc ions in CPA, and suggest a general structural model for this type of inhibition in zinc-dependent metalloproteases.

2. Materials and methods

Bovine CPA was purchased from Sigma and used without further purification. Crystals for the collection of X-ray data were prepared using the dialysis method proposed by Coleman and Vallee [9]. Accordingly, a solution containing 5 mg/ml CPA in 1 M LiCl, 0.02 M Tris-HCl, at 7.5, was dialyzed, using membrane dialysis tubes, against a solution of 0.5 M in LiCl, buffered with 0.02 M Tris to pH 7.5. In a second step, the protein in the membrane dialysis tube was equilibrated against 0.25 M LiCl with the same buffer for 2 days at 4°C. At this point tiny crystals appeared in the dialysis tube, which grew without further stirring to the maximal dimensions of $2 \times 0.5 \times 0.2$ mm^3 after 3 or 4 days of equilibration.

Some of the crystals were separated for subsequent control of the enzymatic activity, the rest of the crystal suspension being equilibrated by dialysis against 0.33 mM $ZnCl_2$, 0.25 M in LiCl, buffered with 0.02 M Tris-HCl to pH 7.5, in order to obtain CPA+Zn crystals [12].

The CPA activity of the crystals in the presence and absence of zinc was analyzed, after dissolution, using hippuryl-L-phenylalanine (HPA) (supplied by Sigma) as a substrate by monitoring the increase in

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absorbance at 254 nm, according to the procedure of Sebastian [19]. Six crystals of native CPA and of CPA+Zn were washed with 0.25 M LiCl, 0.02 M Tris-HCl (pH 7.5) and dissolved in 1 M LiCl, 0.02 M Tris-HCl, pH 7.5. The dissolved CPA+Zn crystals showed only 2% residual activity as compared with the native enzyme crystals, in accordance with previous studies [12].

A complete data set of CPA+Zn X-ray diffraction intensities up to 1.7 Å was recorded at a 100 mm crystal-to-detector distance on a 300-mm MAR Research Imaging plate detector attached to a Rigaku-Denki RU200 rotating CuKα-anode X-ray generator, operated at 5.4 kW, and at 10°C. The diffraction data were processed with MOSFLM v 5.23 [20] and scaled, merged, and reduced using programs of the CCP4 package [21]. Table 1 provides a summary of the data collection and processing. The space group is $P2_1$ with lattice constants $a=46.45$, $b=59.55$, $c=51.23$ and $\beta=97.40°$ compared to $a=51.60$, $b=60.27$, $c=47.25$ and $\beta=97.27°$ of the native enzyme [3]. The coordinates of the protein part of native CPA available from the Protein Data Bank (PDB access code 5cpa [3]) were used to solve the structure. The coordinates were submitted to rigid-body and positional refinements followed by a temperature factor refinement using X-PLOR [22]. After this, the crystallographic R factor dropped from 31.2 to 26.2. The resulting model was inspected and corrected manually against $(2F_{obs}-F_{calc})$ and $(F_{obs}-F_{calc})$ Fourier maps, including water molecules at stereochemically reasonable position using TURBO Frodo (version 5.0a, Biographics, Marseille) on a Silicon Graphics workstation. In the vicinity of Glu-270, the catalytic base of the enzyme located close to the active-site zinc ion, clear (positive) electron density was observed in both Fourier map types which was assigned to a second zinc ion and a chloride ion. The new model was further submitted to crystallographic refinement, with a final R factor of 0.187 and refined against 27835 reflections in the range 6.0 Å to 1.7 Å.

The final model comprises the 307 residues of CPA (labelled 1–307), 192 solvent molecules (labelled W308–W500), two zinc ions (labelled 998 and 999) and one chloride anion Cl^-(501). An inspection of the Ramachandran plot reveals one residue in a disallowed region: Ser-199, unambiguously defined by electron density and with similar main-chain angles to those described for the native CPA structure.

Both figures (1 and 2) are presented in the standard orientation, i.e. looking into the active-site cleft with the catalytic (and inhibitory) zinc ions with a putative substrate binding horizontally from left (N-terminally from the scissile peptide bond) to right (C-terminus with the hydrophobic S1′ pocket).

The final coordinates will be deposited with the Brookhaven Protein Data Bank.

3. Results and discussion

3.1. Three-dimensional crystal structure of CPA+Zn

The binding of a second zinc ion to bovine CPA does not significantly alter the basic crystallographic parameters or the overall three-dimensional structure of the enzyme (Fig. 1). The spatial locations of all the protein residues of the active site of CPA, except Glu-270, are virtually in the same position in the crystal structures of the active enzyme and of the zinc-inhibited enzyme. As seen previously [3], the catalytic zinc ion is coordinated in CPA by the side chains of His-69, Glu-72, His-196, and by an active-site water molecule (W571). Close to these are the residues involved in catalysis and substrate binding, which have been classified in five subsites, S1′–S4 [3,6,23]: Glu-270, Arg-127, Asn-144, Arg-145, Tyr-248, Arg-71, Ser-197, Tyr-198, Ser-199, etc. In the standard mechanism of CPA it is proposed that the nucleophilic attack on the scissile peptide carbonyl of the substrate is made by the coordinated water molecule (W571) which is polarised by the zinc and whose proton is abstracted by Glu-270, the general base [2]. The oxyanion of the resulting tetrahedral intermediate is stabilized by the zinc ion and by Arg-127. Subsequently, a proton is transferred from Glu-270 to the imino group of the substrate peptide bond, which is cleaved.

In the first Fourier map ($2F_o-F_c$ and F_o-F_c) obtained from the CPA+Zn crystal the inhibitory zinc ion (998 numbering in our model) was identified by a strong electron density (5σ, with a temperature factor of $B=23$ Å^2 after final refinement). This second zinc ion is located 3.3 Å from the catalytic zinc (999) (and exhibits a temperature factor $B=11$ Å^2). The catalytic water has been slightly displaced by the inhibitory zinc from its original position, and because of its

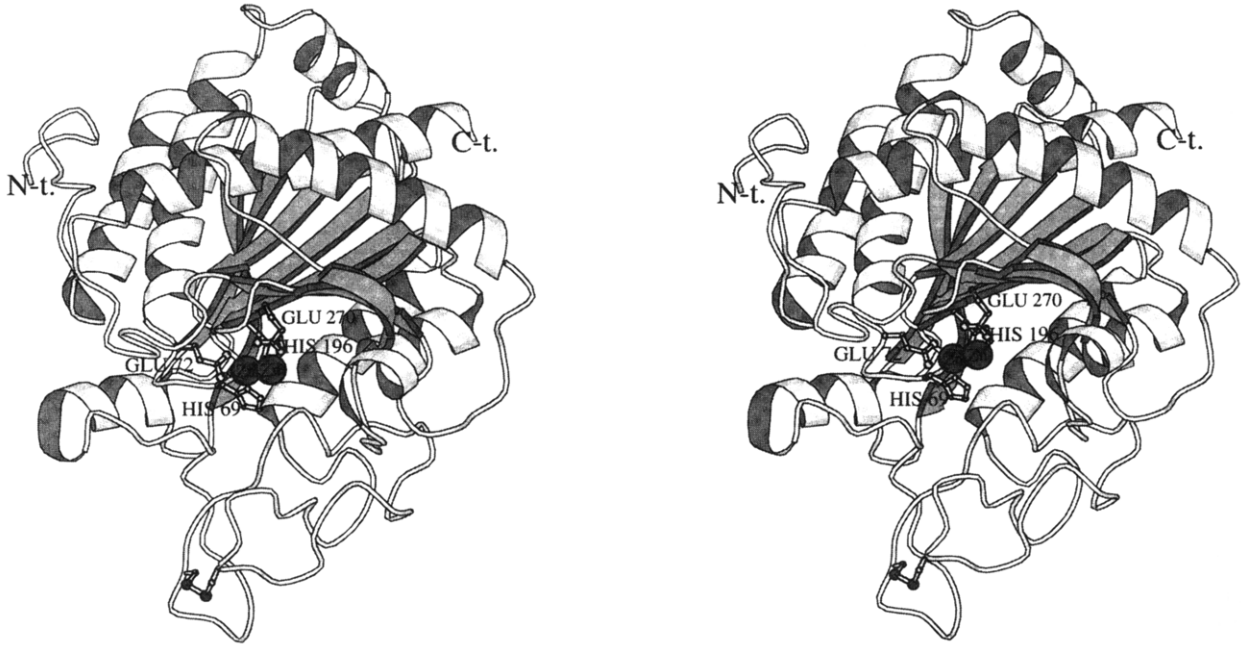

Fig. 1. Ribbon diagram [24] showing the backbone of bovine carboxypeptidase A in the presence of excess zinc. Black spheres represent the two zinc ions bound to the active site. The amino acid residues coordinating both zinc ions are shown in ball-and-stick representation: His-69, Glu-72 and His-296 coordinate the catalytic zinc and Glu-270 the inhibitory zinc.

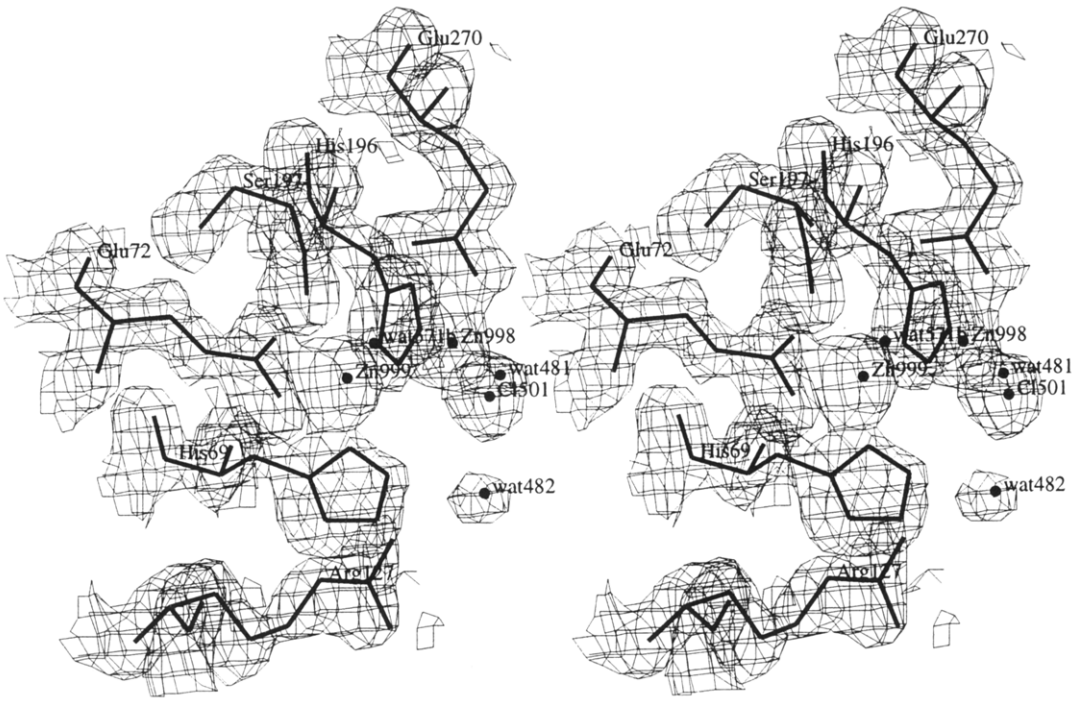

Fig. 2. Stereo drawing showing a region of the ($2F_o-F_c$, contoured at 1σ) difference map of the carboxypeptidase A+excess zinc crystal structure including the main residues of the active site region, the two zinc ions, the coordinating water/hydroxide and water molecules, and the chloride ion.

close proximity to both zinc ions (2.36 and 2.17 Å, see Table 2) it is probably a hydroxide ion and establishes a bridge between the cations with a Zn-O-Zn angle of 114°. The inhibitory zinc is also attached to the side chain of Glu-270, which is rotated 22° with respect to the native structure in order to coordinate in a monodentate manner to the inhibitory zinc through the side-chain oxygen Oε1 (Fig. 2). Additionally, a second strong electron density peak within ligation distance to the inhibitory zinc ion (labelled 998) was assigned to a chloride ion (labelled 501, $B=22$ Å^2). There were two good reasons to consider Cl^- as the coordinating anion, the presence of Cl^- (LiCl and $ZnCl_2$) in the crystallization conditions and the observation that the species Zn(OH)Cl has a net charge of 0, the same as the neutral molecule (W571) that it substitutes. This possibility has also been suggested by Larsen and Auld [12,13] on the basis of enzyme inhibition studies with Zn(II), Pb(II) and Cd(II) by kinetic and electronic absorption spectra approaches. Besides binding to water/hydroxide W571b, Glu-270 Oε1 and Cl^-501, the inhibitory zinc is also bound to water W481, establishing an overall tetrahedral (distorted) coordination with them. The interatomic distances between the inhibitory zinc and the ligands are all around the typical zinc ion-ligand values (1.8–2.4 Å) (Table 2) [25–28]. The angles are also within the ranges reported in the literature. From the point of view of charges in the model that we propose at pH 7.5, the two-zinc ion cluster displays 4 positive charges (due to both metal ions) and three formal negative charges (Glu-72, Glu-270 and Cl^-(501)). The suggested hy-

Table 1
X-ray data collection and processing

Diffraction data	
No. of measured reflections	58729
No. of unique reflections	29901
R_{merge}[a]	0.082
Completeness	
for data (25–1.7 Å)	92.9%
Refinement	
initial R factor	25.2%
final R factor	18.7%
rms deviation from ideal values	
bonds	0.009 Å
bond angles	1.474 Å
planarity	23.974 Å

[a] $R_{merge}=\Sigma\Sigma|I(h)_i-<I(h)>|/\Sigma<I(h)>$; $I(h)_i$ is the observed intensity of the i-th measurement of reflection h, and $<I(h)>$ the mean intensity of reflection h; calculated after loading, scaling, and merging of Friedel pairs with rotavata/agrovata (CCP4 [21]).

Table 2
Crystallographic distances between the catalytic (1) and the inhibitory (2) zinc ions in CPA+Zn and their ligands: comparison with the CPA and CPA+Bzx structures

	CPA [3]	CPA+excess Zn^{2+}	CPA [31]+Bzx[a]
Zn (1)			
Glu-72 O^{e1}	2.18	2.10	2.03
Glu-72 O^{e2}	2.31	2.17	2.75
His-69 N^{e1}	2.12	1.93	2.04
His-196 N^{e1}	2.07	2.08	2.00
W571[2]	205		
W567[2]	3.23		
W571b		2.36	
500 O^{e1}			2.31
500 O^{e2}			2.61
Zn (2)			
Glu-270 O^{e1}		1.86	
Cl^-(501)		2.29	
W571b		2.17	
W481		2.39	

[a] Bzx, substrate-analog inhibitor benzylsuccinic acid [31].
[b] W571 and W567 are alternative sites occupied by a single water [3].

droxide ion W571b compensates the residual charge as is the case in many metal ion clusters [29].

To elucidate the inhibition of CPA by the second zinc, we have overlapped the structures of the enzyme active site derived from CPA+Zn (this work) and from CPA+benzylsuccinic acid [30] where benzylsuccinic acid is a competitive inhibitor of CPA [6] and is supposed to occupy a similar position as the peptide bond to be cleaved. The superimposition of both structures shows that the respective positions of the carbonyl group (500C [30]) of the inhibitor and the inhibitory zinc ion are almost the same (Table 2). The second zinc will thus interfere with the proper positioning of the substrates at the active site of CPA.

It has been previously proposed that Glu-218 and His-303 could be responsible for binding excess inhibitory zinc [31]. We have not found any electron density near these residues which could be ascribed to a bound zinc. In contrast, our results strongly support the experimental results and hypothesis of Larsen and Auld [12,13] which point to the establishment of a monohydroxide bridge between the catalytic and the inhibitory zinc and the coordination of the latter to Glu-270.

3.2. *Inhibition by excess zinc in other metalloproteases*

Recently the crystal structure of the metalloendoprotease thermolysin in the presence of excess zinc has been reported [18]. Despite the great differences in overall folding, zinc binding motifs and active-site topologies between this enzyme and CPA, both main representatives of two large subfamilies – exoproteases and endoproteases – within the zinc proteases, both enzymes have in common an essential – catalytic – zinc (II) ion at the active site. This essential zinc ion is bound in a tetrahedral coordination and plays a similar role in the proteolysis of substrates [32,33]. In thermolysin, the catalytic zinc ion is coordinated by two histidines (His-142 and His-146), one glutamate (Glu-166) and one water molecule (W231).

In the presence of excess of zinc, the crystal structure of thermolysin also shows a second – inhibitory – zinc bound to the active site [18]. A comparison of the features of the second zinc binding site in this structure and in the CPA+Zn structure is noteworthy: (1) the two zincs are at similar distances in both enzymes, 3.2 Å in thermolysin and 3.3 Å in CPA; (2) a water/hydroxide ligand is observed as a bridge of similar geometry with a Zn-O-Zn angle of 116° in thermolysin and of 114° in CPA; (3) in thermolysin the inhibitory zinc is tetrahedrally coordinated to Tyr-157, His-231, W143 and Glu-166. This residue (Glu-166) is also a ligand of the catalytic zinc and, therefore, establishes a second bridge between the two zinc ions. This is not the case in CPA+Zn in which the ligands for the catalytic and the inhibitory zinc are different.

Interestingly, the coordination of the inhibitory zinc in CPA constitutes a new type of zinc binding site, because this metal has always been found coordinated with at least one imidazole group [1,32]. On the other hand, the two inhibitory zinc binding sites, in CPA and thermolysin, share many features with the cocatalytic motif in multizinc enzymes [33], where the participating metal atoms are in close proximity (3–5 Å) and function as a catalytic unit. This is the case, e.g. in alkaline phosphatase, phospholipase C, nuclease P1, lens aminopeptidase, superoxide dismutase [34]. The occurrence of a bridging ligand, aspartate, glutamate, and/or a water molecule or hydroxide ion, is the most significant feature of these cocatalytic sites and is distinctive in differentiating the cocatalytic from the catalytic and structural sites.

It would be of interest to extend these detailed structural comparative studies to other zinc metalloenzymes in order to improve our knowledge of the chemistry of isolated and clustered zincs in these enzymes and their potential involvement in regulatory and toxicological processes. In this respect, it could be added that free Zn^{2+} concentrations in human body ranges from nanomolar to micromolar concentrations in the cytoplasm, in normal states, to much higher concentrations in certain vesicles and fluids or in abnormal/toxicological states [1,35]. The potential influence of these zinc ions on the activity of metalloenzymes in general, and in tissue metallo-carboxypeptidases – involved in important physiological functions [6] – in particular, cannot be neglected.

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