Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 7151–7154, December 1983
Biochemistry

Crystallographic studies on apocarboxypeptidase A and the complex with glycyl-L-tyrosine

(enzyme activity/proteases/protein crystallography/metalloproteins)

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Communicated by Konrad Bloch, August 22, 1983

ABSTRACT **The crystal structures of zinc-free carboxypeptidase A (apocarboxypeptidase A) and the complex of glycyl-L-tyrosine with apocarboxypeptidase A are described and compared to the corresponding structures of the zinc-containing enzyme. Only small conformational changes in the zinc ligands accompany removal of the metal. Interactions between the tyrosine residue of glycyl-L-tyrosine and apocarboxypeptidase A are similar to those observed in the complex with the holoenzyme. However, in the absence of zinc, the carbonyl oxygen of the glycyl moiety now receives a hydrogen bond from the side chain of arginine-127. Although not as yet observed, a similar shift of the carbonyl oxygen of a susceptible bond from the zinc to arginine-127 could stabilize tetrahedral intermediates generated during the hydrolysis of substrates by carboxypeptidase.**

Carboxypeptidase A (CPase A, peptidyl-L-amino acid hydrolase, EC 3.4.17.1) catalyzes the hydrolysis of the carboxyl-terminal residue from peptide and ester substrates (for a recent review, see ref. 1). Waldschmidt-Leitz (2) proposed that a positively charged group in the active site of CPase A could account for the requirement of a free terminal carboxylate group in the hydrolysis of substrates by this enzyme. Inhibition of CPase A by a variety of metal chelators led Smith and Hansen (3) to propose that the active site of the enzyme contains a metal ion, which was later identified as Zn^{2+} (4). However, crystallographic studies revealed that, in addition to the zinc, the side chains of three arginines (residues 145, 127, and 71) extended along the active site of CPase A (5). Although all four positively charged groups may be involved in precatalytic binding interactions with the terminal carboxylate group of the substrate (6, 7), Arg-145 (not the zinc) functions as the binding site for the carboxylate group of the peptide during the hydrolytic step. Instead, the zinc serves to polarize the carbonyl group of the susceptible bond and possibly as the source of a zinc-hydroxyl (or zinc water) species for nucleophilic attack on the substrate during a deacylation step (8, 9).

Removal of the zinc yields an inactive enzyme, apocarboxypeptidase A (apo-CPase A) (10). The conformation of apo-CPase A appears to be similar to that of the native enzyme (11). Zinc is bound quite rapidly by the apoenzyme, with concomitant recovery of activity. Moreover, the recombination of zinc with apo-CPase A is inhibited by peptides, suggesting that these ligands may bind to the apoenzyme (12). The location of bound peptides at the active site in apo-CPase A was demonstrated by difference Fourier studies at low resolution of the complex of apo-CPase A with the dipeptide glycyl-L-tyrosine (Gly-Tyr) (13).

X-ray crystallographic methods are well suited for investigating the structural consequences of zinc removal on the active site conformation and ligand-binding properties of CPase A. Crystal structures of CPase A in the native state and complexed to a variety of ligands have been described (14–16). Direct removal of zinc from crystals of CPase A yields crystals of apo-CPase A that are isomorphous with those of the native enzyme (11). Consequently, sensitive difference Fourier techniques may be used to study the structural changes accompanying zinc removal from CPase A. In this paper, we compare the structures of apo-CPase A and the complex of Gly-Tyr with apo-CPase A to the corresponding forms of the zinc forms. The implications of these structures for the binding and cleavage of substrates by CPase A are discussed.

MATERIALS AND METHODS

Bovine CPase A_α (Cox), 8-hydroxyquinoline-5-sulfonate (HQSA), and Gly-Tyr were purchased from Sigma and used without further purification. Crystals of CPase A were grown by dialysis of CPase A solutions against 0.2 M LiCl/0.02 M Tris·HCl, pH 7.5, at 4°C (11). apo-CPase A was prepared by the standard procedure of soaking the crystalline enzyme in a solution containing chelator (17, 18). Although *o*-phenanthroline is customarily used in the preparation of apoenzyme, some problems with this chelator were noted in early studies involving crystalline CPase A_γ (Anson) (19). Previous crystallographic studies of apo-CPase A (13) utilized HQSA, which is the most effective chelator tested for removing active site metals from CPase A (20). Following this protocol, crystals of apo-CPase A (NaCl) were prepared by soaking CPase A crystals in 5 mM HQSA/0.1 M NaCl/0.04 M Tris·HCl, pH 7.5, at 4°C for 6 days. Crystals of apo-CPase A (LiCl) were prepared by the same procedure substituting LiCl for NaCl. Crystals of the apo-CPase A–Gly-Tyr complex were prepared by soaking apo-CPase A crystals for 2 days at 4°C in fresh HQSA solution containing 50 mM Gly-Tyr in the Tris·HCl/NaCl buffer.

Diffraction data from one crystal each of apo-CPase A (NaCl), apo-CPase A (LiCl), and apo-CPase A–Gly-Tyr complex were collected to 2.4, 2.8, and 2.3 Å resolution, respectively, on a Nicolet $P2_1$ diffractometer at 20°C. The intensity data were processed by using previously described methods (21). Collection of diffraction data to 2.0 Å resolution for native CPase A and the Gly-Tyr complex has been documented (5). The fractional changes in structure factors from the native values for the CPase A–Gly-Tyr, apo-CPase A (NaCl), apo-CPase A (LiCl), and apo-CPase A–Gly-Tyr structures are 12.3, 12.5, 9.5, and 13.8%, respectively.

Difference maps for the various complexes were calculated

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Abbreviations: CPase A, bovine carboxypeptidase A_α; apo-CPase A, zinc-free CPase A; apo-CPase A (NaCl), apo-CPase A prepared in buffer containing NaCl; apo-CPase A (LiCl), apo-CPase A prepared in buffer containing LiCl; HQSA, 8-hydroxyquinoline-5-sulfonate.

by using Fourier coefficients $(|F_{der}| - |F_{nat}|) \exp(i\,\alpha_{calc})$, in which F_{der} and F_{nat} are the structure factors for the derivative and native data sets, respectively, $i^2 = -1$, and α_{calc} is the phase calculated from the coordinates of native CPase A refined at 1.54 Å resolution (22). Diamond's (23) interactive graphics program BILDER, as extensively modified by R. C. Ladner for a VAX 11/780 and the Evans and Sutherland Picture System II, was used to model build the Gly-Tyr molecule and to make adjustments in the conformation of CPase A residues in these complexes. Because the occupancy of Gly-Tyr in these complexes was ≈40% (16), model building was facilitated by the use of electron density maps calculated by using $(5|F_{der}| - 4|F_{nat}|)$ Fourier coefficients. These maps are roughly equivalent to a more traditional $(2|F_{der}| - |F_{nat}|)$ map, which would be appropriate if the complex were fully occupied (24, 25).

RESULTS AND DISCUSSION

The zinc atom in native CPase A is coordinated by four protein atoms (0ε1 and 0ε2 of Glu-72, Nδ1 of His-29, and Nδ1 of His-196) and one water molecule. The carboxylate group of Glu-270, which may act as a nucleophile or general base catalyst during hydrolysis, is hydrogen bonded to the zinc-bound water. The side chains of Arg-71, -127, and -145 line the active site groove and lead into the S_1' binding subsite of CPase A, which accommodates the carboxyl-terminal residue of the substrate. The phenolic hydroxyl of Tyr-248, which may serve as the proton donor in the cleavage of peptide substrates, is located ≈17 Å away from the zinc in the native structure.

The structures of apo-CPase A (NaCl) and apo-CPase A (LiCl) are very similar and indicate few conformational changes in the active site region upon removal of zinc from the crystalline enzyme (Fig. 1). The side chains of His-196 and Glu-270 move away from the original zinc site by small rotations about the χ_1 and χ_2 torsion angles. The similarity in the conformation of these ligands in the native and apo-CPase A structures is consistent with the rapid reassociation of zinc with apo-CPase A. Tyr-248 retains the native conformation in the apoenzyme. In addition, we observe no *cis–trans* isomerization of any of the three *cis* peptide bonds present in CPase A upon removal of zinc. A *cis–trans* isomerization of a peptide bond in concanavalin A upon removal of metal has been suggested (26), although this claim has been questioned (27).

The difference Fourier method reveals only regions of the structure that are altered upon removal of zinc. As an important consequence, the structural interpretations described above would be uninfluenced by the possible presence of residual zinc. Partially occupied crystals may even facilitate the crystallographic analysis, because the approximation of using native protein phases to phase the difference map is more nearly correct in this case (28). This characteristic of difference map studies is a major advantage to solution kinetic studies, in which the presence of residual zinc could contribute an unacceptable background.

In the absence of zinc, it appears unlikely that either Li^+ or Na^+ occupies the metal binding site. The difference electron densities at the zinc site are -1.35 e/Å^3 and -1.24 e/Å^3 for apo-CPase A (LiCl) and apo-CPase A (NaCl), respectively, at 2.8 Å resolution. Were cations binding to the zinc site, a much larger difference in electron density would be expected, because Na^+ has five times as many electrons as Li^+. Both the residual electron density and the motion of the His-196 and Glu-270 side chains away from the metal site are consistent with the binding of a water molecule at this site, because zinc–ligand bond distances in CPase A average 2.1 Å, compared to an average of 2.8 Å for a water–hydrogen bond distance.

The conformational changes accompanying the binding of Gly-Tyr to CPase A have been described (5, 29). Briefly, the carbonyl oxygen of Gly is coordinated to the zinc, while the Tyr side chain is in the S_1' pocket, and the carboxylate group forms a salt bridge to Arg-145. The side chain of Tyr-248 rotates 140° about the χ_1 side chain bond, bringing it from the native "up" position to the "down" position, where the phenol group may hydrogen bond to a carboxylate oxygen of Gly-Tyr. At pH 7.5, the amino nitrogen of Gly-Tyr is statistically distributed between a coordination site on the zinc and a salt bridge with Glu-270.

With the obvious exception of the carbonyl oxygen–zinc in-

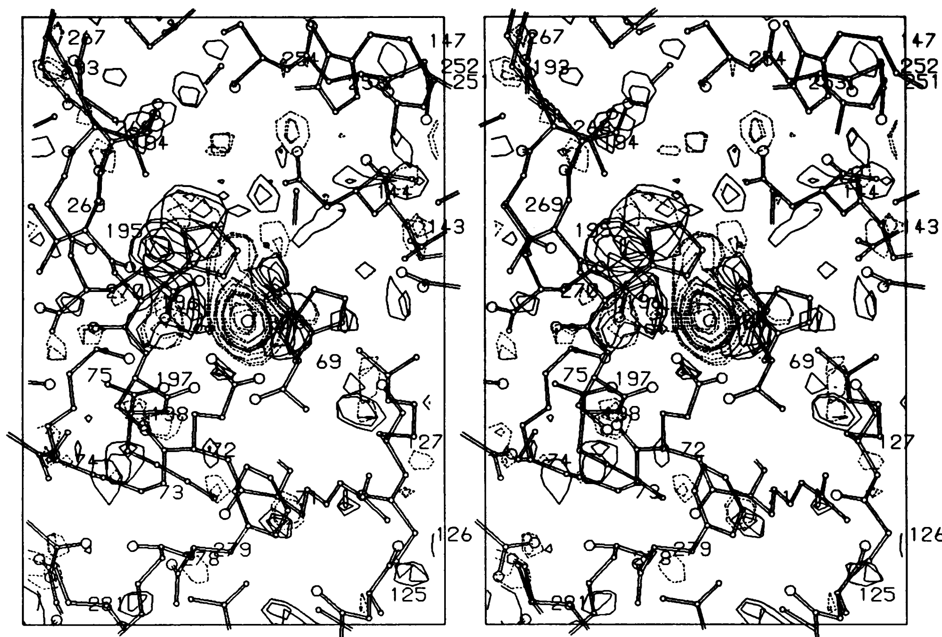

FIG. 1. Difference map illustrating the electron density changes accompanying removal of zinc from CPase A. The native conformations of the CPase A residues are indicated. Contours are at equal and arbitrary levels; negative contours are indicated by dashed lines.

FIG. 2. ($5|F_{der}| - 4|F_{nat}|$) electron density map illustrating the binding of Gly-Tyr to apo-CPase A. The fit of Gly-Tyr to the appropriate density is indicated; CPase A residues are positioned in the native conformation. Electron density is represented only in the vicinity of Gly-Tyr and the original zinc site; the residues of CPase A are not contoured here.

teraction, the binding of Gly-Tyr to apo-CPase A (Fig. 2) in general resembles the binding of Gly-Tyr to the native enzyme. However, in the absence of the zinc, the carbonyl oxygen of Gly-Tyr is now within hydrogen-bond distance (2.9 Å) of the guanidinium group of Arg-127. These two groups are separated by 4 Å in the CPase A–Gly-Tyr complex. Although the carboxylate group of the Gly-Tyr still forms a salt bridge to Arg-145, the Arg-127–carbonyl oxygen interaction and the loss of the interactions of the NH_2 terminus of Gly-Tyr have the overall effect of moving Gly-Tyr ≈1.5 Å along the active site groove (away from the pocket) in the apo-CPase A complex, relative to the CPase A–Gly-Tyr complex (Fig. 3). The side chain of Tyr-248 undergoes a conformational change similar to that observed in the Gly-Tyr–CPase A complex. However, the observed location for Gly-Tyr is quite distinct from that observed in the zinc–enzyme complex, ruling out binding of Gly-Tyr to residual zinc in this study.

A general role for arginines in the binding of substrates to CPase A was suggested by the location of three arginines in the active site region of CPase A (5, 6). Specific roles have previously been assigned to two of these residues: Arg-145 forms a salt bridge to the carboxylate group of ligands bound in the S′ binding site (5), while Arg-71 hydrogen bonds to the carbonyl oxygen of the amino acid residue in the S_2 binding subsite of the complex between CPase A and an inhibitory protein from potatoes (16). Significantly, Arg-127 is the only arginine of the three that does not form specific contacts to the inhibitor in this complex. The hydrogen bond between the carbonyl oxygen of Gly-Tyr and the side chain of Arg-127 in apo-CPase A is the first specific interaction observed for this residue.

Given the undesirable energetic consequences of burying a charged group, it seems surprising that Arg-127 has not been observed to form a hydrogen bond or salt bridge to ligands binding to zinc-CPase A. Although this situation is unfavorable in precatalytic enzyme–substrate complexes, the position of Arg-127 appears suited to facilitate formation of a tetrahedral intermediate and thus accelerate the rate of hydrolysis. This function would be somewhat similar to the role of the "oxy-anion" hole of serine proteases (30). Hydrolysis by CPase A of the ester *o*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenylacetate proceeds by an anhydride mechanism at low temperatures (9). Deacylation of covalent acyl-enzyme intermediates apparently involves attack of the carbonyl carbon by a zinc-bound hydroxyl species (9, 31), which may displace the carbonyl oxygen (unpublished data). If the carbonyl oxygen of the substrate were to shift from the zinc to Arg-127, the metal could readily be perpendicular to the amide bond. Studies on model metal complexes have shown that formation of a tetrahedral intermediate by attack of a metal-bound water on the carbonyl carbon is facilitated by this conformation (32). Decomposition of the tetrahedral intermediate to release free product and enzyme would also be consistent with stereoelectronic considerations (33). In our proposal, Arg-127 would facilitate the hydrolysis of some substrates, not by participating in precatalytic enzyme–substrate binding but by stabilizing the tetrahedral transition state intermediate in the deacylation step. Recognition of the possible involvement of Arg-127 in this tetrahedral intermediate may assist in the design of potential transition-state analogues for CPase A (34–38) and for analogous zinc-containing dipeptidase (39, 40).

Our previous studies have indicated that both ester and peptide substrates are cleaved with the carboxyl-terminal bond spanning the S_1' and S_1 subsites. The finding that Arg-127 may participate in the catalytic mechanism is consistent with this proposal. Contrary to recently published statements concerning our work (41, 42), we wish to emphasize our earlier statements that the rate-determining step and detailed chemical mechanism for hydrolysis of different substrates need not be conserved. Furthermore, from crystallographic studies of CPase A–ligand complexes, several pre- and posthydrolytic binding

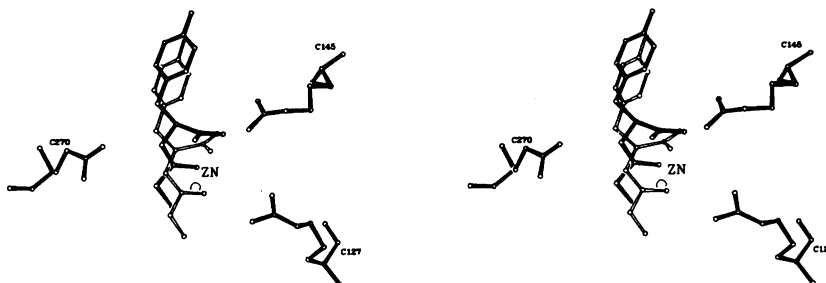

FIG. 3. Comparison of the conformation of Gly-Tyr bound to native CPase A (filled lines) and apo-CPase A (empty lines).

interactions between CPase A and substrates have been identified. The penultimate binding stage of peptide substrates is described, for example, as having the COO^--terminal carboxylate group of the substrate bound to zinc (16). Statements from Vallee's laboratory (41, 42) that our mechanistic proposals require no interaction of peptide substrates with the metal prior to the rate-limiting step, or for peptide substrates to have one fewer steady-state intermediate than esters, are incorrect.

We thank the National Institutes of Health (Grant GM 06920) and the Dreyfus Foundation (D.C.R.) for support.

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