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Binding of a possible transition state analogue to the active site of carboxypeptidase A

(protein crystallography/enzyme–inhibitor complex)

DAVID W. CHRISTIANSON AND WILLIAM N. LIPSCOMB

Gibbs Chemical Laboratories, Department of Chemistry, Harvard University, Cambridge, MA 02138

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ABSTRACT **The mode of binding of the competitive inhibitor 2-benzyl-3-formylpropanoic acid to the active site of carboxypeptidase A has been studied by x-ray diffraction methods to a resolution of 1.7 Å. The actual species bound to the enzyme was determined to be the gem-diol resulting from covalent hydration at the aldehyde carbonyl. Details relating to the process of association of inhibitor with enzyme are unknown at this time: the free aldehyde could initially bind to the enzyme and subsequently undergo catalytic hydration; or, the hydrate itself could be the species initially binding to the enzyme, because it does exist to a high degree (25%) in aqueous solution. Nevertheless, the structure of the complex reported is reminiscent of a possible tetrahedral intermediate that would be encountered in a general base hydrolytic mechanism. Of course, other mechanistic proposals, such as the anhydride pathway, cannot be ruled out simply on the basis of the structure of this enzyme–inhibitor complex.**

The metalloenzyme carboxypeptidase A_α (CPA; peptidyl-L-amino-acid hydrolase, EC 3.4.17.1) is an exopeptidase of molecular weight 34,472, containing one zinc ion bound to a single polypeptide chain of 307 amino acids (1–3). Its biological function is the hydrolysis of COOH-terminal amino acids from polypeptide substrates, and it exhibits preferred specificity toward substrates possessing large hydrophobic COOH-terminal residues. The hydrolytic mechanism of CPA has received considerable kinetic and structural study, and the enzyme has been the subject of many recent reviews (4–8). Important residues for substrate binding and catalysis are arginine-145, tyrosine-248, glutamate-270, and Zn(II); a catalytic role for arginine-127 is also considered in light of its interaction with a substrate in the x-ray structure of an apoenzyme complex (9). However, important details of the hydrolysis remain as yet unclear. Two probable mechanisms include the initial nucleophilic attack of the γ-carboxylate of residue glutamate-270 at the scissile carbonyl carbon of the substrate with subsequent formation of an anhydride intermediate, or the attack of a water molecule at the scissile carbonyl promoted by glutamate-270 and/or Zn^{2+}.

Structural investigations of CPA have been aided in part by x-ray crystallographic studies on the native enzyme (10–12) and its complexes with different inhibitors (12–16). When considering the mechanistic implications of the three-dimensional structure of enzyme–inhibitor complexes, it is important to note the distinction between the use of substrate analogues, products or product analogues, and transition-state analogues as inhibitors. Each of these classes of inhibitors can provide valuable information about the association of substrate with enzyme. However, if one supposes a correlation between inhibitor affinity and actual transition-state resemblance, as presumed from considerations of enzyme–transition-state complementarity (17, 18) and the "induced fit" hypothesis (19), one can conclude that the tightly bound transition-state analogue supplies the most relevant crystallographic information about possible catalytic conformations of the enzyme. For quite some time, transition-state analogues have been presumed to be effective enzyme inhibitors (18, 20), and they have received considerable review (21–25).

Compounds containing the aldehyde component often serve as very effective inhibitors of proteolytic enzymes such as elastase (26), papain (27–29), chymotrypsin (30), amidase (31), asparaginase (32), leucine aminopeptidase (33, 34), and the serine protease SGPA from *Streptomyces griseus* (35). The enhanced electrophilicity at the carbonyl carbon of the aldehyde invites a fourth saturating ligand, such as a water molecule, exemplified by aldehyde–hydrate equilibria in aqueous solution. It is likely that inhibition of enzymes by aldehydes involves the addition of an enzyme-bound nucleophile or a water molecule to the aldehyde upon its binding to the enzyme. The resulting tetrahedral configuration at the formerly carbonyl carbon gives rise to a structure that could resemble tetrahedral intermediates encountered in either acyl enzyme or general base hydrolytic mechanisms. For example, an enzyme hemiacetal complex with an aldehyde inhibitor has been observed by x-ray diffraction methods in the serine protease SGPA (35). Galardy (36) has synthesized and characterized the kinetics of an aldehyde inhibitor of CPA, DL-2-benzyl-3-formylpropanoic acid (BFP; Fig. 1). It is a competitive inhibitor of the enzyme with an apparent K_i of 4.8×10^{-7} at pH 7.5. At neutral pH, it exists as an equilibrium mixture of 75% free aldehyde and 25% hydrated aldehyde in deuterium oxide. Because it is likely that only one species binds to the enzyme, and only one stereoisomer, presumably the L isomer, is preferentially bound to the enzyme, the dissociation constant of the actual species bound is significantly less than the observed K_i.

MATERIALS AND METHODS

CPA [CPA_α; Cox *et al.* (37)] was purchased from Sigma and used without further purification. BFP was the generous gift of R. E. Galardy. CPA was crystallized in space group $P2_1$ (a = 51.60 Å, b = 60.27 Å, c = 47.25 Å, β = 97.27°, crystal habit elongated along the a axis) by dialysis of the enzyme (solubilized in 1.2 M LiCl/0.02 M Tris·HCl, pH 7.4) against 0.2 M LiCl/0.02 M Tris·HCl, pH 7.4, at 4°C (38). CPA crystals were crosslinked with 0.1% (vol/vol) glutaraldehyde (39) for 6 hr after transfer to 0.1 M LiCl/0.02 M Veronal LiOH, pH = 7.5. Crystals of the CPA–BFP complex were prepared by soaking in 0.001 M BFP/0.1 M LiCl/0.02 M Veronal LiOH, pH 7.5, for 5 days. Interestingly, the crystals were severely disordered when soaked under similar conditions in Tris buffer. It must be stressed that the type of CPA

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Abbreviations: CPA, bovine pancreatic carboxypeptidase A_α; BFP, 2-benzyl-3-formylpropanoic acid.

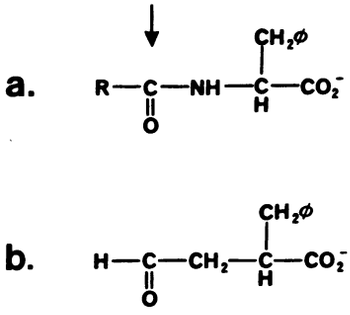

FIG. 1. (*a*) Typical COOH terminus portion (with terminal phenylananine) of a peptide CPA substrate (R = remainder of peptide). Arrow indicates the scissile amide linkage. (*b*) The CPA aldehyde inhibitor 2-benzyl-3-formylpropanoic acid.

used in this crystallographic work, prepared by the method of Cox, exhibits one-third the activity of the enzyme in solution (40). These crystals are very different from those obtained with CPA prepared by the method of Anson (41, 42) (space group $P2_1$; a = 50.9 Å, b = 57.9 Å, c = 45.0 Å, β = 94.7°, crystal habit elongated along the b axis); these crystals of CPA exhibit only 1/300th the activity of the enzyme in solution (43). The less-active crystalline enzyme has often been used for biochemical studies [recently reviewed by Vallee and co-workers (7, 8)]. It is important to make the distinction quite clearly between these two forms when correlating the results of studies in solution with x-ray crystallographic results.

Crystals of the CPA–BFP complex were mounted and sealed in glass capillaries with a small amount of mother liquor. A 1.70-Å resolution data set ($3.2° < 2\theta < 54.0°$) was collected from three of these crystals on a Syntex $P2_1$ four-circle automated diffractometer at ambient temperature, using Ni-filtered CuK_α radiation ($\langle\lambda\rangle$ = 1.54178 Å). These crystals were isomorphous with crystals of the native enzyme and exhibited similar unit cell parameters. These parameters were refined prior to data collection by a least-squares procedure utilizing the setting angles of 24 centered reflections (12 Friedel pairs) in the 2.8- to 3.0-Å range. Data were collected in consecutive spherical shells in the asymmetric unit of reciprocal space to 1.70-Å resolution. Radiation damage to the crystals was evaluated by monitoring four check reflections, and data collection was terminated when the average decay of intensity among these four reflections exceeded 20%. Integrated intensities were estimated by using the Wyckoff step-scan (44) and were corrected for absorption by the method of North (45). Corrections were applied for Lorentz and polarization effects, and a linear correction for decay based on the four check reflections was also performed. Scaling among the five data sets obtained from the three crystals was achieved by allowing for overlap between consecutively collected shells of data. All data sets were scaled and merged. An R factor [$R = \Sigma|I_{hi} - \langle I_h\rangle|/\Sigma\langle I_h\rangle$ (I_{hi} = scaled intensity for reflection h in data set i; $\langle I_h\rangle$ = average intensity calculated for reflection h from replicate data)] of 0.072 was calculated for overlapping data. The final data set to 1.70-Å resolution consisted of 14,616 reflections with intensities exceeding twice the estimated standard deviation.

Structure factors obtained from the corrected intensity data were used to calculate difference electron-density maps using Fourier coefficients ($5|F_o| - 4|F_c|$) and ($|F_o| - |F_c|$) with phases calculated from the atomic coordinates of native CPA refined at 1.54-Å resolution (11), less six water molecules in the active site ($|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively). Given the usual partial occupancy of ligands binding to the active site of CPA, model building was facilitated by the use of electron-density maps calculated with Fourier coefficients ($5|F_o| - 4|F_c|$). These maps are roughly equivalent to the traditional ($2|F_o| - |F_c|$) map, which would be more appropriate were the complex fully occupied. Fast Fourier transform routines (46, 47) were used for all electron-density map and structure factor calculations, and the coordinates of the CPA–BFP complex were adjusted by inspection of the difference electron-density maps. Building of the inhibitor into these maps was performed on an Evans and Sutherland PS300 interfaced to a VAX 11/780. The graphics software was developed by Jones (48) as modified by Pflugrath and Saper (FRODO). The approximate coordinates were then refined by the reciprocal space least-squares method using the stereochemically restrained least-squares algorithm of Hendrickson and Konnert (49). Structure factor derivatives were calculated by using the difference map algorithm of Jack (50). The initial model contained 2437 nonhydrogen atoms of the enzyme, 1 zinc atom, 274 water molecules, and 15 nonhydrogen atoms of the inhibitor. An initial difference electron density map showed that the gem-diol, not the free aldehyde, was bound to the active site. Residue conformations were examined during the course of the refinement by using maps computed with Fourier coefficients as outlined above and calculated phases; only minimal adjustments of atomic positions were necessary. Refinement converged smoothly to a final crystallographic R factor ($R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$) of 0.166 at 1.70-Å resolution. A difference electron-density map calculated with Fourier coefficients ($|F_o| - |F_c|$) and phases calculated from the final model coordinates showed rms residual electron density of 0.05 e/Å^3. The highest peaks in the vicinity of the active site were just under 0.20 e/Å^3—these were principally due to some conformational disorder of the benzyl group of BFP in the hydrophobic pocket of CPA. A rms error in atomic positions of ≈0.2 Å was estimated based on relationships derived by Luzzati (51).

RESULTS AND DISCUSSION

Only one molecule of BFP is observed to bind to the active site of CPA, and it is clearly the L isomer that is bound. A schematic diagram is presented in Fig. 2, and relevant distances are recorded in Table 1. Electron density maps superimposed on the model coordinates are presented in Figs. 3 and 4. Some of the binding interactions observed here are reminiscent of those in other CPA–inhibitor complexes (12–16); the benzyl group of BFP resides in the hydrophobic

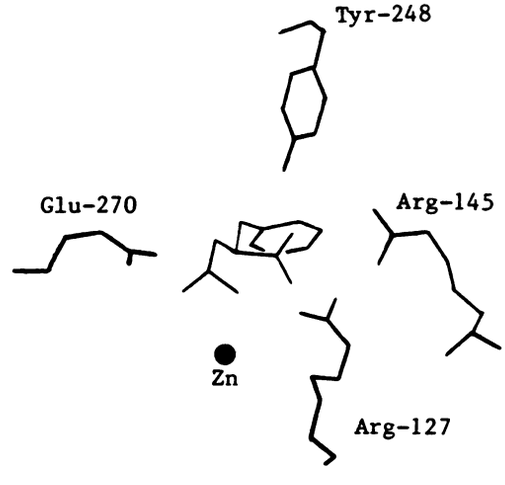

FIG. 2. BFP bound to the active site of CPA. Enzyme residues, in clockwise direction starting with Zn, are Glu-270, Tyr-248, Arg-145, and Arg-127.

Table 1. Selected enzyme–inhibitor distances

Protein	Inhibitor	Distance, Å
Glu-270 Oε1	Hydrate O1	2.4*
Glu-270 Oε2	Hydrate O1	3.2*
Ser-197 carbonyl O	Hydrate O1	3.2*
Tyr-248 phenolic O	Carboxylate O1	2.5*
Arg-127 N1	Carboxylate O1	3.4*
Arg-145 N1	Carboxylate O1	3.1*
Arg-145 N2	Carboxylate O2	3.3*
Zn	Hydrate O1	2.7
Zn	Hydrate O2	2.5

*Possible hydrogen bond.

pocket, and the terminal carboxylate is involved in a salt link with the guanidinium moiety of arginine-145 on the enzyme. The phenolic residue of tyrosine-248 is in the "down" conformation, and the phenolic oxygen is 2.5 Å away from one of the carboxylate oxygens of BFP. This interaction clearly favors the un-ionized state of tyrosine-248.

The coordination polyhedron of the zinc ion, however, is quite different from that of the native enzyme. The zinc ion itself has moved ≈0.4 Å upon the binding of the hydrated BFP inhibitor. The histidine residues serving as zinc ligands (histidine-69, histidine-196) show very slight conformational changes, and all zinc–nitrogen distances remain essentially the same. However, the carboxylate group of glutamate-72, which was bound in bidentate fashion to the zinc ion in the native enzyme, is now nearly singly coordinated to the zinc (within experimental error); zinc–oxygen distances for glutamate-72 are 1.8 and 2.9 Å. This change in coordination is primarily due to the large movement of the zinc ion upon the binding of the inhibitor, although some small conformational changes of glutamate-72 are observed. In addition, the two gem-diol oxygens of the hydrated aldehyde straddle the zinc (again, within experimental error) to bring the coordination number of the metal ion to five. One hydrate oxygen is 2.5 Å from the zinc; the other is 2.7 Å from the zinc in addition to being hydrogen-bonded to glutamate-270 (2.4 and 3.2 Å from Oε1 and Oε2, respectively) and the carbonyl oxygen of serine-197 (3.2 Å; the C=O . . . . O angle is 121°).

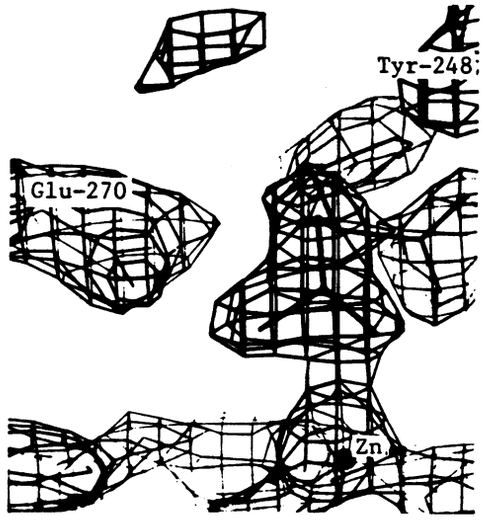

FIG. 3. Portion of a $5|F_o| - 4|F_c|$ difference electron-density map (the viewer is looking into the hydrophobic pocket of CPA). Structure factors were calculated from the native enzyme refined at 1.54-Å resolution omitting active-site water molecules. The refined atomic coordinates are superimposed on the map; Glu-270, Tyr-248, and the Zn ion are indicated. Note the coordination of the gem-diol moiety of BFP to the Zn ion.

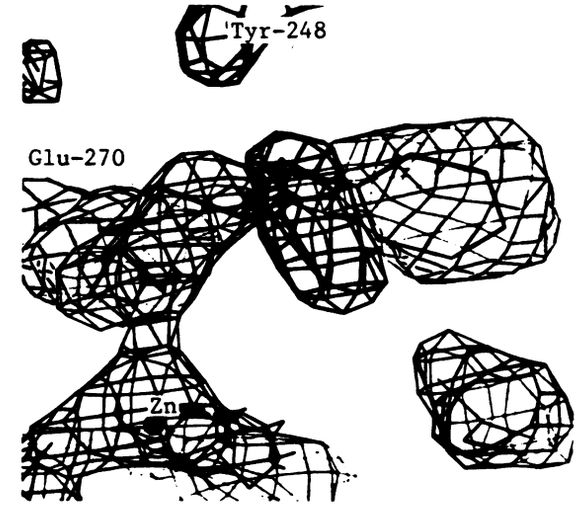

FIG. 4. Same details as Fig. 3, but viewed at a different angle.

Interestingly, serine-197 is in one of the three *cis* peptide bonds found in CPA. It is tempting to identify tentatively one hydrate oxygen as the former zinc-bound water of the native enzyme (10–12). However, it remains to be seen whether the enzyme actually catalyzes the hydration of initially bound free aldehyde (R. E. Galardy, personal communication). In the native enzyme at pH 7.5 refined at 1.54 Å resolution (11), the zinc-bound water was refined at two mutually exclusive sites 1.4 Å apart. One site (water-571) is 2.0 Å from the zinc and is hydrogen-bonded to the γ-carboxylate of glutamate-270; the other site (water-567) is 3.2 Å from the zinc and is hydrogen-bonded to both glutamate-270 and the carbonyl group of serine-197. Perhaps the disordered nature of the zinc-bound water in the native enzyme reflects the possibility of such bi- or trifurcated acceptor hydrogen bond stabilization of an actual reaction intermediate. The structure of the complex resembles what one might expect for the tetrahedral adduct encountered in a general base hydrolytic pathway (Fig. 5).

The structure of the CPA–BFP complex suggests a further role for the positively charged guanidinium group of arginine-127. One of the nitrogens of this group is 3.4 Å away from the terminal carboxylate of BFP, and the other is 3.6 Å away from one of the hydrate oxygens of the aldehyde. Given the possible role of arginine residues of CPA in precatalytic

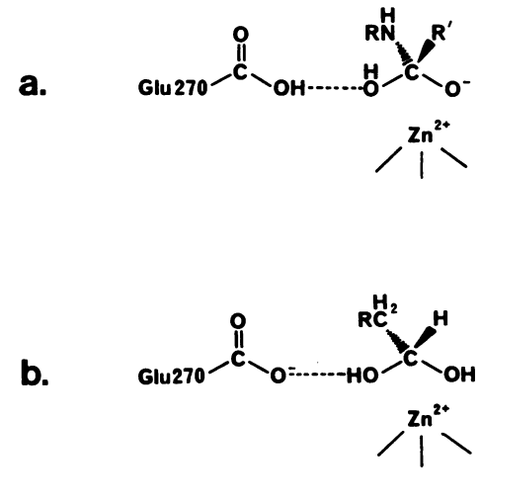

FIG. 5. (*a*) Postulated tetrahedral intermediate of a general base hydrolytic mechanism. (*b*) Schematic diagram of the CPA–BFP interaction; note the resemblance to *a*.

binding interactions with the terminal carboxylate of substrates (52, 53), a vestige of this role for arginine-127 is possibly seen in the proximity of its guanidinium group to the terminal carboxylate of BFP (a hydrogen bond within experimental error). In addition, arginine-127 may also provide a stabilizing hydrogen bond to a tetrahedral intermediate encountered upon nucleophilic attack at a substrate carbonyl. The distance of arginine-127 from the zinc-bound hydroxyl of the gem-diol is rather far to be an actual hydrogen bond. However, the coordination polyhedron of the zinc ion bears a net positive charge in the CPA–BFP complex. For that reason, a close approach of the positively charged guanidinium group of arginine-127 to the positively charged zinc environment would be electrostatically unfavorable. On the other hand, when nucleophilic attack upon an actual peptide or ester substrate occurs, a negative charge develops on the former carbonyl oxygen, thereby rendering the coordination polyhedron, including the zinc ion, electrically neutral. Under these electrostatically neutral conditions, the approach of arginine-127 would favor a hydrogen bond with the tetrahedral intermediate. Thus, an attractive role for arginine-127 would be the transient hydrogen bonding to the developing negative charge on the formerly carbonyl oxygen of the substrate. This role could be the same regardless of whether the mechanism is general base or anhydride.

For quite some time, tyrosine-248 has been postulated as a possible proton donor to the leaving amine or alcohol after the collapse of the tetrahedral intermediate, in view of the large conformational change it undergoes upon the binding of inhibitors to the active site of CPA. In two chemical studies in particular (54, 55), tyrosine-248 has been modified by iodination and diazotization and directly identified. These modified enzymes displayed virtually no peptidase activity, yet dramatically increased esterase activity. These differences have since been attributed to the greater stability of RO^- versus RNH^- as the leaving group after collapse of the tetrahedral intermediate in the absence of proton donation. However, a recent investigation of the substitution of tyrosine-248 by phenylalanine through site-specific mutagenesis (56) of a cDNA clone encoding rat CPA (57) and subsequent expression in yeast has yielded a mutant CPA (78% homologous with the bovine enzyme) that displays both peptidase and esterase activity (58). Hence, an alternative proton donor is required. The proton presumably abstracted by glutamate-270 in the general base mechanism may undergo subsequent donation to the leaving group in the collapse of the tetrahedral intermediate; this proposal was considered by Rees and Lipscomb (16), and recently by Monzingo and Matthews (59) in light of the binding of a novel inhibitor to the active site of the related zinc endopeptidase thermolysin. The distances of 3.4 and 3.2 Å from Oε1 and Oε2, respectively, of the carboxylate of glutamate-270 to the methylene carbon of the BFP molecule make the role of glutamate-270 as proton donor an attractive possibility. The other alternative proton donor is water, possibly activated by its proximity to glutamate-270, the zinc ion, or both.

It remains to be seen whether the enzyme actually catalyzes the direct attack of water at the aldehyde carbonyl. This possibility raises the issue of the direct attack of water on other carbonyl moieties, including the scissile ones of peptides and esters. The anhydride hydrolytic mechanism cannot be ruled out in view of the isolation of an intermediate at low temperature in the case of the ester substrate *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate (60, 61), presumed to be the covalently bound acyl–enzyme complex. A low-temperature crystallographic investigation of this complex may help identify the nature of the spectroscopically observed intermediate.

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