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Biochemistry

Mechanism of carboxypeptidase A: Hydration of a ketonic substrate analogue

(protein crystallography/zinc protease/enzyme–inhibitor complex)

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ABSTRACT **The structure of the complex between carboxypeptidase A_α (EC 3.4.17.1) and the ketonic substrate analogue 5-benzamido-2-benzyl-4-oxopentanoic acid (BOP) has been determined by x-ray crystallographic methods to a resolution of 1.7 Å (final $R = 0.191$). Interestingly, BOP was observed to bind to the active site of carboxypeptidase A_α as the covalent hydrate adduct. Because BOP is probably less than 0.2% hydrated in aqueous solution, this result was unexpected. One possibility is that the zinc-bound water of the native enzyme added to the ketone carbonyl. Alternatively, the enzyme may preferentially scavenge the hydrated ketone as it is continuously maintained at equilibrium in the solution in which the carboxypeptidase A_α crystals were immersed. In either case, this mode of binding of BOP to carboxypeptidase A_α provides an example of the preferred binding of a model of a structure along the reaction coordinate of a hydrolytic reaction.**

The binding of substrates (1), substrate analogues (2), or transition-state analogues (3–6) to the active site of the zinc metalloprotease carboxypeptidase A_α (CPA) has been examined recently by high-resolution x-ray crystallographic methods. The several binding modes observed in these complexes have provided structural insight into possible catalytic conformations that could occur during hydration or hydrolysis reactions. In particular, some carbonyl gem-diol adducts may be bound to CPA as transition-state analogues (3, 5). In these examples, the hydrate species predominates in aqueous solution because of an unusually electrophilic carbonyl carbon, so that the enzyme could have selectively bound the preformed hydrate instead of the free carbonyl species. However, the ketone (−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid (MBP) binds to CPA with an intact carbonyl and not as the gem-diol adduct (2); moreover, its ketone oxygen binds to Arg-127, while the zinc ion retains a bound water molecule. In this study, we have determined the structure of the complex between CPA and the ketone 5-benzamido-2-benzyl-4-oxopentanoic acid (7) (BOP, an analogue of the substrate benzoyl-glycyl-L-phenylalanine; Fig. 1). We find that BOP is bound to CPA as the gem-diol hydrate adduct and is not observed in the ketone form. This was an unexpected result, since both MBP and BOP are present as the ketonic form in aqueous solution to >99%. Nevertheless, they each display markedly different behavior when bound to the active site of CPA. Moreover, the CPA–BOP complex provides an excellent example of an enzyme favoring the binding of a structure along the reaction coordinate of the enzymic reaction.

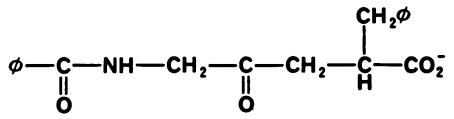

FIG. 1. The CPA inhibitor BOP is depicted here as the ketone. This inhibitor binds to the active site of CPA as the gem-diol species.

MATERIALS AND METHODS

Crystals of CPA were prepared and crosslinked as described (3) and then soaked in a buffer solution (0.2 M LiCl/0.02 M Veronal, adjusted with LiOH to pH 7.4) containing 10 mM BOP for 11 days. Crystals of typical dimensions 0.3 × 0.3 × 0.9 mm were mounted and sealed in glass capillaries along with a portion of mother liquor. A 1.7-Å resolution data set ($3.2° < 2\theta < 54.0°$) was collected from two of these crystals on a Syntex $P2_1$ four-circle automated diffractometer (Nicolet Instruments, Madison, WI). These crystals were isomorphous with crystals of the native enzyme and exhibited similar unit cell parameters ($a = 51.6$ Å, $b = 60.27$ Å, $c = 47.25$ Å, $\beta = 97.27°$; crystal habit elongated along the a axis). These parameters were refined prior to data collection by a least-squares routine 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 1.7-Å resolution. Radiation damage to the crystals was evaluated by monitoring four check reflections, and data collection was terminated when the average decay of these four reflections exceeded 15%. The intensity data were corrected for Lorentz and polarization effects, and a linear correction for decay based on the four check reflections was applied. Structure factors obtained from the corrected intensity data were used to calculate difference electron density maps as described (3). Model building was performed on an Evans and Sutherland PS300 picture system interfaced with a VAX 11/780 computer using graphics software developed by Jones (8) (FRODO). The model was refined against the data by using the stereochemically restrained least-squares algorithm of Hendrickson and Konnert (9). The crystallographic R factor ($R = \Sigma \, | \, |F_o| - |F_c| \, | / \Sigma \, |F_o|$, where F_o and F_c are observed and calculated structure-factor amplitudes, respectively) for the final model was 0.191 at 1.7-Å resolution.

RESULTS AND DISCUSSION

BOP makes the usual contacts in the S_1' subsite described previously for other enzyme–inhibitor complexes (1–6). The unexpected feature in the S_1 subsite is the gem-diol moiety of the inhibitor above the zinc ion (see Fig. 2). Zinc–oxygen distances are 2.9 and 2.5 Å for the two gem-diol oxygens, O-1

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Abbreviations: CPA, bovine carboxypeptidase A_α; BOP, 5-benzamido-2-benzyl-4-oxopentanoic acid; MBP, (−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid.

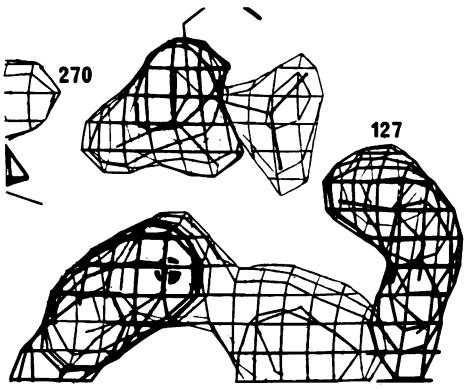

FIG. 2. Difference electron density map calculated with Fourier coefficients 5 | F_o | − 4 | F_c | and phases calculated from the final model less the atoms of BOP, as well as enzyme residues serving as zinc ligands (Glu-72, His-69, His-196) and Arg-127. The viewer is looking into the hydrophobic pocket of the enzyme; refined atomic coordinates are superimposed on the map, while Glu-270 and Arg-127 are indicated by their sequence numbers. The zinc ion appears as a small black sphere in the lower center of the figure. Note the tetrahedral gem-diol moiety of BOP in the region above the zinc ion. Because of disorder of the benzamido portion of the inhibitor, the electron density as contoured does not outline this portion of the inhibitor.

and O-2, respectively; O-1 is 2.6 Å from the carboxylate of Glu-270 and 3.2 Å from the backbone carbonyl oxygen of Ser-197, and O-2 is 3.2 Å from the guanidinium moiety of Arg-127. Both Glu-270 and Arg-127 have moved from their positions in the native enzyme to accommodate the binding of the inhibitor. The benzamido NH group of BOP in the S_1 subsite is disordered in the electron density maps and hence was not included in structure factor calculations during refinement. Although this nitrogen is not clear in electron density maps, its hydrogen bond with Tyr-248 was expected in the light of the interaction of this residue with the amide NH of the P_1 residue of the 39-amino acid inhibitor from the potato, designated PCI (10). In the current study, because of disorder of this section of the BOP molecule, we have no direct evidence for this hydrogen bond.

Hydration Phenomena. Given the negligible amount of hydrated BOP existing in aqueous solution,* the enzyme either has selected the gem-diol as it is continuously formed in solution or has facilitated the hydration reaction. The ability of CPA to facilitate similar hydration reactions is plausible in the light of the ability of carbonic anhydrase, a related zinc enzyme, to catalyze the hydration of certain ketones and aldehydes (13). For CPA, this ability would have a direct bearing on the catalytic mechanism: the three-dimensional structure of the CPA–BOP complex could provide direct chemical evidence for the product of a zinc- and/or Glu-270-promoted attack of water directly at a carbonyl moiety. We then suggest a mechanistic extension to scissile carbonyl moieties of actual substrates. For BOP, the resultant tetrahedral intermediate could proceed no further in the forward direction; however, for an actual substrate, the forward reaction would continue toward the collapse of the intermediate to form products. If a promoted-water mechanism is favored for substrate hydrolysis, then the CPA–BOP complex and the CPA–inhibitor complexes studied previously (3–5) provide a model for the tetrahedral intermediate and the transition states flanking it, if the Hammond Postulate applies (14). The results of these recent x-ray crystallographic studies can be classified according to the orientation of the tetrahedron with regard to the zinc ion, Glu-270, and Arg-127. The distances of these particular interactions for four enzyme–inhibitor complexes are summarized in Table 1. Based upon these four enzyme–inhibitor complexes, there is a considerable variation of possible contacts between an actual tetrahedral intermediate and the enzyme. Furthermore, both enzyme residues and the zinc ion can move somewhat from their positions in the native enzyme to accommodate specific binding modes.

It is intriguing that BOP binds to CPA as the hydrate species, whereas the ketonic substrate analogue MBP binds without forming a tetrahedral adduct with the zinc-bound water molecule (4). There are several steric factors that distinguish the two ketones in their hydrate forms. The *p*-methoxybenzoyl group of MBP has a bulky branching carbon next to the carbonyl, making the carbonyl less accessible to a nucleophile. Furthermore, if the hydrate of MBP is superimposed on the coordinates of BOP in the S_1 subsite on the enzyme, unfavorable interactions occur primarily between the *p*-methoxybenzoyl group and enzyme residue Phe-279. Such steric considerations might explain why the substrate, benzoyl-L-phenylalanine, which is structurally related to MBP, is a poor hydrolytic substrate (7, 15). The free energy of binding of MBP in the CPA–MBP complex might not be sufficient to overcome enough of the structural barriers confronting a hydration pathway. Conversely, the free energy of binding of BOP in the CPA–BOP complex might exceed that of the hydration reaction; the enzyme–inhibitor complex can draw upon this energy to achieve the hydration reaction. If this is the case, however, the apparent inhibition constant (K_i) measured in solution would be greater than the actual dissociation constant (K_d) of the potent inhibiting species (the tetrahedral hydrate)—the smaller the propensity toward hydration, the higher the observed K_i. Hence, the ideal K_d for hydrated BOP is probably much less than the K_i of 4.8×10^{-5} M observed in solution (7).

Mechanistic Considerations. A reasonable promoted-water mechanism might involve the initial hydrogen bonding of a peptide substrate carbonyl to Arg-127. The positively charged guanidinium moiety of this residue (and perhaps the zinc ion) could polarize the scissile carbonyl, making it more susceptible to attack by a promoted water molecule. As the tetrahedral intermediate is formed, or immediately subsequent to its formation, the developing oxyanion could shift

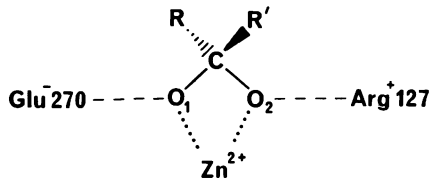

Table 1. Orientations of a possible tetrahedral intermediate

Interaction	Distance ± 0.2 Å			
	CPA–ZGP'	CPA–BFP	CPA–TFP	CPA–BOP
Glu-270–O-1	3.4	2.4	2.6	2.6
O-1–Zn	2.2	2.7	3.4	2.9
O-2–Zn	3.3	2.5	2.6	2.5
Arg-127–O-2	2.7	3.6	3.2	3.2

Observed orientations of tetrahedral inhibitors as represented by distances from enzyme residues Glu-270, Arg-127, and the zinc ion. ZGP' is the hydrolyzed phosphonamidate inhibitor (4), BFP is the hydrated aldehyde inhibitor (3), TFP is the hydrated fluoroketone inhibitor (5), and BOP is the hydrated ketone of the current study.

*The degree of hydration of a related ketonic substrate analogue was reported as <2%—i.e., not detectable within experimental error (11). A more precise study has shown that the simple ketone acetone is 0.2% hydrated in aqueous solution (12). Because of steric effects in the crowded ketonic center of BOP, one might expect it to be hydrated to an even lesser degree as compared with acetone.

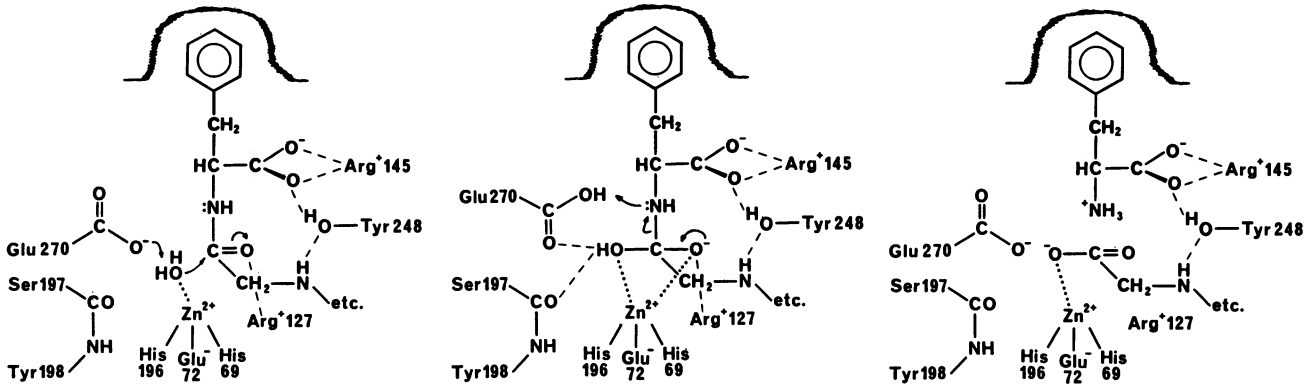

FIG. 3. (*Left*) A typical peptide substrate, with terminal phenylalanine, is shown bound to the active site of CPA. The P_1' phenyl group resides in the hydrophobic pocket, or "specificity pocket," of the enzyme, and both Arg-145 and Tyr-248 provide hydrogen bonds to the terminal carboxylate of the substrate. Tyr-248 also accepts a hydrogen bond from the amide NH of the penultimate peptide bond and thus might provide specificity toward substrates possessing such a penultimate peptide bond. Note the cis-peptide bond between Ser-197 and Tyr-198. Importantly, the scissile carbonyl is polarized by Arg-127 and perhaps only partly, if at all, by the zinc ion. A water molecule, bound to zinc in the native enzyme, is promoted by zinc and Glu-270 for nucleophilic attack at the polarized carbonyl carbon of the substrate. (*Center*) The tetrahedral intermediate resulting from the previous step is illustrated. Subsequent to or concurrent with its formation, the developing oxyanion moves to zinc for greater electrostatic stabilization. However, its contact with Arg-127 might be retained for additional stabilization through a hydrogen bond. The hydroxyl of the tetrahedral intermediate can retain some coordination to zinc, although the negatively charged oxyanion might be drawn closer to the positively charged zinc ion. Additionally, this hydroxyl can donate a bifurcated hydrogen bond to the backbone carbonyl oxygen of Ser-197 as well as the carbonyl of the now-protonated Glu-270. If the tetrahedral intermediate has its two geminal oxygens coordinated to zinc, the postulated mechanism would involve a pentacoordinate zinc ion as a metastable intermediate. This intermediate can collapse to form products, with proton donation by Glu-270 to the leaving amino group as depicted. (*Right*) Hydrolysis products are shown just after the final step, with an intervening proton transfer between the product carboxylate and ammonium group (this proton transfer could be mediated by Glu-270). The product carboxylate can have one oxygen on the zinc ion, while the other can be hydrogen bonded to Tyr-248. Although unobserved as yet, Arg-127 may be involved in a stage of binding the product carboxylate.

from Arg-127 toward the zinc ion. This shift would be a clockwise "rotation" of the tetrahedral center, which is depicted in Table 1. The oxyanion might retain its contact with Arg-127 for additional stabilization; however, the positively charged zinc ion would probably favor an intimate coordination interaction with the negative oxyanion. A proton transfer from the former zinc-bound water to Glu-270 would be facilitated by this clockwise "rotation." Hence, the nucleophilic promotion of the water molecule is a task that could be shared between the zinc ion and Glu-270. The hydroxyl of the tetrahedral intermediate might retain a hydrogen bond with the carboxylic acid carbonyl of protonated Glu-270. In addition to a hydrogen bond with the backbone carbonyl of Ser-197, this hydroxyl could also be coordinated to the zinc ion. Thus, a tetrahedral intermediate (i.e., its two geminal oxygens) could be stabilized through a pentacoordinate zinc ion if the three enzyme residues Glu-72, His-69, and His-196 were counted as single ligands. The collapse of the tetrahedral intermediate would involve a proton transfer from Glu-270 to the leaving amino group. Residue Tyr-248, once thought to be the proton donor in this step, cannot serve this role based on the results of recent site-directed mutagenesis studies (16). A phenylalanine residue was substituted at position 248 in order to make a mutant CPA; this mutant displayed nearly normal peptidase and esterase activity. Therefore, the function of Tyr-248 is not catalysis, but it may add specificity for substrates that have a penultimate peptide bond (i.e., tripeptides or larger), and it may contribute to the hydrophobic nature of the active site of the complex. The NH of the penultimate peptide bond could donate a hydrogen bond to Tyr-248, and the carbonyl oxygen of this penultimate peptide bond could receive a hydrogen bond from Arg-71. Both of these interactions are observed in the binding of product in the CPA–PCI complex (10). Thus, both Tyr-248 and Arg-71 may contribute to the enzyme's specificity toward substrates possessing a penultimate peptide bond. The mechanistic steps and binding interactions described for the promoted-water mechanism are illustrated in Fig. 3.

The results of recent x-ray structural studies of CPA largely imply, but do not prove, that the enzyme favors the promoted-water hydrolytic mechanism (2–6) (Fig. 3). However, chemical data from other laboratories suggest that some ester substrates might proceed through a hydrolytic mechanism involving a mixed anhydride intermediate between the substrate and enzyme residue Glu-270 (17–19). The observation made in some of these low-temperature studies—that an intermediate accumulates that has two nonprotein metal ligands—is consistent with the tetrahedral intermediate of the promoted-water pathway in which the two geminal oxygens are stabilized by the zinc ion. Alternatively, if one or more of these particular ester substrates do indeed follow the anhydride pathway, they may be "forced" to do so because of steric reasons. The bulky chromophore in conjugation with the scissile carbonyls of these substrates may prevent the substrate from achieving the proper transition state structure for the promoted attack of water. The CPA–MBP complex may demonstrate this effect: *p*-methoxybenzoyl group of MBP probably prevents it from binding to CPA as the hydrate, whereas the better substrate analogue BOP can bind to CPA as the hydrate. Perhaps, then, the anhydride pathway may be favored over the promoted-water pathway for those particular substrates that have certain steric limitations. Further x-ray crystallographic, site-directed mutagenetic, and spectroscopic studies may help to resolve these mechanistic queries.

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1. Christianson, D. W. & Lipscomb, W. N. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7568–7572.
2. Christianson, D. W., Kuo, L. C. & Lipscomb, W. N. (1985) *J. Am. Chem. Soc.* **107**, 8281–8283.
3. Christianson, D. W. & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6840–6844.

4. Christianson, D. W. & Lipscomb, W. N. (1986) *J. Am. Chem. Soc.* **108,** 545–546.
5. Christianson, D. W. & Lipscomb, W. N. (1986) *J. Am. Chem. Soc.* **108,** 4998–5003.
6. Christianson, D. W. & Lipscomb, W. N. (1986) in *Zinc Enzymes*, eds. Bertini, I., Luchinat, C., Maret, W. & Zeppezauer, M. (Birkhauser, Boston), pp. 121–132.
7. Grobelny, D., Goli, U. B. & Galardy, R. E. (1985) *Biochemistry* **24,** 7612–7617.
8. Jones, T. A. (1982) in *Computational Crystallography*, ed. Sayre, D. (Clarendon, Oxford), pp. 303–317.
9. Hendrickson, W. A. & Konnert, J. (1981) in *Biomolecular Structure, Function, and Evolution*, ed. Srinivasan, R. (Pergamon, London), pp. 43–47.
10. Rees, D. C. & Lipscomb, W. N. (1982) *J. Mol. Biol.* **160,** 475–498.
11. Galardy, R. E. & Kortylewicz, Z. P. (1985) *Biochemistry* **23,** 2083–2087.
12. Lewis, C. A. & Wolfenden, R. (1977) *Biochemistry* **16,** 4886–4890.
13. Pocker, Y. & Sarkanen, S. (1978) *Adv. Enzymol.* **47,** 149–274.
14. Hammond, G. S. (1955) *J. Am. Chem. Soc.* **77,** 334–338.
15. Hofmann, K. & Bergmann, M. (1940) *J. Biol. Chem.* **132,** 225–235.
16. Gardell, S. J., Craik, C. S., Hilvert, D., Urdea, M. S. & Rutter, W. J. (1985) *Nature (London)* **317,** 551–555.
17. Makinen, M. W., Kuo, L. C., Dymowski, J. J. & Jaffer, S. (1979) *J. Biol. Chem.* **254,** 356–366.
18. Kuo, L. C. & Makinen, M. W. (1982) *J. Biol. Chem.* **257,** 24–27.
19. Suh, J., Cho, W. & Chung, S. (1985) *J. Am. Chem. Soc.* **107,** 4530–4535.