a condensed thiophen ring is involved.

Stereoelectronic effects might also be responsible for the lower activation energy for the rearrangement of **7a**, as the cleavage of the C_2–Si bond can be assisted by the interaction with the singly occupied $2p_z$ orbital on C_3. The radical geometry as inferred from the EPR parameters is such to maximize this interaction.

Acknowledgment. Financial support from "Progetto Finalizzato Chimica Fine e Secondaria" del CNR is gratefully acknowledged. We express our gratitude to Prof. E. Ciuffarin for discussions and helpful suggestions and to G. Bragaglia for his skillful technical assistance.

Appendix

The rates of decay of the unrearranged species, $-d[A^\bullet]/dt$, $-d[A_2]/dt$, and $-d[AB]/dt$ can be expressed as indicated in the following equations:

$$-d[A^\bullet]/dt = k[A^\bullet] + 2k_1[A^\bullet]^2 - 2k_{-1}[A_2] + 2k_1[A^\bullet][B^\bullet] - k_{-1}[AB] \quad (7)$$

$$-d[A_2]/dt = -k_1[A^\bullet]^2 + k_{-1}[A_2] + 2k'[A_2] \quad (8)$$

$$-d[AB]/dt = -2k_1[A^\bullet][B^\bullet] + k_{-1}[AB] + k'[AB] - 2k'[A_2] \quad (9)$$

If we now define $[A_T]$ as the total concentration of the unrearranged A, either as radical or dimer, i.e.,

$$[A_T] = [A^\bullet] + 2[A_2] + [AB] \quad (10)$$

the rate of disappearance of A_T is

$$-d[A_T]/dt = -d[A^\bullet]/dt - 2\,d[A_2]/dt - d[AB]/dt \quad (11)$$

or, by substituting eq 7–9 in eq 11,

$$-d[A_T]/dt = k[A^\bullet] + k'(2[A_2] + [AB]) \quad (12)$$

Condition ii implies that

$$K_{eq} = [A_2]/[A^\bullet]^2 = [B_2]/[B^\bullet]^2 = [AB]/(2[A^\bullet][B^\bullet]) \quad (13)$$

If we now further assume that the reaching of equilibrium is fast compared to the rearrangement of radical $[A^\bullet]$, i.e., $k_1[A^\bullet] >> k$, this seeming true below 370 K for **10a**, the last two terms of eq 11 can be expressed as

$$-2\,d[A_2]/dt = -2K_{eq}\,d[A^\bullet]^2/dt \quad (14)$$

$$-d[AB]/dt = 2K_{eq}\,d\{[A^\bullet]([A^\bullet]_0 - [A^\bullet])\}/dt = -2K_{eq}[A^\bullet]_0\,d[A^\bullet]/dt + 2K_{eq}\,d[A^\bullet]^2/dt \quad (15)$$

Equation 11 then becomes

$$-d[A_T]/dt = -(1 + 2K_{eq}[A^\bullet]_0)\,d[A^\bullet]/dt \quad (16)$$

Similarly, by substituting eq 13 into eq 12, we get

$$-d[A_T]/dt = (k + 2k'K_{eq}[A^\bullet]_0)[A^\bullet] \quad (17)$$

and by combining eq 16 and 17,

$$-d[A^\bullet]/dt = [A^\bullet](k + 2k'K_{eq}[A^\bullet]_0)/(1 + 2K_{eq}[A^\bullet]_0) \quad (18)$$

The decay of radical **10a** should then follow first-order kinetics, with

$$k_{obsd} = (k + 2\,k'K_{eq}[A^\bullet]_0)/(1 + 2K_{eq}[A^\bullet]_0) \quad (3)$$

independently of the relative values of k, k', and K_{eq}.

Registry No. **1**, 719-22-2; **2**, 530-55-2; **3**, 527-61-7; **4**, 60749-72-6; **5**, 4159-04-0; **6**, 56412-33-0; **7a** (MR_3 = $SiPh_3$), 102725-16-6; **7a** (MR_3 = $SiEt_3$), 102725-17-7; **7a** (MR_3 = $GePh_3$), 102725-18-8; **7b** (MR_3 = $SiPh_3$), 72975-18-9; **7b** (MR_3 = $SiEt_3$), 102725-19-9; **7b** (MR_3 = $GePh_3$), 102725-20-2; **8a** (MR_3 = $SiPh_3$), 102725-21-3; **8b** (MR_3 = $SiPh_3$), 87066-11-3; **9b** (MR_3 = $SiPh_3$), 73818-08-3; **10a** (MR_3 = $SiPh_3$), 102725-22-4; **10a** (MR_3 = $SiEt_3$), 102725-23-5; **10a** (MR_3 = $GePh_3$), 102725-24-6; **10b** (MR_3 = $SiPh_3$), 102725-25-7; **10b** (MR_3 = $SiEt_3$), 102725-26-8; **10b** (MR_3 = $GePh_3$), 102725-27-9; **11a** (MR_3 = $SiPh_3$), 102725-28-0; **11a** (MR_3 = $GePh_3$), 102725-29-1; **12a** (MR_3 = $SiPh_3$), 102725-30-4; **12a** (MR_3 = $GePh_3$), 102725-31-5; $Et_3Si^\bullet$, 24669-77-0; $Ph_3Si^\bullet$, 18602-99-8; $Ph_3Ge^\bullet$, 55321-79-4.

The Complex between Carboxypeptidase A and a Possible Transition-State Analogue: Mechanistic Inferences from High-Resolution X-ray Structures of Enzyme–Inhibitor Complexes

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Abstract: The mode of binding of the competitive inhibitor 2-benzyl-4-oxo-5,5,5-trifluoropentanoic 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 is the *gem*-diol moiety resulting from covalent hydration at the ketone carbonyl. The observed structure of this enzyme–inhibitor complex provides one possible model for a tetrahedral intermediate that would be encountered in a promoted water/hydroxide hydrolytic mechanism. Additionally, this structure yields the first direct interaction of Arg-127 with a zinc-bound oxygen of an inhibitor. General mechanistic and structural inferences are drawn based upon the structures of this and other recently determined enzyme–inhibitor complexes. An important realization is that the actual catalytic route of the enzyme may proceed through a sequence of orientations of the tetrahedral intermediate(s). New structural features are discussed, including the change in the zinc coordination polyhedron and the catalytic importance of Arg-127.

The zinc protease carboxypeptidase A (CPA) is no stranger to debate[1-4] about its particular mechanism(s) in the hydrolysis of peptide or ester substrates (promoted water/hydroxide or anhydride pathways). The X-ray diffraction studies implicate Glu-270, Arg-127, Arg-145, Tyr-248, Zn^{2+}, and the zinc-bound

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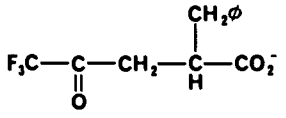

Figure 1. CPA inhibitor 2-benzyl-4-oxo-5,5,5-trifluoropentanoic acid.

water/hydroxide in binding and catalysis. Recently, high-resolution X-ray studies have been made on CPA–inhibitor complexes.[5-7] While these structures do not prove a particular mechanism, inferences from positions of residues and water molecules proximal to an inhibitor, or a reacting substrate, can serve as powerful complements to chemical studies of the enzyme in solution. In this study we examine the complex between CPA and the competitive inhibitor ($K_i = 2 \times 10^{-7}$ M) DL-2-benzyl-4-oxo-5,5,5-trifluoropentanoic acid (TFP; Figure 1)[8] and compare it with structures of other recently studied CPA–inhibitor complexes. The highly electrophilic nature of the carbonyl in TFP bearing an α-trifluoro moiety invites a fourth saturating ligand, such as a water molecule or an enzyme-bound nucleophile, to complete the valence of the carbon atom. This inhibitor is an analogue of the tetrahedral intermediate as it mimics the expected product of nucleophilic addition to the carbonyl moiety. If this tetrahedral intermediate is of high free energy, then, by Hammond's postulate,[9] it will structurally resemble the transition state that precedes it on the reaction coordinate. On the other hand, Albery and Knowles[10] have suggested that intermediates in enzyme-catalyzed reactions will have free energies close to that of the enzyme–substrate complex. In this case, the structure represented by the hydrated fluoro ketone will be somewhat less close to the transition states that flank it. Nevertheless, we expected that either (1) Glu-270 would add to the carbonyl carbon to give a covalently bound inhibitor complex or (2) a water molecule would add to the carbonyl carbon of the ketone, thus giving a hydrated ketone bound to the metal ion at the active site of the enzyme. Our results indicate that (2) is correct.

Experimental Section

CPA_α (Cox) was purchased from Sigma (Type I, crystalline) and used without further purification. The enzyme suspension was solubilized in 1.0 M LiCl, 0.02 M Tris-HCl (pH 7.4), millipored, and then dialyzed against 0.2 M LiCl, 0.02 M Tris-HCl (pH 7.4) at 4 °C. Within 2–4 days crystals appeared that had typical dimensions of 0.3 × 0.3 × 0.9 mm. Crystals were then lightly cross-linked[11] for 3 h in a buffer solution of 0.2 M LiCl, 0.02 M Veronal–LiOH (pH 7.4), containing 0.2% glutaraldehyde (v/v), in order to make the crystals more resistant to gross disorder upon soaking in the solution containing the inhibitor. However, no glutaraldehyde molecules were observed in subsequent electron density maps. Crystals were then soaked in a buffer solution [0.2 M LiCl, 0.02 M Veronal–LiOH (pH 7.4)] containing 0.5 mM TFP for 12 days. In order to prevent dehydration, these crystals were mounted and sealed in glass capillaries along with a portion of mother liquor. Integrated intensities were estimated by using the Wyckoff step scan[12] on a Syntex $P2_1$ automated four-circle diffractometer from three crystals in consecutive spherical shells of the asymmetric unit of reciprocal space to a limiting resolution of 1.7 Å. The intensities were then corrected for Lorentz and polarization effects and for absorption by the method of North.[13] After scaling and merging, an R factor based on intensities[14] yielded a value of 0.061 for replicate data. Electron density maps were calculated as described elsewhere.[5] Model building was performed on an Evans and Sutherland PS300 interfaced with a VAX 11/780 with graphics software developed by Jones[15] as modified by Pflugrath and Saper (FRODO). The enzyme–inhibitor model was refined by the reciprocal space least-squares method employing the stereochemically restrained least-squares algorithm of Hendrickson and Konnert.[16] The crystallographic R factor[17] for the final model is 0.172, and the highest peaks in the vicinity of the active site, as revealed in a final $|F_o| - |F_c|$ map with calculated phases, are under 4σ. An estimate of the rms error in atomic positions, no greater than 0.2 Å, is based on relationships derived by Luzzati.[18]

Table I. Distances of Selected Enzyme–Inhibitor Interactions[a]

protein	inhibitor	distance, Å
Glu-27 $O_\epsilon 1$	hydrate O1	2.6*
Glu-270 $O_\epsilon 2$	hydrate O1	2.9*
Ser-197 carbonyl O	hydrate O1	3.0*
Arg-127 N2	hydrate O2	3.2*
Tyr-248 phenolic O	carboxylate O1	2.8*
Arg-145 N1	carboxylate O1	3.3*
Arg-145 N2	carboxylate O2	3.3*
Zn	hydrate O1	3.3
Zn	hydrate O2	2.6

[a] An asterisk denotes a possible hydrogen bond, as judged from both distance and geometric criteria.

Results and Discussion

Only one molecule of TFP binds to the active site of CPA in these crystals: it is clearly the ketonic hydrate of the L isomer that is bound. Relevant distances of enzyme–inhibitor interactions are recorded in Table I, and an electron density map superimposed on model coordinates is presented in Figure 2. The benzyl group of TFP resides in the hydrophobic pocket (or "specificity pocket") of CPA, and the carboxylate of the inhibitor is engaged in a salt link with the guanidinium moiety of Arg-145. The phenolic residue of Tyr-248 is in the so-called "down" position, and the phenolic oxygen is 2.8 Å away from one of the carboxylate oxygens of TFP, an interaction that clearly favors the un-ionized form of Tyr-248. The hydrate moiety of TFP also makes several contacts with the enzyme. One hydrate oxygen is coordinated by a somewhat lengthy interaction (2.6 Å) with the zinc ion. This same oxygen is hydrogen bonded to one of the guanidinium nitrogens of Arg-127. The other hydrate oxygen is too far from the zinc to be considered an inner-sphere coordination interaction (3.3 Å), but it is hydrogen bonded to Glu-270 and it is within hydrogen-bonding distance to the carbonyl oxygen of Ser-197. Thus, this hydrate oxygen is possibly involved in a bifurcated donor hydrogen bond between these two residues (the alcoholic side chain of Ser-197 points away from the enzyme active site and makes no contact with the bound inhibitor). Although one may be tempted to assign tentatively one of the hydrate oxygens as the zinc-bound water/hydroxide molecule that has been displaced by TFP, we do not know whether the hydrated ketone or the free ketone, subsequently hydrated, binds to the enzyme. Nevertheless, the structure of the observed complex resembles that which might be expected for the tetrahedral adduct arising from the Glu-270- and/or zinc-promoted attack of water at the carbonyl of an actual substrate (Figure 3). This complex is similar to the complex of CPA with the aldehyde inhibitor 2-benzyl-3-formylpropanoic acid (BFP),[19] in which the hydrated aldehyde binds to the enzyme

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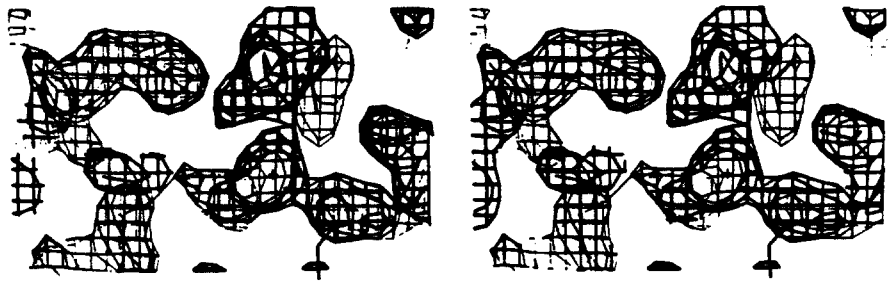


Figure 2. Portion of a $5|F_o| - 4|F_c|$ difference electron density map (the viewer is looking into the hydrophobic pocket of CPA). Structure factors were calculated from the refined model less the atoms for residues Glu-270, Arg-127, and TFP, in order to obtain an unbiased map. Refined atomic coordinates are superimposed on the map; Glu-270 is in the upper left, the zinc ion in the center, and Arg-127 in the lower right corner. Note the clear structure of the α-trifluoro moiety (coming toward the viewer) and the *gem*-diol of the hydrate (in the region above the zinc ion).

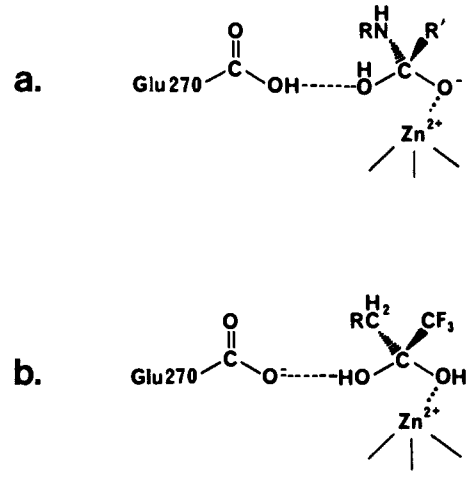
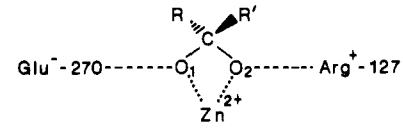


Figure 3. (a) Postulated tetrahedral intermediate of a promoted water hydrolytic mechanism, as illustrated for a peptide substrate. (b) schematic of the CPA–TFP interaction (note the resemblance to (a), with the exception of the direction of the hydrogen bond between Glu-270 and the hydrate oxygen).

Table II. Observed Orientations of Tetrahedral-Like Inhibitors, as Represented by Distances (Å) from Enzyme Residues Glu-270, Zn^{2+}, and Arg-127 (ZGP′ is the hydrolyzed phosphonamidate, BFP is the hydrated aldehyde, and TFP is the hydrated fluoro ketone of the present study)



interaction	structure		
	CPA–ZGP′[a]	CPA–BFP[b]	CPA–TFP[c]
Glu–270–O1	3.4	2.4	2.6
O1–Zn	2.2	2.7	3.3
O2–Zn	3.3	2.5	2.6
O2–Arg–127	2.7	3.6	3.2

[a] From ref 7. [b] From ref 5. [c] Present study.

active site and makes many of the same interactions.[5] However, the hydrogen bond between the zinc-bound hydrate oxygen and Arg-127 found here was not observed in the CPA–BFP complex. The CPA–TFP complex displays the first observed interaction of Arg-127 with a nonprotein zinc ligand. A stereoview of the CPA–TFP complex is presented in Figure 4.

Zinc Coordination Polyhedron. The zinc ligands in native CPA consist of His-69, His-196, the two carboxylate oxygens of Glu-72, and a water molecule. Some inhibitors can bind to the active site without perturbing the zinc environment, such as the substrate analogue (−)-3-(*p*-methoxybenzoyl)-2-benzylpropanoic acid.[20,21] The carbonyl oxygen of this inhibitor is observed to accept a hydrogen bond from the guanidinium moiety of Arg-127 instead of coordinating to the zinc ion.[6] Other inhibitors, however, have been observed to enter the coordination environment of the metal, e.g., BFP, TFP, and the hydrolyzed[7] moieties of the phosphonamidate ZGP′.[22] In each of these CPA–inhibitor complexes the zinc ion is observed to move about 0.4, 0.5, and 0.2 Å, respectively, from the position observed in the native enzyme.[23] The movement of zinc is accommodated by considerably smaller changes in positions of the enzyme residues to which it remains linked. Zinc ligand distances change little, if at all, with one particular exception. The interaction of the zinc ion with Glu-72 tends from bidentate toward unidentate coordination; the zinc ion consistently moves in the direction toward Arg-127. Distances from the zinc ion to $O_\epsilon 2$ of Glu-72 are 2.9, 2.8, and 2.6 Å for BFP, TFP, and hydrolyzed ZGP′, respectively, while the distance is 2.3 Å in native CPA. All other distances from the zinc ion to protein ligands are within 1.8–2.3 Å. The changing metal coordination might be considered in light of the use of Co^{2+}-substituted CPA as a spectroscopic probe of interactions with inhibitors and substrates. For example, Bertini and colleagues[24] concluded through NMR spectroscopy that native Co-CPA displays five-coordination. Furthermore, when treated with the inhibitor β-phenylpropionate, the coordination number remained at 5. These data were used to invoke the presence of a second metal-bound water molecule in the native enzyme. However, the binding of β-phenylpropionate to the metal ion without the displacement of the metal-bound water, accompanied by a movement of the metal ion such that coordination to Glu-72 changed from bidentate to unidentate, would also rationalize the observed spectroscopic results. Conversely, the continuous-wave microwave power saturation method shows native Co-CPA to have a tetracoordinate metal ion (i.e., it "sees" the coordination to Glu-72 as unidentate)[25] and thus is insensitive to changes in coordination of Glu-72 to the metal ion. Co-CPA has the same[26] coordination polyhedron as Zn-CPA and presumably behaves similarly with inhibitors and substrates that interact with the metal ion.

It is not certain whether the movement of Zn^{2+} by about 0.5 Å is necessary for its likely coordination to the substrate carbonyl and/or intermediate oxyanion or whether the initial binding of substrate might trigger the movement of zinc, and its bound water/hydroxide, to a more effective position for a forthcoming catalytic step. Although it would be interesting to observe movement of the metal ion in related zinc metalloproteases, no such effect is reported in the structures of several thermolysin–

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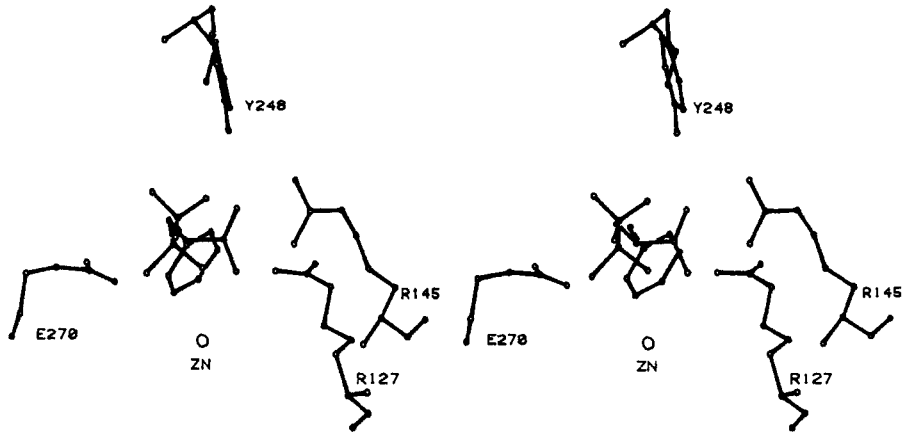

Figure 4. Stereoview of the CPA–TFP complex. Glu-270, Tyr-248, Arg-127, Arg-145, and the zinc ion are indicated.

ligand complexes.[27-31] Thermolysin, like CPA, has one glutamate and two histidine residues serving as metal ligands to an active-site zinc ion.[32] The glutamate residue is, however, monocoordinated to the zinc ion in native thermolysin, whereas it is in bidentate coordination to the zinc ion in CPA. Additionally, the inhibitors studied were not tetrahedral transition-state analogues, except for the unrefined phosphoramidon–thermolysin complex.[28]

Not only are different zinc positions observed in our three CPA–inhibitor structures involving direct inhibitor–zinc interactions but the general orientation of the tetrahedron of atoms configuring the inhibitor is instructively different in each case. This implies that the catalytic route may proceed through a well-defined sequence of orientations of the tetrahedral intermediate(s). The tetrahedron is oriented differently with regard to the nucleophile/base Glu-270, the central zinc ion, and the possible hydrogen-bond donor (electrophile) Arg-127. These three different orientations (Table II) may represent various positions along the free energy profile for the formation or breakdown of a tetrahedral intermediate during the course of hydrolysis. An oxyanion, for example, would be drawn closer to the zinc ion and favor the closer approach of Arg-127 than would a neutral hydroxyl moiety. Additionally, steric interactions within the S_1' hydrophobic pocket, and in other subsites, are known to be of kinetic significance[33,34] and may influence the structure and orientation of the tetrahedral intermediate. One mode of stabilizing the tetrahedral intermediate may involve the binding of two nonprotein ligands to zinc, consistent with cryoenzymological experiments on an ester substrate[35-37] and with our recent study of the binding of an aldehyde hydrate to CPA.[5] This particular mode may occur with concomitant hydrogen bonds from Glu-270 and Arg-127 as well. These considerations do not distinguish between promoted water/hydroxide and nucleophilic catalytic routes, even though the inhibitors studied so far resemble a structure occurring along a pathway in which a general base and/or zinc promotes the attack of water (or hydroxide). Illustrations of the three possible enzyme-stabilized tetrahedral intermediates are presented in Figure 5. The tetrahedral intermediate encountered in a promoted water/hydroxide mechanism for a typical peptide substrate is shown in greater detail in Figure 6, in which all postulated contacts with the enzyme are included.

Arginine-127. A role for Arg-127 was first considered in light of the interaction of this residue with the carbonyl of the dipeptide Gly–Tyr in its complex with the apoenzyme.[38] The proposed role involved a shift of the carbonyl oxygen of a putative anhydride intermediate from coordination with zinc to a hydrogen bond with Arg-127, thus facilitating anhydride hydrolysis by zinc-bound water or hydroxide.[1,38] The resulting tetrahedral intermediate would then have one oxygen on zinc and the other hydrogen bonded to Arg-127. The role of Arg-127 as a hydrogen-bond donor to a carbonyl oxygen is supported in view of the recently determined structure[6] of the complex of CPA with a ketonic substrate analogue, in which this residue is in a position to donate a bifurcated hydrogen bond to the carbonyl oxygen of the inhibitor. In view of the emerging importance of Arg-127 in the binding of inhibitors, and probably substrates, to the active site of the enzyme, it is interesting to consider its role not only in the precatalytic association of substrate with enzyme[39,40] but also as a residue critically important for the catalytic events. Arg-127 is conserved between bovine carboxypeptidases (carboxypeptidase B, 49% homologous with CPA[41]) and is conserved between species (rat carboxypeptidase A, 78% homologous with CPA[42]). Also, the observed movement of zinc upon the binding of inhibitors to CPA in what has previously been noted as the Arg-127 direction[43] seems to suggest that the reaction may proceed in various steps with the electrophilic participation of both Zn^{2+} and Arg-127. For instance, in a promoted water/hydroxide mechanism, the coordination of the substrate carbonyl to zinc would raise the pK_a of the zinc-bound water and make it less "hydroxide-like", and therefore possibly less nucleophilic. A reasonable reaction sequence would involve initial hydrogen bonding of the substrate carbonyl to Arg-127 followed by nucleophilic attack of a potent zinc-bound water/hydroxide. In a subsequent step, the tetrahedral oxyanion intermediate could shift toward direct coordination with zinc. Such a sequence would rationalize the results of chemical studies indicating that one arginine residue is required for catalysis.[44]

The role of hydrogen-bond donors as electrophilic catalysts in proteolytic enzymes, particularly the serine proteases, has been proposed for quite some time (e.g., the "oxyanion hole" of trypsin, chymotrypsin, and elastase[45]). In these cases, two backbone amide

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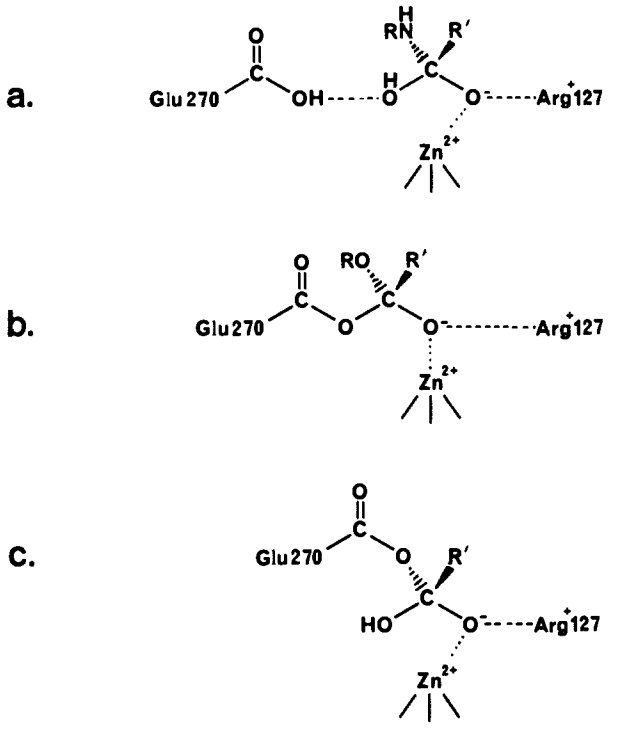

Figure 5. Three possible tetrahedral intermediates in hydrolytic reactions catalyzed by CPA. (a) Tetrahedral intermediate that would be encountered in a promoted water/hydroxide mechanism, as illustrated for a peptide substrate. Stabilization of this intermediate can be achieved by hydrogen bonds with Glu-270 and/or Arg-127, as well as the coordination of either one or two geminal oxygens to the zinc ion. If one oxygen holds a negative charge, it would probably be closer to zinc than the other. (b) First tetrahedral intermediate that would be encountered in an anhydride mechanism, as illustrated for an ester substrate. The zinc-bound water of the native enzyme can move to a nearby "temporary" location, perhaps hydrogen bonded between Glu-270 and the carbonyl oxygen of Ser-197, and return to the zinc coordination polyhedron later in the catalytic cycle. An alternative would involve no such displacement of the zinc-bound water but instead the formation of a five-coordinate zinc ion upon the binding of the ester substrate. Glu-270 could subsequently attack the carbonyl carbon of the substrate to give a tetrahedral intermediate similar to that shown except for the addition of a water molecule to zinc coordination. (c) Second tetrahedral intermediate that would be encountered in an anhydride mechanism. Cryoenzymological experiments show that an intermediate accumulates at low temperature, which involves a five-coordinate zinc ion.[35-37] This intermediate was presumed to be the anhydride with one carbonyl oxygen and a water/hydroxide as the two nonprotein ligands to zinc. The next step in the catalytic cycle for such an intermediate would be the hydrolysis of the covalently bound anhydride by the zinc-bound water/hydroxide. The intermediate pictured depicts the result of such a step. The conformational flexibility of both glutamate and arginine residues allows Glu-270 and Arg-127 to adapt to conformational changes necessary for such steps. Thus, the continuing ability of Arg-127 to provide a hydrogen bond to the tetrahedral intermediate will contribute to its stabilization.

NH protons are thought to provide stabilizing hydrogen bonds to the negatively charged oxygen of a tetrahedral intermediate. Recently, a similar role for His-231 of the zinc metalloprotease thermolysin has been implicated based on the three-dimensional structure of a novel enzyme–inhibitor complex.[27] Likewise, we propose an electrophilic role for Arg-127 in CPA on the basis of our recently determined crystal structures of enzyme–inhibitor complexes. This role is that of a "carbonyl binder", be it the carbonyl of a substrate or a putative acylenzyme intermediate, in order to facilitate the subsequent nucleophilic attack of zinc-bound water/hydroxide. Arg-127 can then assist in the stabilization, by hydrogen bonding or electrostatic stabilization, or both, of the oxyanion of the tetrahedral intermediate. It is very possible

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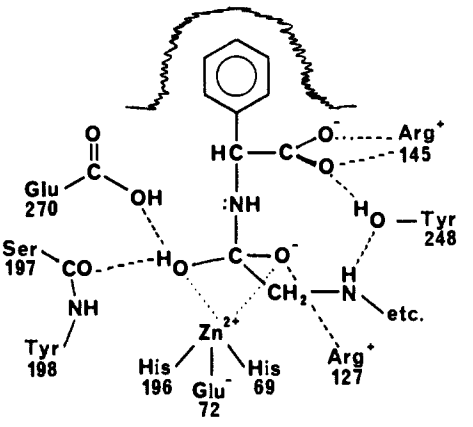

Figure 6. Tetrahedral intermediate for the promoted water mechanism (Figure 5a), illustrating all possible enzyme–substrate contacts in the S_1' and S_1 subsites on the enzyme, based on the results of this and previous structural studies of CPA–inhibitor complexes. The great number of such contacts likely serves to stabilize this postulated tetrahedral intermediate and, by extension, the actual transition state along the reaction coordinate.

that other zinc metalloenzymes possess potentially electrophilic residues (in addition to the metal ion) in their active sites. X-ray crystallographic studies of other zinc proteases will show whether such complementary or auxiliary electrophilic residues are a general feature of these enzymes.

Summary

The exact catalytic conformations of CPA in the hydrolysis of peptide and ester substrates remain, for the most part, open for discussion. These conformations are further complicated by the possibility that a tetrahedral intermediate may proceed through a well-defined sequence of orientations during catalysis. However, the residues involved in binding and catalysis are becoming more clearly identifiable as a result of recent structural and chemical studies. The γ-carboxylate of Glu-270 is either a nucleophile in an "anhydride" mechanism or a general base and/or proton acceptor to a zinc-promoted water/hydroxide. Its role as a catalytic nucleophile is consistent with recent chemical trapping studies that suggest a mixed-anhydride intermediate in both ester and peptide hydrolytic reactions catalyzed by CPA.[46] The zinc-bound water or hydroxide is certainly involved as a nucleophile; the zinc itself may serve as an electrophile in polarizing the carbonyl of a substrate. Additionally, the zinc probably helps to promote its bound water/hydroxide into attacking a substrate or acylenzyme carbonyl. The guanidinium moiety of Arg-127 likely serves as an electrophile by providing a hydrogen bond to a substrate or acylenzyme carbonyl. Arg-145 is important for binding the terminal carboxylate of substrates through a salt link. The phenolic residue of Tyr-248 has been shown to be involved in product binding,[43] and it is also implicated in substrate binding, given its observed role in hydrogen bonding to the terminal carboxylate of inhibitors. This residue was also long conjectured to be a proton donor to a leaving amine or alcohol moiety in the collapse of tetrahedral intermediates. However, such a role has been brought into question by a recently characterized rat CPA, in which a phenylalanine residue was substituted for Tyr-248, which displayed nearly full peptidase and esterase activity.[47] This result implies that Tyr-248 is not a residue imperative for catalysis, regardless of its observed role in substrate and inhibitor binding; however, alternate mechanistic routes cannot be ruled out. The side chain of Glu-270, or a water molecule, may serve as proton donor, depending on the catalytic route; such alternatives have been discussed previously.[5,43] Structural studies of CPA, currently in progress in this laboratory, will help us to further elaborate on

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the nucleophilic and electrophilic roles considered for active site residues.

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Polarized Single-Crystal Absorption Spectra of Co^{2+}-Carboxypeptidase A[1a]

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Abstract: Recent X-ray diffraction studies from this laboratory have shown that the coordination structure of Co^{2+}-reconstituted carboxypeptidase A is essentially identical with that of the native enzyme. The protein donor-ligand atoms are the N_{δ_1} atoms of His-69 and His-196 and the carboxylate oxygens of Glu-72 equidistantly positioned from the metal ion. A water molecule acts as the only nonprotein ligand in the free enzyme. On the basis of NMR proton relaxation enhancement studies of Co^{2+}-carboxypeptidase A, Bertini and co-workers have suggested that two exchangeable water molecules are bound within the inner coordination sphere of the Co^{2+}-enzyme in solution. Here, we compare the polarized single-crystal absorption spectrum of Co^{2+}-carboxypeptidase A to that of the enzyme in solution and show that the crystal spectrum is almost identical in band shape with the spectrum of Co^{2+}-carboxypeptidase A in solution. We conclude that the coordination environment of the metal is the same in solution and in the crystals elongated along the a axis (a = 51.60 Å, b = 60.27Å, β = 97.27°).

In the study of carboxypeptidase A, it is often assumed that the Zn^{2+}-bound water molecule is displaced when a substrate or inhibitor binds. However, a recent 1.54-Å X-ray structure of the complex between the native carboxypeptidase A and the ketonic substrate analogue (-)-3-(*p*-methylbenzoyl)-2-benzylpropionate shows that the ketone carbonyl does not coordinate to the zinc ion and the Zn^{2+}-bound water remains on the metal.[2] Also, electron paramagnetic studies of Co^{2+}-carboxypeptidase A show that a water molecule is retained on the metal in the acyl enzyme reaction intermediate when the ester (*p*-chlorocinnamoyl)-L-phenyllactate is the substrate.[3,4]

The coordination structure of the metal ion in Co^{2+}-carboxypeptidase A is essentially identical with that of the native Zn^{2+}-enzyme.[5,6] The donor-ligand atoms in the free enzyme are the N_{δ_1} atoms of His-69 and His-196, the carboxylate oxygens of Glu-72, and a single water molecule. On the basis of NMR proton relaxation enhancement studies of Co^{2+}-carboxypeptidase A, Bertini and co-workers[7] have suggested that two exchangeable water molecules are bound within the inner coordination sphere of the Co^{2+}-enzyme in solution. In this paper, we compare the polarized single-crystal absorption spectrum of Co^{2+}-carboxypeptidase A to that of the enzyme in solution. The crystal spectrum is almost identical in band shape with the spectrum of Co^{2+}-carboxypeptidase A in solution, indicating that the coordination environment of the metal remains unchanged.

Experimental Procedures

Co^{2+}-carboxypeptidase A was prepared by treatment of the α-form of the native enzyme (Sigma) in solution with *o*-phenanthroline[8] followed by dialysis against buffered solutions of $CoCl_2$ (Specpure, Johnson & Matthey, Inc.). Single crystals were obtained at pH 7.5 in 0.02 M sodium cacodylate in microdialysis tubings (Spectropor) by reducing the concentration of NaCl from 1.0 to 0.3 M. All glassware and quartz cuvettes were washed in acid, and dialysis tubing and plastic laboratory ware were prewashed with buffer containing *o*-phenanthroline. Twice-distilled deionized water was used throughout. All other materials were of ultrapure or analytical grade.

Polarized single-crystal absorption spectra were determined with a custom-built microspectrophotometer.[9] For data collection, crystals were suspended in 0.1 M NaCl solution buffered to pH 7.5 with 0.02 M cacodylate and mounted between quartz slides and coverslips. Identification of the (001) crystal plane was made by microscopic examination of crystals and measurement of interfacial angles based on a description of the $P2_1$ crystals of the α form of Zn^{2+}- and Co^{2+}-carboxypeptidase A provided by Dr. Karl Hardman. We have previously evaluated the effects of scattered light and crystal misalignment on polarization data. For the polarization properties of Co^{2+}-carboxypeptidase A in $P2_1$ crystals, it is readily shown that errors introduced by these factors are less than the experimental uncertainty associated with single-crystal optical density and dichroic ratio measurements.

The solution spectrum of Co^{2+}-carboxypeptidase A in the visible wavelength region was determined at room temperature with a Cary 210 spectrophotometer.

Results and Discussion

Figure 1 illustrates the polarized single-crystal spectrum of Co^{2+}-carboxypeptidase A. The solution spectrum of the enzyme is also shown for comparison. In the upper part of the diagram is illustrated the change in polarization ratio (PR) with wavelength. The PR is defined as the ratio of single-crystal optical densities measured with incident light polarized in two orthogonal directions defined by crystal symmetry. Fluctuations in the PR with wavelength reveal transition moment directions and the symmetry of perturbing influences on degenerate states.[9,10] The absorption

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