of stereochemical imperative governing the decarboxylation of β-keto acids,[28] suggest a general hypothesis: When cryptic chirality is involved,[29] enzymes catalyzing reactions via a particular chiral transition state will not in general be selected over enzymes catalyzing the same reaction via the enantiomeric transition state; enzymes catalyzing reactions via both transition states will be found in nature. In contrast, natural selection in general will distinguish between enzymes catalyzing analogous reactions via diasteromeric transition states, and only a single diastereomeric transition state will be found in naturally occurring enzymes.

Examples of nature of enzymes operating by locally enantiomeric transition states include citrate synthase,[30] certain decarboxylases,[28] and the alcohol dehydrogenases discussed here. Diasteromeric transition states are generally found in nature only when they are needed to make chiral compounds. Thus, while only limited data support this generalization at present, this general statement is worthy of experimental investigation.

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(29) This comment concerning "cryptic" chirality has some significance. In synthesizing molecules that are chiral without isotopic substitution, enzymes must have evolved an active site that synthesizes the desired enantiomer, that is, the enantiomer that is used in the next step of the metabolic pathway. This demand for stereospecificity due to metabolic coupling, discussed at length by Hanson and Rose, imposes an obvious constraint on the structure of enzymatic transition states. While the *particular* chirality may be nonfunctional (e.g, an organism making D-lactate may not be intrinsically less fit than one making L-lactate), the chirality is expected to drift more slowly than the stereospecificity of any individual enzyme, as changing the choice requires the simultaneous evolution of several enzymes that share the chiral compound as a substrate. Analogous arguments might be made to explain the universality of L-amino acids as building blocks of proteins and D sugars as building blocks of nucleic acids.

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# Comparison of Carboxypeptidase A and Thermolysin: Inhibition by Phosphonamidates

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**Abstract:** The binding of the intact phosphonamidate inhibitor *N*-[[[(benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine (ZGP'; $K_i$ = 90 nM), a possible transition-state analogue of the dipeptide substrate Cbz-Gly-L-Phe, to the active site of carboxypeptidase A (CPA) is described. X-ray crystallographic analysis of the enzyme-inhibitor complex provides a well-resolved structure at 2.0-Å resolution. Although our previous study of this compound with CPA yielded the structure of a hydrolyzed phosphonamidate, optimal adjustment of pH now allows the observation of the intact complex. Both phosphonamidate and phosphonate-derived inhibitor designs have realized success toward the inhibition of zinc proteases, and their binding stereochemistry to the active sites of CPA and the related zinc endoprotease thermolysin (TLN) is summarized and considered in light of a common hydrolytic mechanism. Interestingly, for both CPA and TLN those phosphonamidate inhibitors that have $P_1$ glycine residues display anomalous binding modes relative to those inhibitors that have phenylalanine side chains in this position. In the current study with CPA, the CbZ-Gly moiety of ZGP' occupies the $S_1$ hydrophobic side-chain cleft instead of binding in the main active site groove. The anomalous binding mode of ZGP' leads to questions regarding its classification as an analogue of an intermediate or transition state, at least with regard to binding in the $S_1$ subsite.

The zinc proteases comprise a class of enzymes, intriguing from mechanistic and biological perspectives, of which carboxypeptidase A (CPA; peptidyl-L-amino acid hydrolase, EC 3.4.17.1) is perhaps the prototypical example. First isolated and characterized in 1929 by Waldschmidt-Leitz and Purr,[1] this exopeptidase is secreted by the pancreas in mammals, and its biological function is the hydrolysis of C-terminal amino acids from polypeptide substrates. Certain aspects of the catalytic mechanism of CPA have been the object of much discussion and have been emphasized in the reviews.[2-5] Thermolysin (TLN), a zinc endoprotease, is an important zinc enzyme apparently related to CPA through convergent evolution. The high-resolution X-ray structures of native CPA[6] and TLN[7] have allowed detailed analyses and comparisons of the two zinc proteases. Similarities in the active sites of these two otherwise unrelated enzymes have been invoked to imply similarities in their mechanisms of catalysis.[3,8,9]

An understanding of the zinc protease mechanism(s) is important from more than just a scientific standpoint, since several zinc proteases of unknown structure (e.g., angiotensin-converting enzyme, collagenase, enkephalinase) serve as targets for the rational design of therapeutic agents. It is helpful, then, to rely upon the implication of at least a basic similarity among the zinc proteases of known and unknown structure: the relative geometry of catalytically important residues within their active sites is

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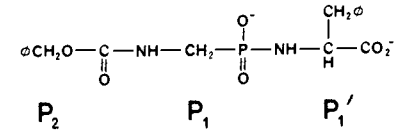


**Figure 1.** CPA inhibitor *N*-[[[(benzyloxycarbonyl)amino]methyl]-hydroxyphosphinyl]-L-phenylalanine (ZGP'; $K_i$ = 90 nM). The $P_2$, $P_1$, and $P_1'$ regions of the molecule are indicated.

probably conserved. Thus, CPA and TLN can serve as models for the other zinc proteases of currently unknown structure, and their interaction with inhibitors may provide models for pharmaceutical target enzyme–drug interactions. Such efforts toward the inhibition of angiotensin-converting enzyme serve as an elegant example of the successful application of this rationale.[10,11]

X-ray crystallography has been applied toward the mechanistic study of CPA[12-19] and TLN[20-27] by the observation of stable enzyme–inhibitor or enzyme–substrate complexes. A common object of study has involved tetrahedral anionic phosphonamidate and phosphonate-derived compounds that may be analogues of intermediates or transition states along the proteolytic reaction coordinate.[14,20,24,25] Although two phosphonamidate inhibitors[28,29] were bound to TLN intact at near-neutral pH,[24,25] the CPA inhibitor *N*-[[[(benzyloxycarbonyl)amino]methyl]hydroxyphosphinyl]-L-phenylalanine (ZGP', $K_i$ = 90 nM; Figure 1),[30] corresponding to the peptide substrate Cbz-Gly-Phe, was observed to bind to CPA as the hydrolyzed phosphonic acid plus phenylalanine at pH 7.5.[14] This was unexpected, since chemical studies of the CPA inhibitor established that the enzyme does not usually catalyze the hydrolysis of phosphonamidate moieties.[30] We now report that a change in pH from 7.5 to 8.5 allows the observation of the intact CPA–ZGP' complex. Even so, the Cbz-Gly portion of the *intact* phosphonamidate binds to the enzyme in an anomalous fashion. Interestingly, phosphonamidate inhibitors of TLN also bind anomalously if the $P_1$ amino acid is a glycine residue,[25] so the conformational freedom conferred by the methylene group of glycine presumably makes additional binding modes accessible.

**Table I.** Distances of Selected CPA–ZGP' Interactions

| enzyme atom | inhibitor atom | distance,[a] Å |
|---|---|---|
| Glu-270 Oε1 | phosphonamidate O1 | 3.1 |
| Glu-270 Oε2 | phosphonamidate O1 | 2.6* |
| Ser-195 carbonyl O | phosphonamidate O1 | 3.3 |
| Arg-127 N2 | phosphonamidate O2 | 2.6* |
| Tyr-248 phenolic O | carboxylate O1 | 2.6* |
| Arg-145 N1 | carboxylate O1 | 3.1* |
| Arg-145 N2 | carboxylate O2 | 3.0* |
| Asn-144 $NH_2$ | carboxylate O2 | 3.2* |
| Tyr-248 phenolic O | carbonyl O | 3.2 |
| Tyr-248 phenolic O | phosphonamidate NH | 3.5 |
| Glu-270 Oε2 | phosphonamidate NH | 2.8 |
| Zn | phosphonamidate O1 | 2.4 |
| Zn | phosphonamidate O2 | 3.5 |

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

These additional binding modes may substantially compete with productive binding of the related proteolytic substrates.

## Experimental Section

$CPA_\alpha$ (Cox) was purchased from Sigma (type I, crystalline) and used without further purification. The enzyme suspension was solubilized in 1.2 M LiCl, 0.02 M Tris-HCl (pH 7.4), filtered with a Millex-GV 0.22 μm filter unit (Millipore Corp., Bedford, MA), and then dialyzed against 0.15 M LiCl, 0.02 M Tris-HCl (pH 7.4) at 4 °C. Within 2–5 days crystals appeared that had typical dimensions of 0.3 × 0.3 × 0.9 mm. These crystals were cross-linked[31] for 6 h in a buffer solution [0.2 M LiCl, 0.02 M Veronal-LiOH (pH 7.4)] containing 0.15% glutaraldehyde (v/v). This process made the crystals more resistant to disorder upon soaking in solutions containing the inhibitor. No glutaraldehyde molecules were observable, however, in the electron density maps of the enzyme–inhibitor complex. Cross-linked crystals were then transferred gradually over a period of 48 h to a buffer solution of 0.15 M LiCl, 0.02 M Veronal-LiOH (pH 8.5), containing 7 mM ZGP'. When solutions were changed more rapidly, the enzyme crystals cracked severely. Crystals were soaked for 5 days at 4 °C. It should be emphasized that these experimental conditions were virtually identical with those of the previous study[14] *except for pH*. Two of the soaked crystals were mounted and sealed in glass capillaries along with a portion of mother liquor in order to prevent dehydration. Data collection was performed on a Syntex (Nicolet; Madison, WI) $P2_1$ automated four-circle diffractometer with intensities estimated by using the Wykcoff step scan.[32] Crystals of the CPA–ZGP' complex were isomorphous with crystals of the native enzyme, so intensity data were collected from two crystals in consecutive spherical shells of the asymmetric unit of reciprocal space to a limiting resolution of 2.0 Å. The usual corrections for Lorentz and polarization effects were applied to these data, and a correction for absorption was made based on the method of North.[33] After three data sets were scaled and merged, an *R* factor based on intensities yielded a value of 0.059 for replicate data. Electron density maps were calculated as described elsewhere.[12] Model building was performed on an Evans and Sutherland PS300 (Salt Lake City, UT) interfaced with a VAX 11/780 (Digital Equipment Corp., Maynard, MA), using graphics software developed by Jones (FRODO).[34] The enzyme–inhibitor model was refined by the reciprocal space least-squares method employing the stereochemically restrained least-squares algorithm of Hendrickson and Konnert.[35] The crystallographic *R* factor for the final model is 0.169, and the highest residual peaks in a final difference electron density map, calculated with Fourier coefficients $|F_o| - |F_c|$ and phases derived from the final model, are under $4\sigma$.

## Results and Discussion

One molecule of ZGP' binds to the active site of CPA; distances of relevant enzyme–inhibitor interactions are recorded in Table I, and a difference electron density map is presented in Figure

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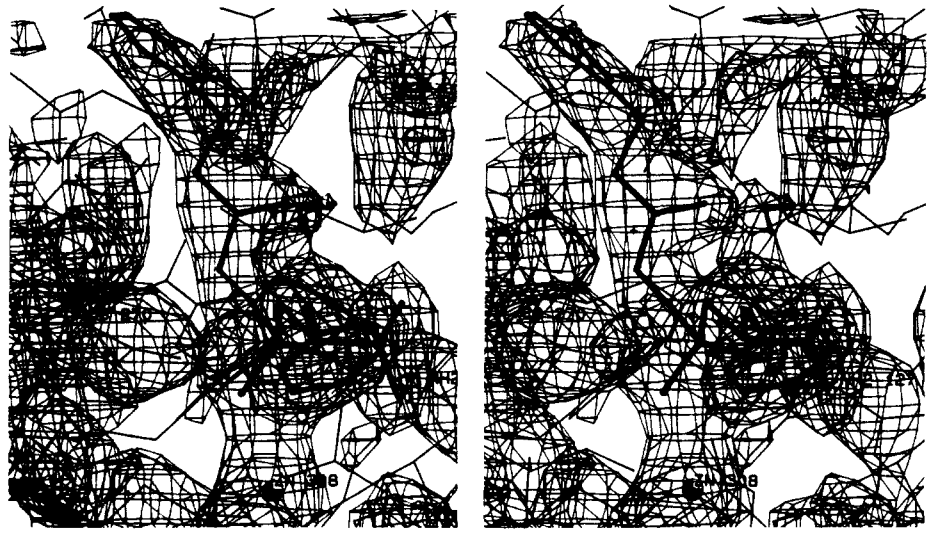

**Figure 2.** Difference electron density map calculated with Fourier coefficients $5|F_o| - 4|F_c|$ and phases derived from the final enzyme–inhibitor model less the atoms of residues Arg-127, Arg-145, and Tyr-248 and the inhibitor ZGP′. Coordinates of the final model are superimposed on the map, and pertinent enzyme residues are indicated by their sequence numbers. Note the location of the Cbz-Gly portion of the inhibitor—this is a hydrophobic cleft normally occupied by the side chains of more complex $P_1$ amino acids or analogues thereof. The Cbz-Gly portion of the inhibitor makes no hydrogen bond contacts with the enzyme.

2. The ZGP′ molecule makes the usual interactions expected within the $S_1'$ subsite on the enzyme: the benzyl groups resides in the hydrophobic pocket, and the terminal carboxylate makes a double salt link with the positively charged guanidinium moiety of Arg-145. Additionally, the phenolic hydroxyl of Tyr-248 and the amide group of the side chain of Asn-144 each donate a hydrogen bond to O1 and O2 (in anti orientation), respectively, of the terminal carboxylate of ZGP′. These three enzyme residues provide elegant specificity for substrates bearing a free C-terminal carboxylate. Additionally, Tyr-248 is thought to provide specificity toward substrates or inhibitors with penultimate peptide bonds, since it has been observed to accept hydrogen bond from the $P_1$ NH of longer substrates[18], inhibitors,[19] and products.[36] However, Tyr-248 does not accept a hydrogen bond from the NH group of the Cbz-Gly portion of ZGP′ (separation, 4.7 Å), nor does the carbonyl of the protecting group hydrogen bond with Arg-71 (separation, 6.8 Å) as previously observed,[18,19,36] since the inhibitor does not follow the expected course of the main chain of a polypeptide substrate. Instead, the Cbz-Gly portion of the inhibitor occupies the $S_1$ hydrophobic cleft of the enzyme, and the phenyl ring of the protecting group tends toward an "edge-to-face" interaction with enzyme residue Tyr-198. Although the interaction here is not optimal as outlined by Burley and Petsko,[37,38] it may contribute to the apparently nonproductive binding of the $P_1$ portion of ZGP′. The Cbz protecting group makes no other polar or weakly polar interaction with the enzyme (or with the symmetry-related enzyme in the unit cell), with the possible exception of the carbonyl. The carbonyl oxygen of the protecting group is 3.2 Å from the phenolic hydroxyl of Tyr-248. Since Tyr-248 presumably donates a hydrogen bond to the terminal carboxylate of the inhibitor, its additional interaction with the protecting group would involve a bifurcated hydrogen bond at best.

We must stress that a normal binding mode of ZGP′ would not be sterically hindered by contacts within the crystal lattice. Indeed, the ketonic analogue of the substrate *t*-Boc-L-Phe-L-Phe binds to CPA normally, and it is not affected by contacts within the crystal lattice.[19] Additionally, because no glutaraldehyde molecules are observable in the electron density maps, we conclude that cross-linking the enzyme does not force ZGP′ (which, of course, is observable in the electron density maps) to adopt an unusual conformation. Instead, the anomalous binding of the Cbz-Gly portion of ZGP′ may result from the additional conformational flexibility conferred by a methylene group at the $\alpha$-carbon of $P_1$ Gly. The $C_2$–$N_1$–$C_{\alpha 1}$–$P_1$ (subscripts indicate atomic locations, i.e., $P_2$, $P_1$, or $P_1'$ inhibitor residues) dihedral angle ($\phi_1$) of −109° and $N_1$–$C_{\alpha 1}$–$P_1$–$N_{1'}$ dihedral angle ($\phi_2$) of 32° found in the CPA–ZGP′ complex would be inaccessible for a $P_1$ L-amino acid (or analogue) with a side chain. For example, the ketomethylene analogue "BBP" of the peptide substrate (*tert*-butoxycarbonyl)-L-phenylalanine-L-phenylalanine, which binds as the ketonic hydrate, requires that corresponding dihedral angles $\phi_1(C_2$–$N_1$–$C_{\alpha 1}$–$C_1) = -148°$ and $\phi_2(N1$–$C_{\alpha 1}$–$C_1$–$C_{1'}) = -31°$ in order for the enzyme to accommodate both the $P_1$ side chain and the N-protected peptide backbone; $\phi_1$ ; −109° and $\phi_2$ = 32° would not be allowed for the binding of BBP.[19] Thus, the CPA–ZGP′ binding mode would be improbable for inhibitors or substrates with large $P_1$ amino acids such as phenylalanine, or even one as small as alanine. A stereoview of the CPA–ZGP′ complex is presented in Figure 3, where the binding conformation of ZGP′ is also compared with that of BBP.

**Phosphonamidate and Phosphonate Binding.** The phosphonamidate moiety of ZGP′ coordinates to the active site zinc ion of CPA in asymmetric fashion, and it is oriented differently relative to all TLN–phosphonamidate complexes. Differences in phosphonamidate and phosphonate binding modes between the two enzymes are depicted in Table II. Differences in the orientations of the tetrahedral phosphonamidate inhibitors, as measured by contacts with the active site base (CPA, Glu-270; TLN, Glu-143), zinc ion, and electrophile (CPA, Arg-127; TLN, His-231), may indicate that these inhibitors mimic different stages of the hydrolytic reaction catalyzed by the two enzymes. In CPA, the tetrahedral phosphorus groups of a phosphonamidate and phosphonate are "cocked" toward the base (Glu-270; a slight counterclockwise rotation of the phosphorus center in Table II), whereas in TLN the phosphonamidates and phosphonate are cocked toward the electrophile (His-231; a slight clockwise rotation of the phosphorus center in Table II). It is possible that the anomalous binding of ZGP′ in the $S_1$ subsite of CPA necessitates a different orientation of the tetrahedral phosphonamidate. Even so, the measured $K_i$ for ZGP′ against CPA activity is 90 nM,[30] assuming that the anomalous binding mode predominates in aqueous solution. If a bulkier $P_1$ amino acid were substituted for the $P_1$ Gly portion of ZGP′, two additional hydrogen bonds with the enzyme could be exploited between the protecting group and enzyme residues Arg-71 and Tyr-248. These additional interactions, if attainable, would probably result in an even more potent inhibitor. It is interesting to note that the phosphonate inhibitor (2-*R*,-*S*)-2-benzyl-3-phosphonopropanoic acid (Figure 4), synthesized and assayed by Galardy and colleagues,[39] displays a nearly

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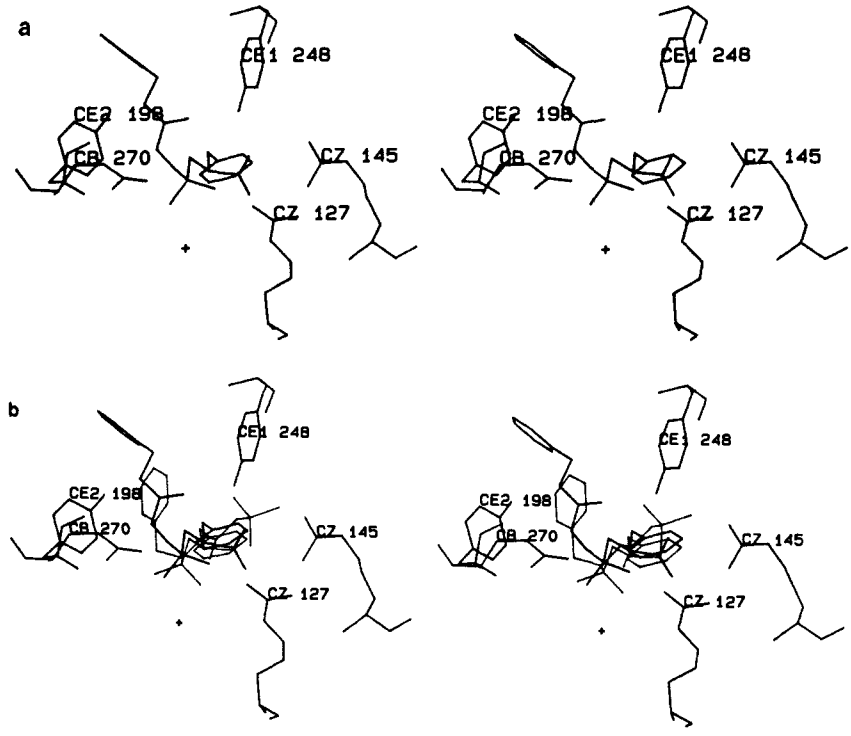
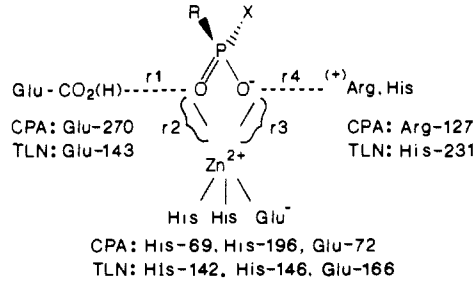



**Figure 3.** (a) Stereoview of the CPA–ZGP′ complex. Important active site residues are indicated by their sequence numbers (Glu-270, Tyr-248, Arg-145, Arg-127, Tyr-198). The zinc ion appears as a star. (b) Same stereoview as in part a, but with the coordinates of the inhibitor BBP (a ketonic analogue of the peptide substrate *t*-Boc-L-Phe-L-Phe) superimposed (thin bonds). Note that the Cbz-Gly portion of ZGP′ occupies the region of the $P_1$ side chain of BBP and does not occupy the main active site groove complementary to the extended dipeptide. The *tert*-butyl group of BBP indicates the region expected for binding of the penultimate peptide bond.

**Table II.** Phosphon(amid)ate–Zinc Interactions




| enzyme | inhibitor | X | distance,[a] Å r1 | r2 | r3 | r4 | ref |
|---|---|---|---|---|---|---|---|
| CPA | Cbz-Gly-$PO_2^-$-NH-Phe | NHR′ | 2.6 | 2.4 | 3.5 | 2.6 | this work |
| CPA | Cbz-Gly-$PO_3^{2-}$ + Phe | $O^-$ | 3.4 | 2.2 | 3.3 | 2.7 | 14 |
| TLN | Cbz-Gly-$PO_2^-$-NH-Leu-Leu | NHR′ | 2.5 | 3.0 | 2.1 | 2.9 | 24 |
| TLN | Cbz-Phe-$PO_2^-$-NH-Leu-Ala | NHR′ | 2.3 | 2.6 | 2.2 | 2.7 | 25 |
| TLN | Cbz-Gly-$PO_2^-$-O-Leu-Leu | OR′ | 2.7 | 3.0 | 2.1 | 3.0 | 24 |

[a] Note the general difference in orientation of phosphon(amid)ates (measured by contacts with zinc as r2 and r3) between CPA and TLN.

identical $K_i$ (220 ± 50 nM for the stereoisomeric mixture, hence, ca. 110 nM for the predominating stereoisomer) even though it lacks a $P_1$ Cbz-Gly portion. The phosphoramidate *N*-phospho-L-phenylalanine (Figure 4), stabilized and assayed by the same investigators, displays a $K_i$ of 240 ± 50 nM. The virtual equipotence of these smaller inhibitors with ZGP′ may indicate that the additional interactions supplied by the Cbz-Gly moiety of ZGP′ are negligible even in solution. This idea was proposed by Galardy and colleagues[39] and is now confirmed in the current X-ray study. The distances of active sites bases Glu-270 (CPA) and Glu-143 (TLN) from the anionic phosphonamidates suggest that the glutamate residues of the enzymes may be protonated in order to accommodate the binding of the inhibitors without undue electrostatic destabilization. This possibility for TLN has been recently discussed.[23,24] In CPA, the role of Glu-270 as a possible catalytic proton donor was first considered by Rees and Lipscomb,[36] and subsequently this role received detailed elaboration from Monzingo and Matthews.[23]

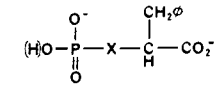



**Figure 4.** The phosphonate 2-benzyl-3-phosphonopropanoic acid, for X = $CH_2$. A competitive CPA inhibitor, it displays a $K_i$ of ca. 110 nM, virtually identical with that of ZGP′. For X = NH, this is the phosphoramidate *N*-phospho-L-phenylalanine, which displays a $K_i$ of 240 ± 50 nM. The virtual equipotence of these smaller inhibitors with ZGP′ suggests that the Cbz-Gly portion of ZGP′ provides little advantage to binding, a conclusion consistent with the results of the current X-ray study. Since $pK_{a_2}$ of a phosphonic acid generally ranges between 7 and 8, in this pH range of 7–8 the enzyme may select either the mono- or dianionic form of the inhibitor at the phosphonate moiety.

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The tetrahedral phosphonamidate ZGP′, as it is bound to the active site of CPA, may be an analogue of a tetrahedral intermediate or transition state of the proteolytic reaction. In particular, it may provide a model for a structure that occurs along the reaction coordinate of a promoted-water hydrolytic pathway rather than one along the putative pathway involving nucleophilic attack of an enzyme carboxylate. The mechanistic significance of the binding of these inhibitors has been discussed and analyzed in view of several enzyme–inhibitor structures.[15] In our perspective, the reversibly reactive substrate analogues, such as the aldehyde and ketones,[12,15,17,19] may best represent the ultimate binding of reaction intermediates (or flanking transition states), since the binding of these compounds invokes reversible chemistry analogous to that of the first catalytic step. We now designate these reversibly reactive substrate analogues as *reaction coordinate analogues*, and we feel that this designation aptly reflects their analogous reactivity with substrates. Of course, aldehydes and ketones are not substrates for hydrolysis, so their hydration indicates a "dead-end" attempt by the enzyme to perform a hydrolytic reaction. The binding of these compounds to CPA indicates the degree of selectivity that the enzyme displays toward three possibilities: (1) intact carbonyl, (2) nucleophilic adduct with Glu-270, or (3) hydrated carbonyl. The fact that CPA exclusively prefers the third possibility over the second possibility provides some unique structural evidence in support of a promoted-water proteolytic mechanism. Bartlett and Marlowe[40] have suggested that substrates and their possible transition-state analogues are linearly related by $K_M/k_{cat}$ and $K_i$. This linear relationship is elegantly demonstrated in their accompanying study of TLN substrates and inhibitors. However, this relationship is not observed in the analysis of ZGP′ and the shorter phosphoinhibitors of Figure 4, as indicated by Galardy and colleagues.[39] Perhaps the discrepancy in the analysis arises from the uncertain protonation state of the smaller phosphonates as they bind to CPA, i.e., as mono- or dianions at the phosphonate moieties. If they bind as dianions, their comparison to the necessarily monoanionic phosphonamide would not be valid. These issues may be resolved in further chemical studies of CPA–inhibitor complexes.

When the structure of the intact CPA–ZGP′ complex is compared with that of the hydrolyzed complex previously reported,[14] significant differences are observed in the location of the two halves of the molecule. The root mean square (rms) positional change in the $P_1'$ phenylalanine moiety of the inhibitor is 2.5 Å, and the rms change for the Gly-$PO_3(H)^-$ portion is 0.9 Å (not including the coordinates of the Cbz protecting group, since it was disordered in the hydrolyzed ZGP–CPA complex). These changes are prominent and are much greater than the approximate rms coordinate error (ca. 0.2 Å) in either enzyme–inhibitor model. In the hydrolyzed complex, Glu-270 is further away from the zinc-bound phosphonic acid group, perhaps due to local electrostatic effects with the possibly divalent anion. Tyr-248 was disordered in the hydrolyzed complex, whereas it unequivocally occupies the "down" position in the intact complex. The approach of a hydrolytic water molecule to the intact phosphonamidate would be more hindered, but not inaccessible, with Tyr-248 in the down position. In the hydrolyzed ZGP′ complex, the $P_1'$ phenylalanine is further out of the hydrophobic pocket of the enzyme, and perhaps its location represents a point along the trajectory of product release (especially since it is not blocked by Tyr-248). Because of these crystallographically observed differences between the cleaved and intact ZGP′ complexes, we maintain that structures of the two complexes indeed reflect chemistry that probably ocurred at the enzyme active site. At pH 7.5, the phosphonamidate was cleaved at the active site (noncatalytically) during the time frame of X-ray data collection sufficiently enough to allow the observation of a unique ternary product complex consisting of zinc-bound phosphonate and cleaved phenylalanine.

**Carboxylate Binding.** The binding of compounds exploiting zinc–carboxylate interactions has received considerable X-ray crystallographic study.[18,21,23,25,26] Phosphon(amid)ates and carboxylates can exploit favorable electrostatic interactions with the active site metal ions of zinc proteases, and stereochemical considerations of anion–cation association are related among these species. Indeed, the bisubstrate analogue and carboxylate-derived CPA inhibitor 2-benzylsuccinate ($K_i$ = 450 ± 80 nM)[41] inspired the design of 2-benzyl-3-phosphonopropanoic acid ($K_i$ = ca. 110 nM; Figure 4).[39] Hence, the latter compound may represent a bisubstrate analogue *or* an analogue of an intermediate or transition state along the reaction coordinate of proteolysis. Successful application of the carry-over design of the TLN inhibitor *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan,[42] shown by Monzingo and Matthews[23] to bind with the N1 carboxylate serving as a bidentate zinc ligand, to an angiotensin-converting enzyme inhibitor[11] resulted in a drug now used in the control of hypertension. The carboxylate moiety, of course, comprises one product of the proteolytic reaction catalyzed by the zinc proteases, and the negative charge of the carboxylate can be used to exploit a favorable electrostatic interaction with the active site zinc ion. The most efficient charge interaction will occur when the carboxylate or phosphon(amid)ate is bound to the metal ion in bidentate fashion. In fact, the carboxylate of Glu-72 in native CPA is a bidentate zinc ligand.[6] The active site zinc ion of TLN is also liganded by a carboxylate (Glu-166), but the interaction in this enzyme is unidentate. Other important factors, such as steric effects in each interacting enzyme subsite, will additionally influence the orientation of anion coordination to an active site metal ion.

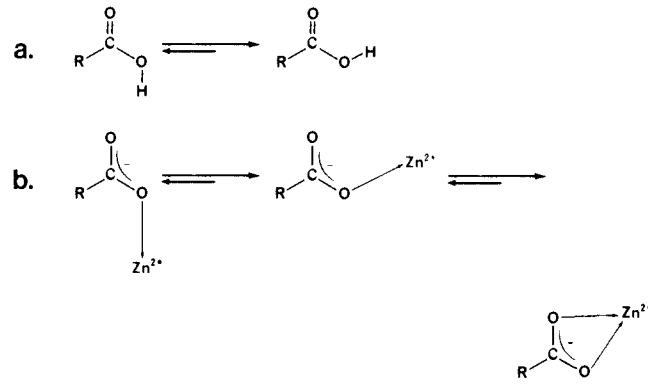


**Figure 5.** (a) *Syn*-carboxylic acids are more stable than their anti counterparts by 4.5 kcal/mol. (b) Likewise, we suggest that carboxylates prefer syn coordination to metal ions and that this coordination is optimally bidentate for the most efficient electrostatic interaction. Of course, steric requirements governing the overall association of enzyme and inhibitor will influence the orientation of a metal-bound carboxylate.

In four out of five examples, the metal coordination of the carboxylate-bearing enzyme inhibitor to CPA or TLN displays syn stereochemistry with respect to the carboxylate. It is well-known that carboxylic acids are less acidic as the syn-protonated stereoisomers (Figure 5).[43-45] This effect is due to the favorable opposition of the C=O and H—O bond dipoles in the *syn*-carboxylic acid.[44] A similar argument can be presented for carboxylate–zinc coordination interactions. A carboxylate coordinated to zinc in anti fashion would unfavorably align the C=O and O—Zn bond dipoles. The more favorable situation would instead be one of syn coordination, where C=O and O—Zn bond dipoles oppose. This complex will realize further electrostatic stabilization if bidentate coordination geometry is attainable. Only the carboxylate of benzylsuccinate in its complex with TLN shows anti coordination stereochemistry, so perhaps here the greater steric

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Table III. Carboxylate-Zinc Interactions

| enzyme | ligand | distance, Å O1-Zn | distance, Å O2-Zn | coord stereochem | ref |
|---|---|---|---|---|---|
| CPA | Bz-Phe | 2.7 | 2.2 | syn | 18 |
| CPA | "potato" inhibitor | 3.2 | 1.8 | syn | 36 |
| TLN | *N*-(1-carboxy-3-phenylpropyl)Leu-Trp | 2.4 | 2.0 | syn | 23 |
| TLN | Cbz-Phe | 3.0 | 2.1 | syn | 26 |
| TLN | benzylsuccinate | 4.1 | 2.1 | anti | 21 |

demands of the rest of the benzylsuccinate inhibitor require a nonoptimal anion-cation interaction. The geometries of carboxylate-zinc interactions as observed in CPA and TLN are compared in Table III.

## Summary

The phosphonamidate ZGP′ binds to CPA as an analogue of a possible intermediate or transition state along a promoted-water hydrolytic pathway. The tetrahedral phosphonamidate moiety, which straddles the active site zinc ion, is oriented differently when compared to the tetrahedral *gem*-diol(ate) CPA inhibitors[12,15,17,19] or related TLN-phosphon(amid)ate complexes[24,25] recently reported. This may be due to the fact that ZGP′ displays an anomalous binding mode to CPA where the Cbz-Gly portion of the inhibitor occupies the hydrophobic cleft normally occupied by the side chains of $P_1$ amino acids or analogues thereof.[18,19,36] This alternative strong binding mode may bear some relevance to general phenomena involving slow-binding enzyme inhibitors, as has been demonstrated for phosphonamidates that bind to TLN.[25] When anionic inhibition of CPA and TLN by phosphon(amid)ates is compared with that of related carboxylate-derived compounds, it is seen that zinc-carboxylate interactions prefer syn-coordinated carboxylates. This coordination appears to tend toward bidentate if no overriding steric factors persist. This stereochemical feature is important in the design of inhibitors derived from carboxylates and phosphon(amid)ates targeted toward those zinc proteases of known and unknown structure.

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# *Communications to the Editor*

## Simultaneous Observation of O-O and Fe-$O_2$ Stretching Vibrations of Fe(TPP)$O_2$ in Dioxygen Matrices by Resonance Raman Spectroscopy

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The observation of $\nu(O_2)$ and $\nu(Fe-O_2)$ ($\nu$: stretching) vibrations of oxyiron porphyrins is highly important in elucidating the nature of the O-O and Fe-$O_2$ bonds in oxygen transport/storage proteins such as hemoglobin (Hb) and myoglobin (Mb). Although Caughey and co-workers[1-4] observed the $\nu(O_2)$ of $HbO_2$ and $MbO_2$ at ~1155, ~1130, and ~1105 $cm^{-1}$ in IR spectra, the corresponding $\nu(Fe-O_2)$ vibrations have not been observed by IR spectroscopy. On the other hand, attempts to observe the $\nu(O_2)$ of these heme proteins by resonance Raman (RR) spectroscopy have been unsuccessful although the $\nu(Fe-O_2)$ of $HbO_2$ was readily observed at 567 $cm^{-1}$ by Soret excitation.[5] Different from oxyiron porphyrins, the $\nu(O_2)$ and $\nu(Co-O_2)$ of oxycobalt porphyrins can be resonance-enhanced simultaneously, and extensive RR studies have already been carried out on Co(II)-substituted heme proteins[6,7] and their model compounds.[8,9] Thus far, RR observation of the $\nu(O_2)$ of oxyiron porphyrins has been successful only for those containing axial thiolate ligands[10,11] although the reason for this is not clear. In this communication, we report the first

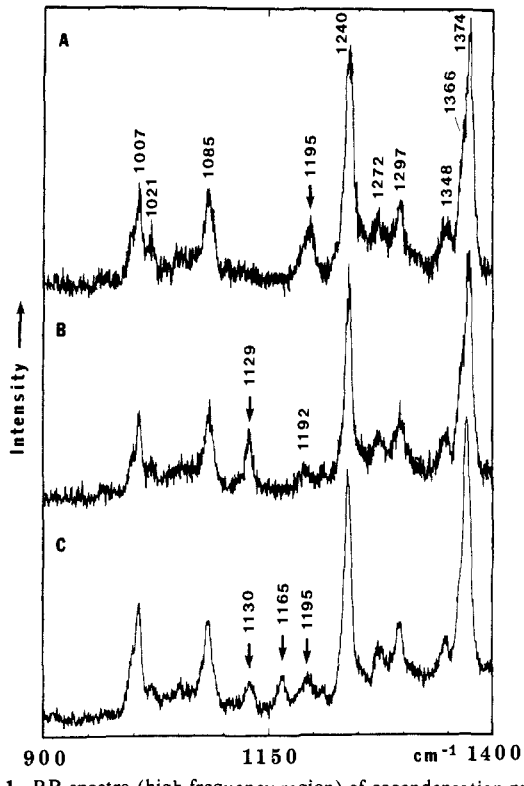


**Figure 1.** RR spectra (high frequency region) of cocondensation products of Fe(TPP) with (A) $^{16}O_2$, (B) $^{18}O_2$, and (C) scrambled dioxygen ($^{16}O_2/^{16}O^{18}O/^{18}O_2$ in an ca. 1:2:1 ratio) at ~25 K (406.7-nm excitation).

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