Mechanically Significant Diastereoselection in the Sulfoxime Inhibition of Carboxypeptidase A*

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The diastereomers of L-α-[S-(2-phenylethyl)sulfinimidoyl][methyl] benzenepropanoic acid bind differentially to carboxypeptidase A. These putative transition state-analogue inhibitors show unique and interpretationally significant pH dependences for $K_i$, as well as for the visible absorption spectra of their $E$-1 complexes in the case of the cobalt-substituted enzyme. From the geometry of the enzymically preferred isomer, it may be concluded that the mechanism of peptide scission by the enzyme entails addition of a nucleophile to the $s_i$ face of the bound-substrate prochiral carboxamide linkage. New interpretational constraints on the mode of action of the enzyme are thereby imposed.

The carboxamide functional group, as found in peptides, is a prochiral unit. Each such residue possesses a $re$ and a $si$ face, which in principle may have a different susceptibility to nucleophilic carbonyl addition, as occurs in enzymic proteolysis for example. Indeed, because of the stereospecificity which is the hallmark of enzymic processes, it can be asserted that one or other of these two faces in a substrate must be attacked exclusively in the catalytic process at the active site of any given proteolytic enzyme. However, all trace of this stereoselection is dissipated in the final products of enzymic peptide scission, so that this aspect of enzyme mechanism has been largely ignored in studies directed at explaining the mode of action of proteases. Of course this information is known in the case of certain enzymes as a consequence of crystallographic evidence and an established mechanism, notably for the serine proteases, in which an enzyme-attached nucleophile has restricted scope for attacking the bound substrate. However, for other enzymes of uncertain mode of action, in particular carboxypeptidase A, an independent answer to this question of nucleophile stereopreference could yield a definitive indication of mechanism. The challenge is to provide a suitable experimental approach, so as to discern the geometry and the energetics of the essential enzymic interactions involving the tetrahedral adduct that is created, along with its associated transition states, in the course of peptide scission.

Various carboxyl hydrate- and phosphorus-containing substrate analogues (1, 2) have been prepared as enzyme inhibitors for carboxypeptidase A and for the similar metalloprotease thermolysin, with the intention of approximating a tetrahedral adduct intermediate (1–5). However, crystallographic examination of enzyme complexes of such species has not resolved the mechanistic question. For several of these probes the tetrahedral moiety occupying the locus of a scissile linkage of a typical substrate (e.g. RCO–NHCH₂ZCO₂H) seems to coordinate variably in a mono- or bidentate fashion with the active site zinc ion, in what appears generally to be mixed inner and outer sphere complexation (6, 7).

In this article we describe the inhibitor characteristics with regard to carboxypeptidase A for a diastereomeric pair of sulfoximine-containing substrate analogues, 3 and 4, that are found to be active in the micromolar concentration range. The sulfoximine group nitrogen atom (but not the oxygen) is sufficiently basic to coordinate strongly with a metal ion. The sulfoximine functionality also possesses a stable tetrahedral configuration, which in the cases shown presents a chiral ligand to the zinc ion within the enzyme. Consequently, the preferred geometry for a tetrahedral adduct analogue interacting with the active site may be revealed by a comparison of these two species as enzyme inhibitors.

MATERIALS AND METHODS

Carboxypeptidase A (ZnCpA)1 was supplied by Sigma (No. C0386). The Allen form was chosen for its reported greater solubility. It was recrystallized by dialysis according to established procedures before use (8). Cobalt carboxypeptidase A (CoCpA) was prepared by repeated extraction of a suspension of the crystalline enzyme with aqueous 1,10-phenanthroline with subsequent reconstitution with cobaltous chloride, following established procedures (9, 10). Enzyme concentrations were estimated using $\epsilon_{280} = 6.42 \times 10^4$ M$^{-1}$ cm$^{-1}$. Buffers employed in this work for kinetic analysis were (0.05 M each) as follows: 2-amino-2-methyl-1,3-propanediol, pH of 8.75–10.0; Tris, pH of 7.25–7.75; Mes, pH of 5.5–7.75; 2,6-pyridinedimethanol, pH of 5.0–5.5. All enzyme work was done with solutions 1.0 M in sodium chloride. Solutions at pH of 6.5 or lower were made (0.5–1.0) $\times 10^{-4}$ M in additional zinc or cobaltic ion as required. The assay substrate for kinetic work was $N$-[3-(2-furyl)acryloyl]L-phenylalaninyl-L-phenylalanine.

1 The abbreviations used are: ZnCpA, CdCpA, and CoCpA, bovine pancreatic carboxypeptidase A (native, cadmium-, and cobalt-substituted, respectively); BzI, benzyI substituent; FAPP, $N$-[3-(2-furyl)acryloyl]L-phenylalaninyl-L-phenylalanine; Mes, 2-(N-morpholino)ethanesulfonic acid; BMBP, (−)-2-benzyl-3-(p-methoxybenzoyl)propanoic acid.

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nine (FAPP, $K_i = 10^{-4}$ M) (11) from Sigma. The synthesis, resolution, and absolute configuration of inhibitors 3 and 4 have been described (12); sulfone 6 was similarly prepared by peracid oxidation of the corresponding thioether intermediate from the preparation of 3 and 4, IR 1120 and 1300 cm$^{-1}$.

Kinetic Analysis—The pH dependence of $K_i$ for inhibitors with both ZnCpA and CoCpA was determined at 25.0 ($\pm0.1$) °C in buffers previously listed with FAPP as assay substrate at an initial concentration of less than $K_m$, with spectrophotometric (328 nm, 1-cm path length) analysis and the method of initial rates. Enzyme concentration was maintained well below inhibitor concentration in $K_i$ determinations (100-fold). Values of $K_i$ were obtained at each pH by a nonlinear least-squares fit of data from perturbation of $k_{cat}/K_m$ to the appropriate inhibition equation. All pH values in this article are calibrated pH meter readings uncorrected for ionic strength effects. Tolerances listed are standard errors from least-squares analysis.

Spectral Titrations—Visible absorption spectra for concentrated solutions of CoCpA (0.17-0.69 mM, 1-5-cm path length) were recorded conventionally at various values of pH in the range 6-10 in the presence of the inhibitors 3 or 4. It was verified that [I] $\gg K_i$ at all pH values so examined. The intrinsic $pK_a$ for the sulfoximine group has not been reported hitherto. We have secured a value for dimethyl sulfoximine by fitting its $^1$C NMR chemical shift (6 relative to CH$_2$SOCH$_3$, or (CH$_3$)$_2$SO$_2$, internal reference) against the previously devised $H_3$ acidity function scale (13) in the alkalinity range of 0.1-15 N NaOH (an appropriately sigmoidal +4 ppm shift of signal was noted, corresponding to formation of the N-anion). A $pK_a$ value of 15.8 ($\pm0.1$) was thereby obtained.

RESULTS

Design of Inhibitors—The primary catalytic specificity of carboxypeptidase A is for cleavage from peptides of individual C-terminal L-amino acid residues having a hydrophobic side chain. There also seems to be a secondary lipophilic interaction of the enzyme with the penultimate amino acid residue, for among the more susceptible peptides are acyl-PhEPh species such as 5, the assay substrate herein employed (in which the free L configuration carboxyl substituent is also an essential specificity feature). With this knowledge, 3 and 4 were constructed in which the benzyl groups (BzI) correspond spatially to those in 5, and in which the sulfoximine group resides in the scissile (\(\text{***}\)) carboxyl position. Furthermore, 3 and 4 have been resolved into their respective enantiomers, with relative configurations unambiguously established by crystallography (12). As a control, the substrate analogue 6 has also been prepared. The sulfone oxygens are noncoordinating, so that an inhibition comparison with 3 and 4 should reveal the significance of any sulfoximine-metal ion interaction at the enzyme active site. Due to extensive previous structural investigations of carboxypeptidase A complexed with similar substrate analogues, the potential mode of ligation of the inhibitors to the enzyme can be identified with some confidence.

Inhibition Characteristics—In fact, absolute configurational assignments for 3 and 4 correlate with the enzyme’s ability to recognize only an L configuration for the C-terminal amino acid residue in a peptide substrate. The more tightly binding enantiomers of 3 and 4, which by their synthesis possess an identical chirality at the methine carbon center, also have the same spatial arrangement next to the carboxyl group as has L-phenylalanine (12). Hence, they are given the L designation.

The enzyme affinity of D-3 as measured by the competitive $K_i$ is at least 47-fold poorer than for L-3 at a pH of 7. The observed $K_i$ for D-4 is likewise greater than for L-4 (factor of 12). However, our synthesis of D-4 apparently yields some contamination with the extremely tight binding 1-3, so that the true affinity of D-4 is probably masked. Subsequently described inhibition studies employ only the L-enantiomers, which are hereafter simply designated 3 and 4, and which have the absolute configurations depicted.

Both 3 and 4 have been shown to be strictly competitive, rapid equilibrium inhibitors in the enzymic hydrolysis of peptide substrate FAPP (5) by carboxypeptidase A. With 3 at concentrations of 0.58 or 1.78 $\mu$M at a pH of 7.6, there was a substantial retardation of $k_{cat}/K_m$, but no perturbation of $k_{cat}$. Similar results were obtained with 4. Illustrative experimental competitive inhibition constants (pH 6.0-6.2) are $3$, $K_i = 0.26 (\pm0.01) \mu$M; $4$, $K_i = 2.31 (\pm0.10) \mu$M; and (L)-6, $K_i = 231 (\pm18) \mu$M. The much weaker affinity of 6 for carboxypeptidase A tends to confirm that the mildly basic sulfoximine nitrogen of 3 or 4 (p$K_a$ of 5.3 for conjugate acid, 14) does interact with the active site metal ion. The sulfone oxygens are so weakly basic (estimated p$K_a$ of 11 for conjugate acid, 15) that no coordination to a metal ion in competition with solvent water seems possible; indeed, 6 binds to the enzyme scarcely more strongly than does the "specificity fragment" 3-phenylpropanoic acid. Consequently, an explicit enzymic interaction involving the nitrogen (but not the sulfoximine oxygen) of 3 or 4 seems indicated.

pH Dependence for $K_i$—The inhibitors 3 and 4 exhibit rather different dependence upon pH in their binding to carboxypeptidase A, and allowance must be made for this in comparing them. As shown in the Dixon plots presented in Fig. 1, the $K_i$ value for the more tightly bound inhibitor 3 appears invariant as pH increases up to a value of 9, above which enzyme affinity for 3 diminishes (p$K_a$ decreases). In contrast, 4 exhibits a gradually increasing $K_i$ with increasing pH. Greatest affinity of 4 for the enzyme is seen at a pH of ~6 (p$K_a$ maximum), and at a pH of 9 the values of $K_i$ for 3 and 4 differ by 1,000-fold. Similar data have been collected for cobalt carboxypeptidase A (CoCpA), the corresponding enzyme in which Co$^{2+}$ has been substituted for Zn$^{2+}$ in the active site (Fig. 2). This disparity in behavior for
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Fig. 2. Log-log plots of pH dependence for $k_{cat}/K_m$ and for $K_i$ with CoCpA. Top: (open circles) log($k_{cat}/K_m$) versus pH for hydrolysis of FAPP (5, 80 μM) in buffered saline solution. Interpretation as for Fig. 1. ($k_{cat}/K_m$) = $2.65 \times 10^{-17} M^{-1} s^{-1}$, $pK_a = 5.92 (\pm 0.07)$ and 9.20 (±0.06). Bottom: $pK_i$ versus pH for 3 (filled circles) and for 4 (filled squares), interpretation as for Fig. 1 ($K_i$ values for pH of 5.55 disregarded in fit).

Table 1

| Kinetic parameters fitting pH dependence of $K_i$ for the inhibition of carboxypeptidase A by 3 and 4 (according to Scheme 1) |
|---|
| E-1 | (K$\text{lim}_{H}$ | $K_1$ | $K_2$ | $pK_a$ |
|---|
| ZnCpA-3 | 0.30 (±0.01) | — | — | 9.17 (±0.04) |
| CoCpA-3 | 0.19 (±0.03) | 5.99 | 6.24 (±0.08) | 9.21 (±0.09) |
| ZnCpA-4 | 1.12 (±0.07) | 6.09 (±0.05) | 6.52 (±0.04) | 9.2 |
| CoCpA-4 | 1.20 (±0.11) | 6.11 (±0.07) | 8.65 (±0.07) | 9.2 |
| ZnCpA-6 | 231 (±18) &* |

* Apparently $pK_2 = pK_1$: therefore, no measurable inflection in pH profile for either.

† Inserted as a fixed value (corresponding to the lower $pK_2$ in $k_{cat}/K_m$) in fitting data to Scheme 1, in order to limit the number of adjustable parameters.

‡ Inserted as a fixed value (corresponding to the higher $pK_2$ in $k_{cat}/K_m$ and in $K_i$ for 3) in fitting data to Scheme 1 in order to limit the number of adjustable parameters.

§ Single point determination at pH of 5.98; $K_i$ increases with pH (14), a value of 2.3 mM was measured for 6 at a pH of 7.6.

diastereomeric inhibitors differing only in configuration at sulfur is potentially very significant mechanistically. Consequently, an endeavor at rationalizing the pH dependences is justified.

A manner in which this is to be done for the apparently complex pH dependence exhibited by 4 has been suggested previously (14). There are well-known enzymic deprotonations characterized by $pK_a$ values of ~6.1 and ~9.2, which affect catalytic behavior of the enzyme. The log($k_{cat}/K_m$) versus pH profile depicted at the top of Figs. 1 and 2 indicates a quenching of enzymic activity in acidic as well as in alkaline medium, as has been repeatedly observed elsewhere. The acidic limb $pK_a$ is known to be attributable to deprotonation of a water molecule which is coordinated to the active site metal ion in the absence of substrate (16). The alkaline limb $pK_a$ is likewise associated with deprotonation of an active site metal ion ligand, most certainly the imidazole ring of His-196 in the free enzyme (17). The former assignment follows from the unambiguous reverse protonation inhibition characteristics of an analogue of 3 or 4 that presents a phenolate ligand to the active site metal ion; the latter assignment derives from NMR spectral evidence with CoCpA. Because these prototropic changes are so intimately involved in the processing of substrates by the enzyme, similar involvement in the binding of 3 and 4 should be anticipated. Indeed, analogous breaks in the $pK_i$ versus pH profiles for 3 (alkaline limb) and for 4 (acidic limb) are cleanly observed. However, the gradual fall-off in apparent $pK_i$ exhibited by the curves for 4 indicates an additional deprotonation occurring specifically in the $E$-$I$ complex, with an intermediate $pK_i$ value. The fitted curve for 4 postulates a double inflection on the alkaline limb, with the same downward tilt at a pH of ~9.2 as seen for the other pH profiles in the figures but with a compensatory upward inflection at slightly lower pH (specifically that of an $E$-$I$ complex, 18). Scheme 1 provides a diagrammatic summary of our interpretation, while Table I lists parameters ($K_i \text{lim}$, $pK_a$, $pK_b$, $pK_c$) fitting the pH dependence of $K_i$ to the appropriate kinetic equation, also given in Scheme 1.

$$EH\cdot H_2O \quad \frac{(K_i \text{lim})}{H} \quad EH\cdot H \quad \frac{(K_i \text{lim})}{(+ H_2O)}$$

$$K_f \quad H^+ \quad K_f \quad H^+$$

$$EH \quad OH \quad EH \quad \text{or} \quad E\cdot H$$

$$K_f \quad H^+$$

Scheme 1

The $pK_i$ versus pH profile for 3 with ZnCpA is seemingly more plain, with only $pK_a$, the alkaline limb $pK_a$ discernible (Fig. 1). However, we shall suggest that the curves for 3 and 4 actually have the same form, and that $pK_a$ and $pK_c$ are merely hidden for 3 because they coincidentally have nearly the same value. If the intermediate $pK_i$ inflection noted in Fig. 1 for the $E$-$I$ complexes were in the case of $E$-$3$ shifted upward and to more acidic pH values, specifically to the vicinity of $pK_i$, a cancellation would occur and the overall curve should be leveled as is observed. By this hypothesis both curves may be reconciled with a common explanation (Scheme 1). The $pK_i$ versus pH profile for 3 with CoCpA is similar (Fig. 2), and in this instance we are able to show a small double inflection on the acidic limb in order to demonstrate the point. (Although the data here are ambiguous as regards the small inflection, $pK_a$ as obtained from the $k_{cat}/K_m$ profile is known to have a lower value for CoCpA.) Fortunately, additional evidence exists that the $E$-$I$ deprotonation detected by kinetic means for 4 (namely, $pK_a$) does in fact take place also in the $E$-$I$ complexes of 3 in more acidic solution.

Visible Spectra of CoCpA Complexes—Direct examination of $E$-$I$ complexes is feasible in the case of cobalt-substituted carboxypeptidase A because the enzyme with Co$^{3+}$ chelated at the active site has a characteristic visible spectrum due to the metal ion (9). The cobalt enzyme by itself in saline solution has a broad absorption maximum centered on 550 nm ($\sim 150 M^{-1} cm^{-1}$) with shoulders near 500 and 570 nm, as depicted in Figs. 3 and 4 (dotted line). The spectrum is noticeably perturbed upon addition of 1 molar equivalent of 3 or 4 but not further with extra increments of inhibitor. Moreover, the spectra of the $E$-$I$ complexes are conspicuously pH-dependent. In the case of 3, the overall spectral intensity is enhanced upon addition of inhibitor, as may be seen in Fig. 3, and the longer wavelength shoulder becomes a distinct maximum at 567 nm in neutral solution. However, the latter peak clearly shrinks (absolutely as well as relative to the shorter wavelength maximum) as the pH is lowered, with an apparent isosbestic near 587 nm (change reversible on pH restoration). The absorption versus pH profile has been sat-
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Reconciliation of Spectra with Kinetic Data—What is the identity of the functionality yielding the pH-induced spectral perturbation? That residue clearly regulates inhibitor binding, for in each case an inflection in the $pK_v$ versus pH profiles ($pK_v$ values for 3-CoCpA, 6.24, and for 4-CoCpA, 8.65) correlates with those obtained spectrosopically ($pK_v$ values for 3-CoCpA, 6.65, and for 4-CoCpA, 8.54). The most reasonable explanation is that the sulfoximine NH of 3 or 4 is strongly acidified in consequence of coordination to the active site metal ion; i.e. the pertinent proton dissociation may be formulated as $\text{(Enz)M}^{+2+}=\text{NH} = \text{S(O)R}_2 = (\text{Enz})\text{M}^{+} = \text{N=S(O)R}_2 + \text{H}^+$. Such an ionization should perturb the Co$^{3+}$ visible spectrum and could be expected to show an influence from inhibitor configuration. Moreover, it rationalizes the pH profiles for $K_i$ with the inhibitors. As seen most clearly with 4, $pK_v$ diminishes above a pH of 6 as a consequence of the fact that hydroxide is the external ligand completing the coordination sphere of the active site metal ion above that pH in the absence of inhibitor or substrate. (As previously noted, the $pK_v$ of ~6 seen in the $k_{\text{cat}}/K_M$ profiles of Figs. 1 and 2 has been shown to be due to $(\text{Enz})\text{M}^{+2+}=\text{OH}_2^-$ (16, 17)). The comparatively weakly basic sulfoximine nitrogen of 4 cannot itself compete effectively with OH$^-$ as a ligand, but the conjugate base $\text{R}_2\text{S(0)=N}^-$ is able to do so. Therefore, the apparent value of $pK_v$ decreases until the medium is sufficiently alkaline to deprotonate the metal ion-activated sulfoximine group within the E-I complex, whereupon the pH profile levels off. This occurs near a pH of 8.5 in the case of 4, but closer to a pH of 6 with 3. While such strong acidification of an NH group might seem questionable, it is entirely consistent with the potent Lewis acid character of the active site metal ion. The $pK_v$ of ~6 observed for $(\text{Enz})\text{M}^{+2+}=\text{OH}_2^-$ in the absence of inhibitor means that a solvent water molecule (intrinsic $pK_v$ of ~15.7) has been acidified >$10^9$-fold by coordination to the metal ion. Independent determination of the acid dissociation constant for dimethyl sulfoximine (by $^{13}$C NMR spectral perturbation, employing a basicity function extrapolation based on overlapping indicators) yields an almost identical intrinsic $pK_v$ of 15.8. It is not at all unreasonable that the enzyme should similarly augment the acidity of a bound $\text{R}_2\text{S(O)=NH}$ group.

**Discussion**

Although several tetrahedral adduct-analogue inhibitors for carboxypeptidase A have been described, a significant differential in enzyme affinity corresponding to the stereochemistry of the metal ion ligating moiety has not been reported previously. In the present instance, we find diastereomer 3 to bind from 10- to 1,000-fold more tightly than 4, depending on pH. Particularly revealing is the 100-fold greater acidification of the sulfoximine NH proton for 3 in consequence of nitrogen coordination to the metal ion. It appears that the active site metal ion exhibits a considerably greater Lewis acidity toward one diastereomer, for the ease of N-deprotonation ought to correlate directly with the dative electron-withdrawing capacity of the metal ion engaged with the sulfoximine nitrogen. This apparent Lewis acid anisotropy within the active site has ramifications for the catalytic mechanism, for it seems as if the acidity of the metal ion becomes focused on a single apex of the pyramidal species that is presented to it.

Independent kinetic evidence suggesting that the active site metal ion functions as a Lewis acid in the course of peptide scission exists in the form of the synergism test (20). We refer to the finding that the native enzyme (ZnCpA) is a poor...
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For example, it has been suggested that Glu-270-CO$_2$ functions as a base in the enzyme-catalyzed, stereospecific enolization-H.D-exchange of ketone-containing inhibitor BMBP (24). However, a revised crystal structure for the BMBP-carboxypeptidase A complex (25) reveals noncoordination of substrate ketone to metal ion, and that the more strongly basic metal-bound hydroxide is closer to the exchangeable CH$_2$ group of BMBP than is Glu-270-CO$_2$. The observed pH dependence of enzyme-induced proton exchange within substrate also suggests that (Enz)Zn$^{2+}$OH$^-$ is the operative base, when the pH profile is properly interpreted (14, 16). Finally, our hypothesized process (Zn$^{2+}$-OH$^- + ArOCOCD,R \rightleftharpoons Zn^{2+}$-OD + ArOCODHR) constitutes a valid intramolecular mechanism for proton exchange completely within the E-S complex. The original proposal that Glu-270-CO$_2$ abstracts the substrate deuterium explains nothing by way of catalysis, since in that case a second, intermolecular exchange step of the carbonyl with external solvent would be required prior to reintroduction of a proton into substrate, which step would not be expected to compete with intramolecular reprotonation of substrate enolate by Glu-270-CO$_2$: i.e., it is only a diprotic acid intermediate such as Zn$^{2+}$-OHD which allows rapid label exchange entirely within an E-S complex.

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**Fig. 5. Stereo角度看 active site region for carboxypeptidase A.**

Structure (enzyme crystallographic coordinates, 26, 27) is shown complexed with 3 (bold bonds) in manner of peptide substrates, with sulfoximine N-coordinated to Zn$^{2+}$ (filled circle). Arg-145 and Asn-144 are to the right, interacting with the carbonate of the inhibitor; Glu-270 is to the left, immediately behind the sulfoximine group and phenethyl substituent of the inhibitor in this projection. Arrows point to sulfoximine oxygen and to adjoining proximal oxygen of inhibitor carboxylate group.

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Other investigators have apparently arrived independently at this same conclusion (21).
opposed and inaccessible to Glu-270.

How then is a nucleophile delivered to substrate? One proposal finding recent advocacy is a four-center mechanism, in which the substrate carbonyl supposedly enters the coordination sphere of the metal ion while a metal-bound hydroxide is concurrently transposed to the carbonyl (27–29). This scheme has been advanced by crystallographers on the finding that certain carbonyl hydrate containing inhibitors (e.g. 1) bind to the enzyme with both oxygens more or less equivalently coordinated to the active site metal ion. Such a process is compatible with the synergism test previously alluded to, and may be given stereochemical definition by the sulfoximine-binding results with 3 and 4. However, we have a problem with the chemical plausibility of such a scheme. It seems impracticable to ask a metal ion simultaneously to activate a nucleophilic water molecule (by facilitating deprotonation, 30) while also providing significant Lewis acid catalysis to the substrate carbonyl. Ligand hydroxide should so activate a nucleophilic water molecule (by facilitating deprotonation, 31) while also providing significant Lewis acid catalysis to the substrate carbonyl. Ligand hydroxide is only incipient (28). In other words, this proposal fails to explain the central problem of enzyme mechanism, what is the origin or the kinetic acceleration?

We should like to introduce an alternative idea, which appears not to have previously been advanced. Examination of Fig. 5 reveals that the sulfoximine oxygen in the bound inhibitor (which we contend approximates the position of the introduced nucleophile in the tetrahedral adduct for substrate hydrolysis) resides in close proximity to one of the substrate carbonylate oxygens. We shall explore the proposition that it is the substrate carbonate that serves to deprotonate a water molecule, promoting nucleophilic addition to the adjacent carboxylate, which is activated by conventional coordination to the metal ion. It is well known that this carbonate group is an essential specificity feature for recognition of substrates by carboxypeptidase A, and that for productive binding it forms a salt link with the side chain of Arg-145. However, this by no means precludes an active role as general base in the catalytic mechanism as well (precedent 31). We suggest that after 20 years of largely unsupported speculation that the carbonate of Glu-270 plays such a role, some consideration be given to the possibility that the equally puissant carbonate of substrate may be the actual proton transfer agent in the hydrolytic cycle. Our mechanistic suggestion is summarized in Fig. 6. We offer the following arguments in favor of this hypothesis: (i) As previously designated, addition to the scissile linkage is from the proper (ii) face as indicated by the preferential binding and acidification of 3. (ii) The acidic (Zn²⁺) and basic (CO₂⁻) catalytic groups are separate entities, unlike the four-center mechanism wherein the metal ion must fulfill a dual role. The enzymic catalysis may consequently be partly attributed to the conventional counter-entropic effect of gathering and precisely orienting reactive functionality. (iii) In the latter regard, it is worth noting that for the carbonate-containing side chain of Glu-270, nominally free rotations about no less than three carbon-carbon single bonds would have to be frozen (>CH₂–CH₂–CH₂–CO₂⁻), were it to be brought into a fixed position to serve as a general base proton acceptor as in the conventional mechanism. In contrast, the substrate carbonate is rigidly oriented. It is of course directly connected to the reaction center via the substrate backbone, with the conformation of the latter locked by virtue of interactions with the enzyme. Of the two oxygen atoms in the substrate carbonate, the one distal to the reaction center is hydrogen bonded to an NH of the side chain carboxamide of Asn-144; this should tend to freeze rotation about the substrate >CH₂–CO₂⁻ bond, and to hold the proximal (catalytic) oximin in a fixed position. When the Bürgi-Dunitz (32) reaction coordinate for addition to carbonyl groups is projected onto the scissile carbonyl of the substrate (on the si face), using crystallographic coordinates for the enzyme-GlyTyr complex (22), the nucleophile passes within 2.7 Å (H-bonding distance) of the proximal oxygen atom of the substrate carbonate precisely at the point where pyrimidization of the carbonyl group commences. Hence, there can be little question that the substrate carbonate is suitable to serve as a general base assisting H₂O addition. In contrast, there are no obvious conformational constraints on the side chain of Glu-270, such as would be needed to orient it rigidly in the manner that seems to be a prerequisite for effective intramolecular general base catalysis (33). However, were the Glu-270 carbonate to have only a charge-neutralization role, electrostatically satisfying electron deficiency alternately in the metal ion or in the substrate carbonyl group at various
stages of the catalytic cycle, this conformational flexibility could be of benefit in allowing optimal interaction guided by electronic attraction. (iv) A potential objection is that the geometry of the substrate carboxylate requires that the purportedly less basic anti lone pair of electrons on the carboxylate oxygen be a proton acceptor (34). However, it is not known how great the anti/syn basicity differential is in aqueous solution, and in any event the conjugate acid subsequently would serve to protonate the nitrogen of the scissile linkage prior to dissociation of the tetrahedral adduct (Fig. 6). This latter step can be rate limiting in model amide hydrolyses (29), and so a stronger acid here might be advantageous overall. (v) There is additional suggestive evidence that the substrate carboxylate plays more than the merely passive role of binding the reactant to the enzyme. A heterocyclic tetrazole 5-membered ring has been shown to be a useful surrogate for the carboxyl group in various biochemical analogues. It has a similar pK_a so that it provides a coprogenic anion when incorporated via its carbon atom into a synthetic pseudosubstrate not in pair of a carboxylate. In a many an analogues the carboxylate is accepted normally by its biochemical receptor. Such a substrate analogue for carboxypeptidase A has been examined (35). In contrast to a rapidly cleaved carboxylate-containing model substrate, the corresponding peptide linkage was not hydrolyzed at all within the tetrazolate. This may indicate that the substrate-CO_2^− normally does more than merely provide for recognition by Arg-145. We note that the anionic charge on a tetazo hold is distributed over four nitrogen atoms rather than the two oxygens of a carboxylate, and judging by the product orientations obtained upon N-alkylation of tetrazole anions, electron density on the 2- and 3-position nitrogens is greater than for the 1- and 4-positions (which would be the obligatory catalytic proton acceptor at the active site) (36). Therefore, it may be that the heterocyclic ring is an incompetent general base for the catalytic mechanism. (vi) Finally, a potentially serious objection to the proposed essential role of the substrate carboxylate must be met. The metalloenzyme thermolysin is homologous in many respects to carboxypeptidase A, and it is an endopeptidase which does not require a free C-terminal carboxylate on its substrates. Were the mechanisms of these enzymes identical (which ought not to be a foregone conclusion), the substrate carboxylate could not have the pivotal role prescribed for carboxypeptidase A. However, the active site of thermolysin contains an additional histidine residue (His-231), and its imidazole ring appears suitably positioned for proton acceptance on Nε from a nucleophile attacking the appropriate face of a bound substrate. Furthermore, the imidazole is “backed-up” by a carboxylate (Asp-226) which is H-bonded to Nε in the fashion of the serine proteases (37). An imidazole moiety is the more suitable general base for an enzyme operating at neutral pH, as demonstrated by its prevalence in enzymology (38). Our suggestion is that carboxypeptidase A makes do with a carboxylate base only because its proximity within the substrate never allowed an evolutionary replacement. The side chain of Glu-270 has similarly been conserved because it functions as an obligatory anion, but not necessarily as a proton acceptor.

In conclusion, we have attempted to initiate a case for a hitherto ignored mechanism for carboxypeptidase A. Our purpose is not to disenthrone completely Glu-270 from its place of prominence in conventional mechanistic speculations. Rather, we merely observe that another carboxylate is invariably present within the active site when substrates are productively bound. Because kinetic evidence fails to implicate Glu-270 in a protopic capacity (i.e. the pK_a of ~6.1 observed in enzyme kinetics is not attributable to that residue and such a pK_a need not represent an enzymic general base in any event), we suggest that an open mind should embrace all possibilities and give consideration equally to proposals involving either carboxylate in a catalytic capacity. Our introduction of the sulfoximine as an active site stereochimical probe is only the beginning of a hoped-for discrimination between these alternatives.

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