Positive ion FAB mass spectra were obtained with a Kratos MS50RF (Kratos Analytical, Ltd., Manchester, U.K.) double-focusing instrument with a mass range of 10000 amu at full accelerating voltage (8 kV). The instrument was fitted with a Model B11NF saddle-field fast atom gun (Ion Tech, Ltd., Teddington, U.K.) and a post accelerator detector (PAD). The PAD was operating at 14 kV. Xenon was used to bombard the samples at 8 kV. The samples were dissolved in methanol and applied to a gold FAB probe in a matrix of 3-nitro-benzyl alcohol. The mass spectra were acquired at a scan rate of 30 s/decade with a resolution of 1 in 2500. All data were acquired and processed with the Kratos DS-90 data system.

NMR spectra were obtained on a Varian XL200 spectrometer in [$^2H_5$]pyridine solution following reduction by stannous chloride;[14] 3–5 mg of sample was added to 6–9 mg of $SnCl_2$ in a volume of 0.5 mL. Typically, 256 free induction decays were collected with an accumulation time of 4 s for 1D spectra. COSY spectra were taken with the use of a 1024 by 1024 data matrix with 256 t_1 increments of 16 free induction decays each.[15] Prior to NOESY or difference nuclear Overhauser effect studies, the solutions were degassed by three freeze–exhaust–thaw cycles. Phase-sensitive NOESY spectra were obtained by the method of States et al.,[16] in overnight runs, with the use of 1024 by 1024 data matrices and a mixing time of 0.8 s. This allowed collection of 256 t_1 increments each with 64 free induction decays. To provide difference nuclear Overhauser effect spectra, selected resonances were irradiated for 4 s before the accumulation of 6000 free induction decays for 1 s, which were then subtracted from free induction decays obtained with the irradiating frequency far removed. Fourier transforms of the differences provided spectra in which the irradiated peak and those showing interactions were 180° out of phase.

Conclusions

The reaction of ferrous deoxymyoglobin with $BrCCl_3$ lead to the formation of three major non-protein-associated modified heme products and a protein-bound heme metabolite. The non-protein-associated products were identified as Iβ-carboxyvinyl, Iα-hydroxy-β-(trichloromethyl)ethyl, and Iα,β-bis(trichloromethyl)ethyl derivatives of the prosthetic heme moiety and appear to result from the initial regiospecific attack of the trichloromethyl radical on the I-vinyl group. These findings add to our knowledge of the general reactivity of radicals toward hemoproteins and may aid in understanding the process by which CCl_4 inactivates cytochrome P-450.

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Sulfoximine and Sulfodiimine Transition-State Analogue Inhibitors for Carboxypeptidase A

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Abstract: New substrate analogues [(±)-2-carboxy-3-phenylpropyl]methylsulfoximine and [(−)-2-carboxy-3-phenylpropyl]methylsulfodiimine have been prepared and shown to be potent competitive inhibitors of the zinc enzyme carboxypeptidase A (limiting values of K_i = 2.7 and 0.22 μM, respectively). A complicated pH dependence for K_i is explained by deprotonations occurring on the enzyme, the inhibitor, and the enzyme–inhibitor complex. The mode of inhibitor binding is also characterized by visible absorption and ^1H NMR spectra of the cobalt-substituted enzyme. Mechanistic consequences are considered; no support is found for a concerted mechanism of acyl substitution occurring within the coordination sphere of the active-site metal ion.

Current mechanistic theory suggests that stable substrate analogues which structurally resemble high-energy intermediates along the reaction path for an enzymic reaction should function as potent inhibitors. For the prototypical metalloprotease carboxypeptidase A (ZnCpA), a number of putative illustrations of this principle have been recorded.[1] By now it is apparent that tight-binding, synthetic competitive inhibitors for this enzyme contain the specificity features of a typical substrate (i.e., the terminal carboxylate plus adjacent hydrophobic side chain in proper stereochemical relationship, as in a hydrolytically susceptible *N*-acylphenylalanine peptide, for example), as well as a suitable metal ion ligating group in place of the scissile carboxamide linkage.[1-3] Should this latter moiety bear an appropriate structural similarity to a *carbonyl hydrate*, then an especially favorable match with the active site would be anticipated according to the theory of transition-state mimicry.[4] Enzymic peptide hydrolysis presumably entails a similar intermediate, and the active site ought to attain complementarity to such a metastable species in the course of catalysis, in order that binding energy may be most effectively channeled into kinetic acceleration.[5] Among the more prominent examples of this principle are inhibitors containing a phosphonyl anion at the scissile locus (e.g., CH_3-PO_2^-NHCHBzCO$_2$H);[1a-d] the tetrahedral nature of this functional group appears to provide an excellent steric fit to the active site, while anion coordination satisfies the electron deficiency of the metal ion present therein.

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The biochemically rarely enlisted sulfoximine **1** and sulfodiimine **2** functional groups are chemically stable and are also tetrahedrally hybridized.[6,7] Furthermore, the imino nitrogen atoms are sufficiently basic to allow metal ion coordination in a unidentate or potentially (in the case of **2**) in a bidentate fashion. This article explores the inhibition characteristics for carboxypeptidase A of substrate analogues incorporating these novel functionalities.

1 2

Experimental Section

Carboxypeptidase A, EC 3.4.17.1, (ZnCpA) was supplied by Sigma (No. C0386). The Allan form was chosen for its reported greater solubility. It was recrystallized by dialysis according to established procedures before use.[8] Co(II) carboxypeptidase A (CoCpA) was prepared by dialysis of the enzyme against 1,10-phenanthroline with subsequent reconstitution with cobaltous chloride, following established procedures.[9] Enzyme concentrations were estimated with $\epsilon_{278} = 6.42 \times 10^4\ M^{-1}\ cm^{-1}$. Buffers employed in this work for kinetic analysis were (0.05 M each) as follows: Ammediol, pH 8.75–10.0; Tris, pH 7.25–8.75; Mes, pH 5.3–7.25; 2,6-pyridinedimethanol, pH 5.0–5.3. All enzyme work was done with solutions 1.0 F in sodium chloride. Kinetic assay solutions at pH 6.5 or lower were made (0.5–1.0) $\times 10^{-4}$ M in additional zinc or cobaltous ion as required. The assay substrate for kinetic work was *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-L-phenylalanine (FAPP, $K_m = 9 \times 10^{-5}$ M)[10] from Sigma.

Synthesis. Inhibitors **3–5** were prepared from 2-benzyl-3-(methylthio)propionic acid.[11] Resolution of that precursor into enantiomers was accomplished as follows. The precipitate obtained from addition of 2.5 g of (*S*)-(−)-α-methylbenzylamine to 4.2 g of the racemic acid in 200 mL of ethyl acetate was recrystallized (6×) to constant melting point from ethyl acetate, yielding 2.27 g of the amine salt of a single enantiomer: mp 148–149 °C; $[\alpha]_D$ −7.98° (*c* = 6.14, MeOH). The amine was extracted from the combined mother liquors by washing with dilute hydrochloric acid, and (*R*)-(+)-α-methylbenzylamine was then added as before. Repeated recrystallization yielded 2.44 g of the enantiomeric amine salt: mp 148–149 °C; $[\alpha]_D$ +8.06° (*c* = 6.58, MeOH). For preparation of L-**5**, 1.33 g (3.3 mmol) of the latter salt in 100 mL of chloroform was washed (4×) with 50 mL of 2 N hydrochloric acid and then with water. The chloroform solution was dried and concentrated to 6 mL. To a cold solution of 1.34 g (10 mmol) of *N*-chlorosuccinimide in 50 mL of acetonitrile was slowly added the chloroform solution of the resolved acid with simultaneous passage of anhydrous ammonia through the solution (NH_4Cl precipitate). The mixture was then stirred at 0–25 °C overnight. The precipitate was removed, and the filtrate was evaporated to yield solid succinimide plus an oil. The residue was extracted with methanol, and the solute was submitted to preparative plate silica chromatography (EtOAc–MeOH, 3:2). Material with an R_f value of ca. 0.2 was recovered by extraction with methanol. Recrystallization from methanol–acetone yielded 0.28 g (35%) of [L-(−)-2-carboxy-3-phenylpropyl]methylsulfodiimine (**5**): mp 134 °C dec; $[\alpha]_D$ −35.9° (*c* = 5.9, MeOH); IR (KBr) 1390 and 1580 cm^{-1} ($-CO_2^-$); ^1H NMR (D_2O) δ 2.73–2.97 (m, 3 H), 3.24 (s, 3 H), 3.45 (d, *J* = 13.7 Hz, 1 H), 3.90 (dd, *J* = 11.4 and 13.7 Hz, 1 H), and 7.12–7.27 (m, 5 H); ^{13}C NMR (D_2O) δ 38.33, 44.06, 45.16, 59.27, 126.83, 128.70, 129.17, 138.30, and 178.99. Anal. Calcd for $C_{16}H_{16}N_2O_2S$: C, 54.98; H, 6.71; N, 11.66; S, 13.34. Found: C, 54.91; H, 7.06; N, 11.39; S, 13.21.

Similarly obtained from the (*S*)-(−)-α-methylbenzylamine salt was the enantiomer (D-**5**): mp 134 °C; $[\alpha]_D$ +36.8° (*c* = 6.3, MeOH). Racemic **5** possessed mp 116 °C.

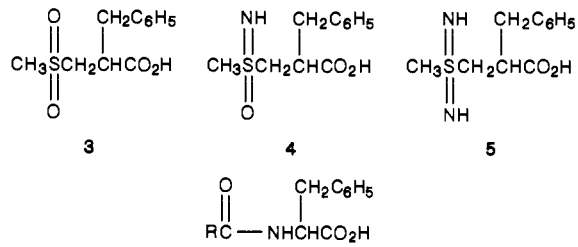
Preparation of racemic (2-carboxy-3-phenylpropyl)methylsulfoximine (**4**) has been described.[11] For the preparation of (±)-2-carboxy-3-phenylpropyl methyl sulfone (**3**), the corresponding carboxy thioether was heated in acetic acid with an excess of 30% hydrogen peroxide, giving after recrystallization from chloroform–heptane a 79% yield of racemic **3**: mp 107 °C; IR (KBr) 1140, 1300 (>SO_2), and 1700 ($-CO_2H$) cm^{-1}. Anal. Calcd for $C_{11}H_{14}O_4S$: C, 54.52; H, 5.82; S, 13.23. Found: C, 54.81; H, 5.96; S, 13.10.

Kinetic Analysis. For **3–5** the pH dependence of K_i with both ZnCpA and CoCpA was determined at 25.0 (±0.1) °C in buffers previously listed with FAPP as assay substrate at an initial concentration of $0.12K_m$, with spectrophotometric (328 nm, 1–5-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 perturbations 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 indicated in this article are standard errors from least-squares analysis.

Proton NMR. Paramagnetically shifted signals for the metal ion coordinated ligands may be observed for Co(II) carboxypeptidase A (0.89 mM) complexed with excess **5** (1.36 mM) for the spectral region δ > 10.[12,13] A series of 200-MHz spectra were obtained of **5**·CoCpA in deuterium oxide solution (1.0 F NaCl, buffered with Mes and Tris, 5.0 mM each), covering the region δ 0–80, at room temperature and at various pD values, by the Fourier transform technique, using a presaturation pulse sequence in order to suppress the signal of residual HDO. For estimation of pD values with the glass electrode, the usual correction was applied (pD = pH meter reading + 0.4). Changes in the spectrum upon adjustment of pD were shown to be reversible.

Results

Characterization of New Inhibitors. The series of structurally related potential inhibitors **3–5** appears to offer a unique opportunity to examine coordination chemistry for the essential metal ion within carboxypeptidase A. Each structure may be regarded as an analogue of an *N*-acylphenylalanine substrate **6**, with the sulfur-containing functional group occupying the location of the enzymically cleaved carboxamide linkage. The latter is believed to interact in Lewis acid–base fashion with the active site metal ion in the course of peptide hydrolysis. These substances have been prepared by us, and the sulfodiimine **5** has also been resolved into its enantiomers. The sulfoximine **4** may exist as two diastereomers, but apparently our synthesis yields a single (racemic, *RR*:*SS*) isomer, according to an NMR NOE study.[11] Although **4** has a propensity to cyclize to a lactam at a pH of 3 as has been previously described,[11] less acidic solutions (which are employed in this study) are adequately stable, as is also the case for **3** and **5**.



3 4 5

6

Because interest focuses on the metal ion ligating properties of the sulfur–imine moieties, an effort was made to establish the *proton affinity* of this functionality in the cases of **4** and **5**. For the inhibitor **5** a ^{13}C NMR spectral titration (Figure 1) in aqueous solution provided pK_a values for both the carboxylic acid (pK of 3.20) and the protonated sulfodiimine group (pK of 5.85). The value for N-protonation is in excellent agreement with literature values for dialkylsulfodiimines.[7] For the inhibitor **4** a corresponding attempt at ^{13}C NMR titration was frustrated by lactam cyclization and precipitation of the substrate near a pH of 3. However, the spectrum appeared pH invariant at higher and lower acidities, and since literature precedent suggests a pK_a of ca. 3

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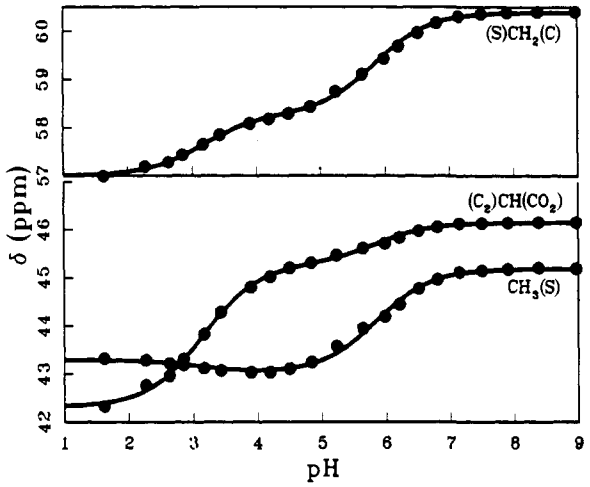

Figure 1. ^{13}C NMR titration curves for **5**. Biphasic sigmoidal fit of δ vs pH for signals from **5** in 1.0 F NaCl solution (10% D_2O, dioxane internal reference), yielding pK_a values of 3.20 (±0.04) and 5.85 (±0.04) for $-CO_2H$ and $>SN_2H_3^+$ groups, respective. Off-resonance decoupling employed to aid in spectral assignments.

for the protonated sulfoximine functional group,[6,7] it is likely that both carboxylic acid and protonated sulfoximine groups have nearly the same pK_a in **4**. The chemical behavior would then be associated with an isoelectric point, as seems reasonable. The sulfone functional group of **3** should be devoid of proton affinity; the literature suggests a pK_a of −11 for its conjugate acid.[14] No beneficial interaction of the oxygen atoms of this group with an active site metal ion would be anticipated. Therefore, **3** should be suited to serve as a *control* in evaluating the enzyme inhibitory behavior of **4** and **5**. By inference, the single oxygen atom of the sulfoximine group in **4** should also not coordinate with metal ions.

Inhibition Patterns. The separated enantiomers of sulfodiimine **5** differed considerably in their inhibition of the catalytic activity of carboxypeptidase A. At a pH of 7.45, the dextrorotatory form of **5** yielded a competitive K_i of 0.42 (±0.01) mM, which is 153 times *greater* than that for the levorotatory form of comparable optical activity. From this it may be concluded that the resolution was substantially (>99%) complete and that the tighter binding form, L-**5**, has the same configuration as have typical substrates containing L-phenylalanine as the C-terminal (cleaved) residue (**6**). For the inhibition studies subsequently described, the designation **5** shall refer to the levorotatory (L) form exclusively. No resolution was carried out on **3** or **4**, which have been employed as the racemates, with an adjustment to the apparent value of K_i where appropriate (as described subsequently).

Both **4** and **5** were shown to be strictly competitive inhibitors in the enzymic hydrolysis of peptide substrate FAPP by carboxypeptidase A. With **5** at concentrations of 1.6 or 4.0 μM, there was a substantial retardation of k_{cat}/K_m, but no perturbation of k_{cat}. Similar results were obtained with **4**. For a pH of 7.45–7.50, the relative competitive inhibition constants are as follows: **5**, K_i = 2.73 (±0.11) μM; **4**, K_i = 28.4 (±1.0) μM (corrected for inhibition by L isomer only); **3**, K_i (with $S \ll K_m$ in assay also) = 10.3 (±0.5) mM (no correction for configuration, since both L and D isomers might be expected to be active in this concentration range).

pH Dependence for K_i. The inhibitors **3–5** exhibit a complex 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 Figure 2, K_i values with ZnCpA achieve their lowest values (pK_i maximum) around a pH of 6, with diminished affinity of the inhibitors for the enzyme at higher pH. In Figure 3 analogous plots are shown for **4** and **5** with CoCpA [i.e., the fully catalytically competent enzyme in which Co(II) has been substituted for Zn(II) as the active-site metal ion]. In the cases of **4** and **5** with both enzymes the pH profiles are not

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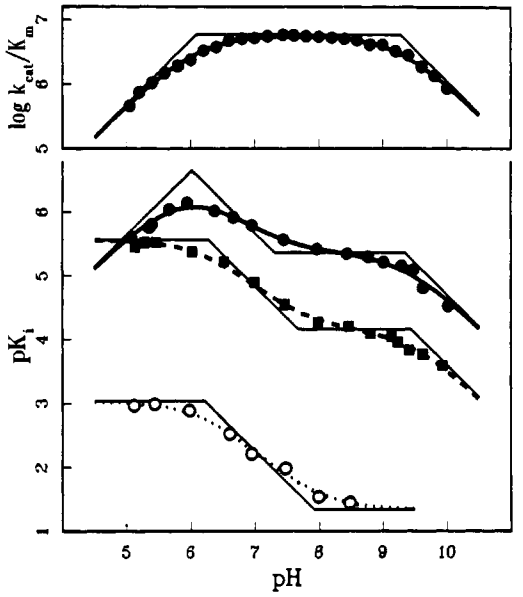


Figure 2. log–log plots of pH dependence for k_{cat}/K_m and for K_i with ZnCpA. Top: log (k_{cat}/K_m) vs pH for hydrolysis of FAPP (10 μM) in buffered saline solution. Bottom: pK_i vs pH for **5** (filled circles, solid line), **4** (filled squares, dashed line), and **3** (open circles, dotted line). Breaks in asymptotic straight lines (slopes −1, 0, and +1) denote pK_a values obtained by least-squares fitting of data to appropriate equations as described in text.

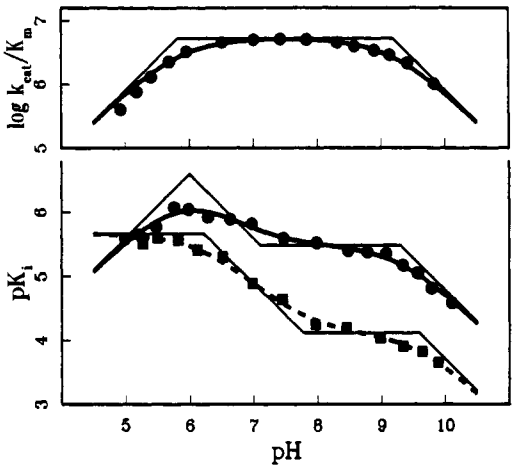


Figure 3. log–log plots of pH dependence for k_{cat}/K_m and for K_i with CoCpA. Top and bottom symbol codes and asymptotic lines as for Figure 2.

simple, and an input of external evidence is required to warrant fitting a curve to the data as has been done in the figures. One such extrakinetic factor is the pK_a value for the sulfur–imine functional groups as cited previously (specifically, a pK_a of 5.85 for **5**). Additionally, there are functional groups on the enzyme, deprotonation of which perturbs the kinetics of substrate hydrolysis and which might be expected to influence binding of **3–5**. The relevant evidence is the pH dependence of k_{cat}/K_m, the second-order rate constant for peptide hydrolysis at extreme substrate dilution, which is also shown as Dixon plots in Figures 2 and 3. As is well-known, the enzyme activity diminishes on both the acidic and alkaline limbs of the pH profile, with the following characteristic kinetic parameters as recorded in our laboratory:[13] ZnCpA, $(k_{cat}/K_m)_{lim} = 5.89 \times 10^6$ M^{-1} s^{-1}, pK_1 = 6.09, and pK_2 = 9.28; CoCpA, $(k_{cat}/K_m)_{lim} = 5.27 \times 10^6$ M^{-1} s^{-1}, pK_1 = 5.82, and pK_2 = 9.19. A factual assignment of the enzymic residues responsible for the pH perturbations has recently appeared.[13] Some similarities in the curves for hydrolysis and inhibition may be noted. For example, the pK_i vs pH profile for **5** likewise diminishes at the extremes of pH, although the fitted curve appears to have an additional concavity near neutral pH, which requires explanation.

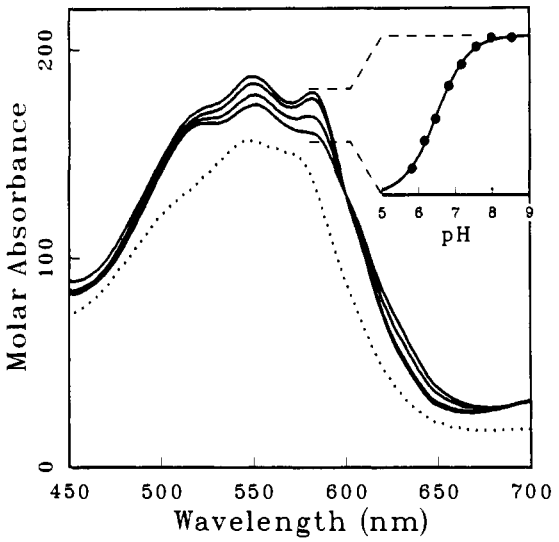


Figure 4. Visible absorption spectra of CoCpA. Dotted line: free enzyme (0.58 mM, referenced against air). Solid lines: same enzyme in the presence of an excess of **5** at pH values of 5.81, 6.45, 7.16, and 7.56. Inset: Least-squares fit of A_{582} (×3) vs pH to sigmoidal expression, yielding a pK_a value of 6.48 (±0.02).

Spectra of 5·CoCpA Complex. Since the pK_i vs pH profiles necessarily display influences on *equilibrium binding*, deprotonations occurring within the EI complex as well as the free enzyme and the inhibitors will affect the apparent value of K_i. Consequently, spectral examination of enzyme complexes with **4** and **5** was undertaken. This is feasible in the case of CoCpA, because the enzyme with Co(II) chelated at the active site has a characteristic visible absorption, as well as certain unique NMR signals, which respond to the presence of external ligands occupying the active site. For example, we have recently described a series of synthetic inhibitors for carboxypeptidase A, which have been designed to present a *phenolate ion* for coordination to the active-site metal ion.[3] Examination of the spectra of EI complexes of CoCpA in those cases revealed a dramatic perturbation of the coordination sphere of the metal ion, best interpreted as a change in ligand number from five to four in consequence of inhibitor binding.[13]

When **5** binds to CoCpA, the spectral consequences are more subtle, as shown in Figure 4. The cobalt enzyme by itself in buffered saline solution has a broad absorption maximum centered on 550 nm ($\epsilon \sim 150$ M^{-1} cm^{-1}) with a shoulder near 500 nm (Figure 4, dotted line). This spectrum is noticeably perturbed upon addition of 1 molar equiv of **5** but not further with extra increments of inhibitor. Complexation of **5** to the enzyme yields CoCpA absorption bands at 519, 544, and 582 nm (ϵ only slightly enhanced). The overall shape of the spectrum of the EI complex is similar to that of CoCpA itself. In comparison with results of phenolate ligation to the enzyme,[13] **5** does not appear to perturb the coordination geometry of the active-site metal ion. In fact, the spectrum by itself does not conclusively establish that the sulfodiimine group coordinates to the Co(II) ion. Therefore, the influence of pH upon the visible absorption spectrum was examined.

For spectral titrations, an excess of **5** was present in the CoCpA solution to ensure that binding of inhibitor to enzyme remained complete throughout the pH range of 5.5–8.5. Appropriate computations with the experimental K_i values confirm that this is so. As may be seen from the series of visible absorption spectra in Figure 4, an increase in pH from 5.81 to 8.53 progressively enhances a shoulder near 519 nm as well as the maxima at 544 and 582 nm, while concurrently reducing the shoulder near 625 nm (changes reversible on pH decrease). An isosbestic point was observed at 611 nm. Fitting the absorption intensity at 582 nm vs pH results in a sigmoidal curve and provides a spectrophotometric pK_a of 6.48 (±0.02) for the complex of **5** with CoCpA (Figure 4, inset). We shall subsequently suggest that this pK_a is also manifested in the pK_i vs pH profile (Figure 3).

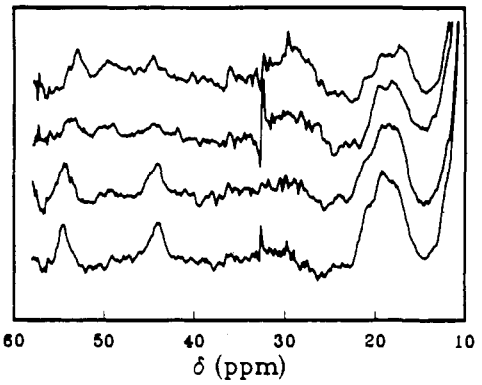


Figure 5. ^{1}H NMR spectra (200 MHz) of complex of CoCpA and **5** in buffered saline–deuterium oxide solution. Top to bottom: pD values of 6.3, 6.7, 7.0, and 7.9, respectively. For interpretation of paramagnetically shifted signals, see text.

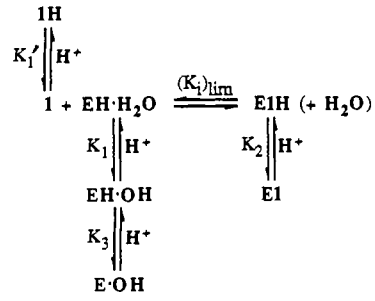
A visible absorption spectrum of CoCpA complexed with inhibitor **4** was also obtained (not shown). It is generally similar to the complex with **5**; however, no pH-induced spectral perturbations were ascertainable in that case.

In a attempt to identify the proton-bearing functional group in the **5**·CoCpA complex which yields the perturbation in the visible absorption spectrum, an NMR investigation was undertaken. Due to a pronounced spectral shift induced by paramagnetic effects of unpaired electrons within the Co(II) ion bound at the active site, resonances from ^{1}H nuclei within the immediate environment of the metal ion occur well downfield from their normal spectral positions and are not enveloped by signals from the rest of the hydrogen atoms of the enzyme.[12] As a useful example, we have recently been able to assign the alkaline-limb pK_a value noted in the k_{cat}/K_m vs pH profiles (Figures 2 and 3) to an enzymic *imidazole* ligand of the active-site metal ion (His 196), on the basis of certain pH-induced ^{1}H NMR spectral perturbations of paramagnetically shifted signals from these residues for CoCpA.[13] In Figure 5 is presented the downfield portion of the ^{1}H NMR spectrum of the CoCpA complex with **5** in D_2O saline solution, at several different pD values. At a pD value of 7.9, two well-resolved NMR signals at δ 54.5 and 43.8 are seen, attributable to the C(4)H imidazole ("meta" position) ring hydrogens of active-site residues His 69 and His 196, respectively.[13] It is N(1) of these histidine side chains (together with Glu 72) which hold the metal ion at the active site. Coordination to the paramagnetic Co(II) species explains the extreme downfield position of the signals and their broadness. An additional ^{1}H NMR signal at δ 18.6 is also observed, which we attribute to the CH_3 and CH_2 moieties flanking the sulfodiimine functional group of **5** bound to CoCpA. In the uncomplexed enzyme this signal is absent (or much diminished; the CH_2 next to the side-chain carboxylate of the third enzymic metal ion ligand, Glu 72, may also fall in this region). Upon lowering pD, the signal at δ 18.6 decreases in intensity, and another signal at δ 28.7, presumedly assignable to the same group in the protonated EI complex, appears progressively. This change is reversible upon increase of pD. Unfortunately, lower pD values than displayed could not be employed for a quantitative estimation of the pK_a for the transition, because of enzyme instability in acidic solution and line broadening of NMR signals. The observation that the inhibitor so strongly experiences a paramagnetic influence of the Co(II) ion tends to confirm that the sulfodiimine functional group indeed coordinates to the active-site metal ion of the enzyme. The pronounced pH dependence for δ also indicates the occurrence of ionization of a substituent within or very near to the coordination sphere of the metal ion, which influences both visible absorption and NMR spectra, and very probably K_i as well.

Discussion

The purpose for preparation of inhibitors **3**–**5** and our extensive examination of their interaction with carboxypeptidase A was an

Scheme I[a]



[a] $(K_i)_{apparent} = (K_i)_{lim}(1 + H/K_1')(1 + K_1/H)(1 + K_3/H)/(1 + K_2/H)$.

anticipation that sulfur–imine-containing functional groups would interact in an informative way with the active-site metal ion of the enzyme. Indeed, a high degree of specificity is shown in the binding of **4** and **5**, suggesting that the novel functional groups may find a useful role if suitably placed within inhibitors targeted for more biomedically significant proteases. Since **4** and particularly **5** are several orders of magnitude more efficacious than **3** for inhibiting carboxypeptidase A, sulfur–imine ligation to the metal ion seems probable. However, before a detailed interpretation of the mode of binding for **4** and **5** to carboxypeptidase A can be offered, some explanation of the complicated pH dependence for K_i as manifested in Figures 2 and 3 must be provided.

Beginning with **5**, the experimental value for pK_i appears to peak (tightest binding) near a pH of 6. This is readily explained. The pK_a of the water molecule which coordinates to the active-site metal ion in the absence of a substrate has approximately this value; i.e., above a pH of 6 *hydroxide* is the predominant external ligand completing the coordination sphere of that ion (L_nM^+–OH). Convincing evidence for this catalytically significant assignment (e.g., the acid-limb pK in the k_{cat}/K_m vs pH profiles, Figures 2 and 3) has been presented in studies of the *reverse protonation inhibition* of carboxypeptidase A by specifically designed phenolate-containing inhibitors.[3,13] In addition, we have unambiguously shown that the pK_a of the *sulfodiimine* group in **5** also has approximately this value (Figure 1). Quite obviously, effective coordination of a nitrogen atom in **5** to the metal ion in ZnCpA or CoCpA would require the *N* to be unprotonated (pH ≥6), while L_nM^+–OH would have to exist as its conjugate acid (pH ≤6). In more acidic solutions the enzyme must compete with protons for the sulfur–imine (apparent pK_i decreases); in more alkaline solutions hydroxide ion competes with **5** for the metal ion (apparent pK_i also decreases). In confirmation of this interpretation, the pK_i vs pH profile for **4** does not show a drop-off on the acid limb; protonation of its sulfur–imine should occur only at a pH of ≤3 (which is an enzymically inaccessible region).

The extreme alkaline limb of the pK_i vs pH profiles for **4** and **5** may similarly be rationalized by comparison with the pH dependence of catalytic activity (k_{cat}/K_m). The fall-off in the rate of hydrolysis of peptide substrates at pH values of >9 has been shown to correlate with the deprotonation of an *imidazole* ligand (most probably His 196) which bears the role of helping to hold the metal ion at the active site (NMR evidence[13]). Since a strongly electron-donating *imidazolate* coordinated to the metal ion would substantially reduce the Lewis acidity of Zn(II) or Co(II) toward additional external ligands, it is entirely expected that the affinity of the sulfur–imines for the enzyme should diminish (pK_i decrease) above a pH of 9, as is observed.

All that remains to be explained is the gradual decrease in the experimental values of pK_i between a pH of 6 and 9. Our interpretation, subsequently to be justified, is that deprotonation of an EIH complex occurs in this region, such that the slope of the pK_i vs pH profile (the gradient of which according to the preceding explanation should attain a value of −1 at a pH of <6) is effectively leveled out. The collective interpretation expressed above may be summarized diagramatically as in Scheme I. According to this interpretation there are two relevant ionizations of the *free enzyme*: that of the metal-bound water, K_1, and that

Table I. Kinetic Parameters Fitting pH Dependence of K_i for **3–5** (According to Schemes I and II)

EI	$10^6(K_i)_{lim}$ (M)	pK_1	pK_2	pK_3
ZnCpA·**5**	0.22 (±0.01)	6.02 (±0.04)[a]	7.31 (±0.06)	9.34 (±0.07)
CoCpA·**5**	0.26 (±0.02)	6.00 (±0.06)	7.11 (±0.08)	9.30 (±0.08)
ZnCpA·**4**	2.7 (±0.4)[b]	6.28 (±0.10)[c]	7.68 (±0.07)	9.44 (±0.08)
CoCpA·**4**	2.2 (±0.5)	6.23 (±0.15)	7.78 (±0.09)	9.59 (±0.10)
ZnCpA·**3**	910.0 (±80.0)	6.27 (±0.09)	7.92 (±0.09)[d]	

[a] For **5**, pK_1' parameter (for protonation of sulfodiimine group) forced to equal pK_1 of enzyme (Scheme I). [b] For **4** (racemic material), K_i values corrected to reflect activity for L isomer only. [c] For **4**, pK_1' parameter ~3.0 (fixed). [d] For **3**, pK_2 attributed to bound water molecule in $E\cdot H_2O\cdot I$ (see Scheme II and text); pK_1' nonexistent.

of metal-coordinated imidazole, K_3, both of which adversely affect binding of inhibitor. An as yet unidentified deprotonation of the *EIH complex*, K_2, partially negates one of these. In the case of **5**, *N*-protonation of the *sulfodiimine*, K_1', also disfavors effective inhibition. Shown also in Scheme I is the corresponding equation which yields an expected pH dependence of the observed value for K_i in terms of a *limiting value* for K_i and the appropriately cited acid dissociation constant parameters. The success of this interpretation is demonstrated by the curved lines in Figures 2 and 3, which are a least-squares fit of the data to that equation. Breaks in the asymptotic straight lines denote the fitted pK values. The derived parameters (limiting value for K_i plus pK_a values) are listed in Table I. Such a multiparameter treatment of a restricted data set would ordinarily be scientifically dubious; however, in this instance it is justified because each of the introduced pK values has *independent substantiation.* In the low-pH region, K_1 and K_1' (which for **5** are so close in numerical value that they must be set identical with one another for statistical analysis) correspond reasonably to a mean value between the known inhibitor ionization (**5**, pK_a of 5.8, ~pK_1') and the enzymic deprotonation seen in the acid limb for k_{cat}/K_m (ZnCpA·H_2O, pK_a of 6.1; CoCpA·H_2O, pK_a of 5.8; both ~pK_1). Likewise, K_3 on the alkaline limb is in each case in reasonable agreement with the high-pH proton dissociation which is independently seen in k_{cat}/K_m (imidazole metal ligand: ZnCpA, pK_a of 9.3; CoCpA, pK_a of 9.2; both ~pK_3). The intermediate deprotonation, K_2, must be that of an EI complex, according to the direction of the inflection it imparts to the curve.[15] Indeed, we have noted in the pH dependence of the visible absorption spectrum for **5**·CoCpA just such a perturbation, yielding an appropriate sigmoidal curve (Figure 4). The discrepancy between the kinetic pK_a of 7.1 and the spectrophotometric pK_a of 6.5 is not regarded as significant; the measurements are taken with grossly different enzyme concentrations, the shape of the pK_i vs pH profile in this region can support a lower pK_a value nearly as well, and the spectrophotometric pK_a arises from a rather small change in absorption. Furthermore, the ^1H NMR spectrum of **5**·CoCpA also exhibits pH-induced spectral perturbations which appear to correlate with this pK_a (Figure 5). What is the group responsible for this EIH deprotonation? Clearly a functionality in the vicinity of the metal ion is responsible. Metal ion ligands contributed by the *enzyme* apparently may be excluded; the $L_nM^{2+}\cdot OH_2$ water molecule which ionizes in the free enzyme has in all likelihood been displaced by substrate, and the histidine ligands of the metal ion do not display an ^1H NMR perturbation in Figure 5, as they do when one of them suffers deprotonation in the free enzyme.[13] By elimination, we are left with deprotonation of the *imino* ligand of the inhibitor within the coordination sphere of the metal ion: $(S)=NH\rightarrow M^{2+} \rightarrow (S)=N-M^+ + H^+$. While we find it surprising that the active-site metal ion should so strongly acidify an NH group (extension of the ^{13}C NMR titration in Figure 1 up to a pH of 13 reveals no indication of N-deprotonation), such an ionization would indeed tend to strengthen binding of inhibitor to the enzyme, and result in a leveling-off of the pK_i vs pH profile, as is observed (Figures 2 and 3). This phenomenon is perhaps another manifestation of the Lewis acidic potency of the active-site

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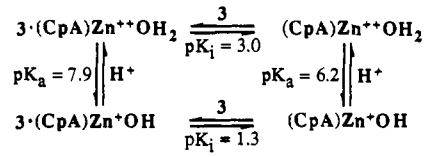
Scheme II



metal ion, an observation which carries mechanistic significance for the hydrolysis of peptide substrates.[3]

The pH dependence of K_i for **4** is explained analogously to that of **5** and requires no special consideration. Not previously commented upon is the pH dependence of pK_i for the sulfone **3**. Although of much weaker affinity for carboxypeptidase A, it has a profile which bears a superficial similarity to that of **4** and **5**. The data are shown fitted to a simple sigmoidal curve (Figure 2). There is no expectation that the sulfone group should coordinate to the active-site metal ion (which is presumably why **3** binds relatively weakly). In fact, the pK_i vs pH profile for **3** is well precedented; comparably weak inhibitors such as 3-phenylpropionic acid, D- and L-3-phenyllactic acid,[16] and the ketone-containing substrate analogue BMBP[17] all exhibit similar curves. To the best of our knowledge a complete and satisfactory explanation of the nature of this pH dependence has not previously been given.[3] We believe the key is that no member of this collection of inhibitors displaces the water molecule bound to the active-site metal ion of the enzyme (as has specifically been shown to be the case for BMBP[18]). However, we hypothesize that occupancy of the active site by **3** *perturbs* the pK_a of $L_nM^{2+}\cdot OH_2$, raising it from a value of 6.2 in the free enzyme to a value of 7.9 in the EI complex. This is chemically not unreasonable and would yield the sigmoidal curve shown in Figure 2, in which the high and low limiting values for pK_i correspond to binding to the $L_nM^{2+}\cdot OH_2$ and the L_nM^{+}–OH forms of the enzyme, respectively. This simple interpretation, summarized in Scheme II, also has mechanistic significance. The *initial* act of complexation of a peptide substrate with carboxypeptidase A (i.e., before the peptide linkage has had time chemically to modify the active-site metal ion) should similarly perturb the pK_a of $L_nM^{2+}\cdot OH_2$, perhaps raising it by as much as 2 units. This realization should make our previously postulated reverse-protonation mechanism for enzymic activation of peptide substrates more palatable, since the kinetic disadvantage of reverse protonation is substantially dissipated by a pK_a perturbation of this nature in the process of enzyme–substrate complexation.[3] In short, a pK_a of 6.2 for $L_nZn^{2+}\cdot OH_2$ in the *free* enzyme does not require that L_nZn^{+}–OH be the catalytically active species; nor is there even a probability that should be so for peptide hydrolysis, since the pK_a value would be elevated upon initial substrate complexation.[19]

Finally, what may be said about the *geometry* of the sulfur–imine interaction with the active-site metal ion for **4** and **5**? A major motivation in preparing these inhibitors was a hope that **5**, which is potentially capable of *bidentate* coordination to the metal ion (using both nitrogens), would thereby bind more tightly than **4**, which contains only a single basic ligand donor. Others have attached much significance to the high affinity of phosphonyl-containing inhibitors for metalloenzymes, suggesting that the ligation of a $>PO_2^-$ group mimics a "four-center" concerted transition state for peptide hydrolysis. In that mechanism incipient ligand hydroxide, initially attached to the metal ion, attacks and migrates to the scissile carboxamide, *as* the amide carbonyl oxygen enters the coordination sphere of the metal ion.[1h,i,20] While we hold no disapprobation of this speculation,[21] we find no substantiation of its interpretation within our data. Although **5** binds exceedingly well to carboxypeptidase A (and comparably as well as structurally similar phosphonamidate inhibitors), inspection of $(K_i)_{lim}$ values in Table I reveals that its affinity for the enzyme is only 8–12 times that of **4**. Since the nitrogen atoms of **5** have higher *basicity* than that of **4** *by a greater margin* than this (comparison of pK_a values, even with a statistical allowance for two nitrogens in **5**), the additional stabilization with **5** may be explained adequately with unidentate ligation and without recourse to an additional input from the second nitrogen on sulfur. Furthermore, the visible absorption spectrum of **5**·CoCpA (Figure 4) also provides no indication of a unique geometry for the metal ion, neither in comparison with the uncomplexed enzyme nor as a consequence of prototopic change involving the ligated nitrogens. The stereochemical question for **5** can probably only be firmly answered crystallographically. However, even should **5** prove to show some bidentate participation, our conclusion would remain; *no energetic advantage* accrues to this mode of coordination relative to that obtainable by an analogous unidentate ligand of similar pK_a. Consequently, **5**, considered as a transition-state mimic which ought to complement the active site, does not exhibit any special enzyme affinity which would support a "four-center" mechanism for peptide hydrolysis.[21]

In conclusion, the chemical mechanism by which carboxypeptidase A cleaves peptide linkages remains unresolved, although an establishment of the protonation state of critical active-site residues[13] and the changes which substrate complexation induces in them, along with the binding energy comparison of **4** and **5**, ought to provide some restraints on mechanistic speculation. In a practical vein, attention is drawn to the high affinity which the sulfodiimine functionality confers toward a metalloenzyme: **5**, $(K_i)_{lim}$ = 0.22 μM [comparable to *N*-(methylphosphonyl)-phenylalanine, K_i = 0.12 μM[1c]]. This easily introduced functional group is not only chemically unreactive under biological conditions; with a pK_a of 5.8 it also would be *uncharged* at physiological pH (in contrast with the phosphonyl group). Hence, it is potentially capable of allowing inhibitors in which it is contained to traverse membrane barriers. Incorporation into substrate analogues targeted to interact with medicinally important enzymes is therefore attractive.

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(20) Sayre, L. M. *J. Am. Chem. Soc.* **1986**, *108*, 1632–1635.
(21) It is not intuitively apparent to us why a "four-center" mechanism should be energetically more favorable than any of a number of nonconcerted alternatives. Coordination of the nucleophile (hydroxide) to the active-site metal ion can only reduce the latter's Lewis acidity in its role of activating the substrate carboxamide. Any gain in promoting the nucleophile ought to be negated by diminished capacity to active substrate.