pH-Dependent Properties of Cobalt(II) Carboxypeptidase A-Inhibitor Complexes

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Received September 24, 1991; Revised Manuscript Received January 28, 1992

ABSTRACT: ^{1}H NMR spectroscopy of the isotropically shifted signals in cobalt carboxypeptidase, CoCPD, permits a direct and selective detection of protons belonging to the residues liganded to the metal. The chemical shift of these protons in the free enzyme and enzyme-inhibitor complexes with changing pH monitors the state of ionization of the ligands directly and of other residues in the active center indirectly. The ^{1}H NMR spectrum of CoCPD at pH 6 shows three well-resolved isotropically shifted signals in the downfield region at 62 (a), 52 (c), and 45 (d) ppm which have been assigned to the NH proton of His-69 and to the C-4 H's of His-69 and His-196, respectively. Titration of signal a with pH is characterized by a pK_a of 8.8 which is identical to that seen in prior electronic absorption and kinetic studies. The fact that the signal reflecting the NH of His-69 is still observed at pH 10 and no major shifts occur for the signals reflecting the C-4 H's indicates the alkaline pK_a in carboxypeptidase A catalysis, pK_{EH}, cannot be ascribed to ionization of the histidyl NH of either His-69 or His-196. Binding of L-Phe shifts this pK_a to 7.7 while not greatly perturbing the downfield ^{1}H NMR signals that reflect the ligation shell of the cobalt coordination sphere. These results indicate the pK_a of 8.8 in CoCPD and the pK_a of 7.7 in the CoCPD·L-Phe adduct reflect ionization of the same group. In conjunction with previous kinetic studies of L-Phe inhibition, it can now be ascertained that the protonated α-amino form of L-Phe (IH) binds 50-fold tighter to the ionized form of the enzyme (E). L-Phe binding as its protonated α-amino group likely disrupts the Glu-270 carboxylate–metal water interaction by forming a salt link between the Glu-270 carboxylate and the α-amino group. ^{1}H NMR shows N_3^- binds to the protonated adduct, EHIH, while a second D-Phe binds to the ionized adduct EHI. The roles of the ionization of the metal-coordinated water and Tyr-248 in these processes are discussed.

Carboxypeptidase A_α (ZnCPD)[1] is an exopeptidase containing a zinc ion bound to His-69, Glu-72, and His-196 of a single polypeptide chain of 307 amino acids (Rees et al., 1986). While the pancreatic bovine enzyme has been the most extensively studied, the sequences of carboxypeptidase E or enkephalin convertase (Fricker et al., 1986), carboxypeptidase N (Gebhard et al., 1989), and carboxypeptidase M (Tan et al., 1989), human enzymes which process or regulate hormones, all have potential metal binding sites very similar to those of the carboxypeptidases A and B from bovine, rat, mouse, and crayfish sources (Vallee & Auld, 1990).

A number of kinetic and spectroscopic investigations on the pancreatic enzyme have been aimed at characterizing the molecular mechanism of its catalytic activity (Vallee et al., 1983; Auld & Vallee, 1987). The crystal structure of the native enzyme (Lipscomb et al., 1968) and of several adducts with inhibitors and a pseudosubstrate has been determined (Rees et al., 1983; Christianson & Lipscomb, 1989). Replacement of the spectroscopically silent zinc(II) ion with high-spin cobalt(II) provides a derivative which retains the structural features (Hardman & Lipscomb, 1984) and the catalytic properties of the native enzyme (Auld & Vallee, 1970) and is at the same time suitable for spectroscopic investigations (Vallee et al., 1983; Bertini & Luchinat, 1984). ^{1}H NMR spectroscopy of the isotropically shifted signals in CoCPD has proven to be particularly useful since it permits a direct and selective detection of the protons belonging to the residues liganded to the metal. The chemical shift of these protons with changing pH monitors the state of ionization of the ligands directly and of other residues in the active center indirectly (Bertini & Luchinat, 1983, 1986).

Previous ^{1}H NMR, EPR, electronic spectroscopy, and kinetic studies have led to the proposal of a two-site model for anion binding in which the mutual relationships between the metal binding site and a second "trigger" site are stressed (Williams & Auld, 1986; Bicknell et al., 1988; Bertini et al., 1988a,b, 1990a,b; Luchinat et al., 1988; Martinelli et al., 1989; Bal et al., 1990). Direct binding of anions to the metal occurs with very low affinity unless the trigger site is occupied by amino acids or carboxylate inhibitors. The access of the anions to the metal is probably hindered by the presence of a Glu-270 hydrogen bond to the water coordinated to the metal; only by displacing the carboxylate group of the latter residue is the metal made more accessible to anions.

A crucial point of carboxypeptidase chemistry which still needs clarification is the understanding of its acid–base properties pertaining to binding substrates and inhibitors. The dependence on pH of the catalytic activity of carboxypeptidase A has been extensively investigated (Auld & Vallee, 1970, 1971; Bunting & Chu, 1976; Makinen et al., 1984; Auld et al., 1986; Mock & Tsay, 1988). The enzyme clearly exhibits the presence of two crucial pK_a's respectively located at pH 6 and pH 9; the nature of the essential ionizable groups which are responsible for these acid–base equilibria is still controversial (Gardell, 1985; Hilvert et al., 1986; Mock & Tsay,

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[1] Abbreviations: ZnCPD, native carboxypeptidase A; CoCPD, cobalt(II)-substituted carboxypeptidase A; Mes, 3-(*N*-morpholino)ethanesulfonic acid; N_3^-, azide; Z, carbobenzoxy; ClCin, chlorocinnamoyl; OPhe, phenyllactate.

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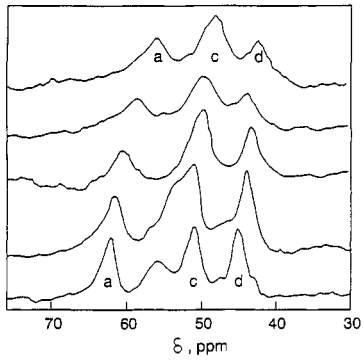

FIGURE 1: ^{1}H NMR spectra of CoCPD (1 mM) in 1 M NaCl/20 mM Mes, pH 6.0 at 25 °C, at the bottom and increasing pH values of 7.3, 8.3, 9.2, and 10.0.

1988; Auld et al., 1989). Therefore, to shed further light on the pH-dependent properties of the enzyme, we have undertaken an extensive spectral investigation of CoCPD and some of its binary and ternary adducts with inhibitors, through visible and ^{1}H NMR spectroscopy, in the pH range 6–10. Analysis of the differential pH-dependent behavior of structurally distinct derivatives provides a deeper insight into the acid–base properties of the system. The results of these spectroscopic studies permit identification of some of the general features of acid–base reactivity which are in good agreement with the two-site model describing enzyme reactivity toward anions.

Experimental Procedures

Bovine carboxypeptidase A prepared by the method of Cox et al. (1964) was purchased from Sigma Chemical Co. and further purified through affinity chromatography on CABS–Sepharose to remove protease contaminants (Bicknell et al., 1985). All reagents used were of analytical grade.

Metal removal and cobalt(II) replacement were performed as previously described (Auld, 1988). Protein crystals were dissolved in 0.02 M Mes buffer, pH 6.0, in the presence of 1 M NaCl. Formation of the cobalt(II)-substituted enzyme was monitored through electronic absorption spectroscopy. The various derivatives of CoCPD were prepared as previously reported (Bertini et al., 1988a). The pH was adjusted to the correct value by addition of small amounts of sodium hydroxide.

The electronic absorption spectra were run at room temperature on a double-beam Cary 17 D and on a Perkin Elmer Lambda 9 spectrophotometer. ^{1}H NMR measurements were performed at 90 MHz on a Bruker CXP 90 instrument, at 300 K, using the modified driven equilibrium Fourier transform (MODEFT) pulse sequence for water suppression (Hochmann & Kellerhals, 1980). Some of the ^{1}H NMR spectra were run on a Bruker MSL 200 instrument. The ^{1}H NMR samples were usually 1 mM in protein.

Results

CoCPD. Visible absorption and ^{1}H NMR spectroscopy of the pH dependence of CoCPD have been able to detect pH-dependent variations consistent with a pK_a of 8.8 (Latt & Vallee, 1971; Bertini & Luchinat, 1983). Owing to the improved spectral resolution, we have performed again the ^{1}H NMR spectra of CoCPD in the pH range 6–10 (Figure 1). The ^{1}H NMR spectrum of CoCPD, at pH 6, shows three well-resolved isotropically shifted signals in the downfield region, located at 62 (a), 52 (c), and 45 (d) ppm, and a broader signal at 56 ppm (b). Signals a, c, and d have been assigned to the NH proton of His-69 and to the C-4 H's of His-69 and

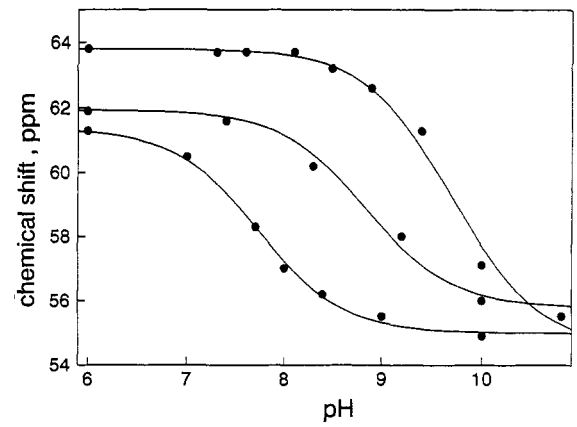

FIGURE 2: Dependence of the ^{1}H NMR signal a on pH for CoCPD (middle) and the cobalt enzyme in the presence of L-Phe (100 mM) (bottom) and of L-Phe (100 mM) plus N_3^- (100 mM) (top). The lines are theoretical pH titration curves using the parameters for the pK_a and limiting values of the chemical shifts (ppm) for the acid and basic forms of 8.84, 61.94, and 55.78 (CoCPD), 7.73, 61.39 and 54.99 (+L-Phe), and 9.71, 63.80, and 54.56 (+L-Phe + N_3^-).

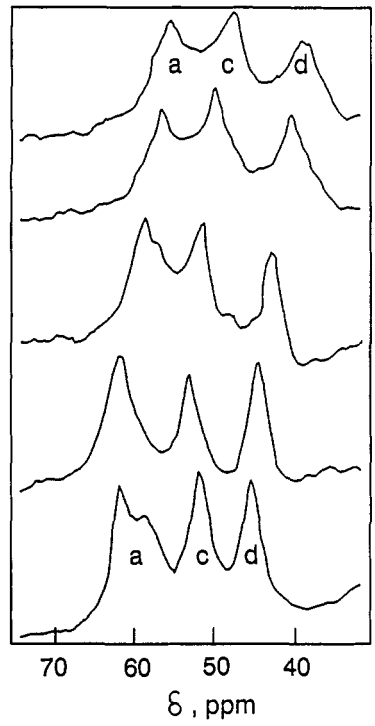

FIGURE 3: ^{1}H NMR spectra of CoCPD (1 mM) at pH 6.0 in the presence of L-Phe (100 mM) (bottom) and at increasing pH values of 7.0, 7.7, 8.4, and 10.0.

His-196, respectively (Bertini et al., 1988a). The spectrum is virtually pH independent up to pH 7.5. For higher pH values, the chemical shift of both signals a and c, assigned to the same histidine residue, decreases progressively with increasing pH. This behavior is indicative of the occurrence of an acid–base equilibrium between two species which is fast on the NMR time scale. The pK_a value which is obtained from best-fitting analysis of the spectral variations of signal a is 8.84 ± 0.1 (Figure 2). The marked broadening of all the lines that is observed around pH 9 suggests a borderline situation between fast and quasi-fast exchange conditions.

CoCPD·L-Phe. The mode of interaction of L-Phe with CoCPD has been investigated through visible absorption (Latt & Vallee, 1971; Bertini et al., 1990a) and through ^{13}C (Luchinat et al., 1988) and ^{1}H NMR spectroscopy (Bertini et al., 1988a). L-Phe binds CoCPD in a 1:1 fashion with an affinity constant of 300 M^{-1}, at pH 6. Only the broad signal b of the

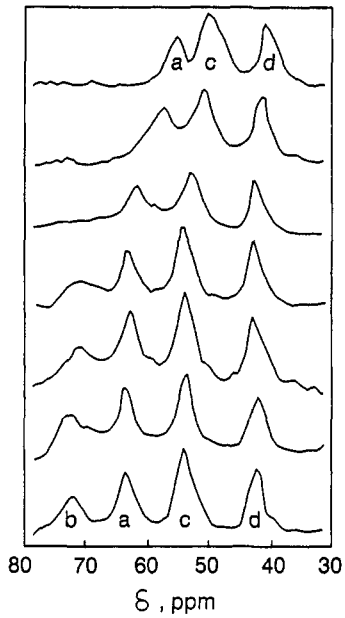

FIGURE 4: ^{1}H NMR spectra of CoCPD (1 mM) in the presence L-Phe (100 mM) and N_3^- (100 mM) at pH 6.1 (bottom) and at increasing pH values of 7.6, 8.5, 8.9, 9.4, 10.0, and 10.8.

^{1}H NMR spectrum undergoes a slightly increased chemical shift, 58.6 ppm, to become a shoulder on signal a (Figure 3). As in the case of the uncomplexed cobalt enzyme, increasing pH is correlated with a decrease in the chemical shift values of signals a and c. In fact, the observed pattern of spectral variations with increasing pH is almost identical to that observed for CoCPD (Figure 1). From the analysis of the pH-dependent variations in signal a, the ^{1}H NMR experiments yield a pK_a of 7.73 ± 0.1 (Figure 2), a value essentially identical to that for the visible absorption studies (pK_a = 7.6 ± 0.1) (Bertini et al., 1990a) but more than 1 unit lower than that of CoCPD alone.

CoCPD·L-Phe·N_3^-. The binding of anions to carboxypeptidase is synergistically promoted by the presence of an amino acid in the active site of the enzyme. In the presence of L-Phe, N_3^- forms a tight ternary complex with CoCPD with well-defined electronic and ^{1}H NMR spectral properties (Bicknell et al., 1988; Bertini et al., 1988a). The ^{1}H NMR spectrum of this ternary adduct, at pH 6, shows four isotropically shifted signals. Signals a (64 ppm), c (55 ppm), and d (43 ppm) are only slightly shifted from that observed in CoCPD or in its complex with L-Phe, while signal b (72 ppm) is increased markedly (Figure 4). The spectrum does not exhibit any major change with increasing pH up to pH 9 except for a broadening of signal b. For higher pH values, the ^{1}H NMR transforms into the spectrum of the alkaline form of the CoCPD·L-Phe adduct, suggesting N_3^- has been released from the enzyme. Under the conditions used, the latter process reaches completion for pH values >10.5 with an apparent pK_a of 9.7 (Figure 2).

CoCPD·D-Phe and CoCPD·(D-Phe)$_2$. In contrast to L-Phe, D-Phe is able to bind CoCPD both in a 1:1 and in a 2:1 ratio. The apparent affinity constants for the formation of the 1:1 and the 2:1 complexes, at pH 6.0 in the presence of 1 M NaCl, are 700 and 73 M^{-1} as determined by ^{1}H NMR (Bertini et al., 1988a). Taking advantage of the different stability constants of the 1:1 and the 2:1 adducts, the two derivatives can be selectively prepared by using appropriate concentrations of D-Phe at pH 6.0. However, it must be stressed that both K_1 and K_2, describing the apparent affinity constant of the first and the second D-Phe moiety, respectively, likely markedly

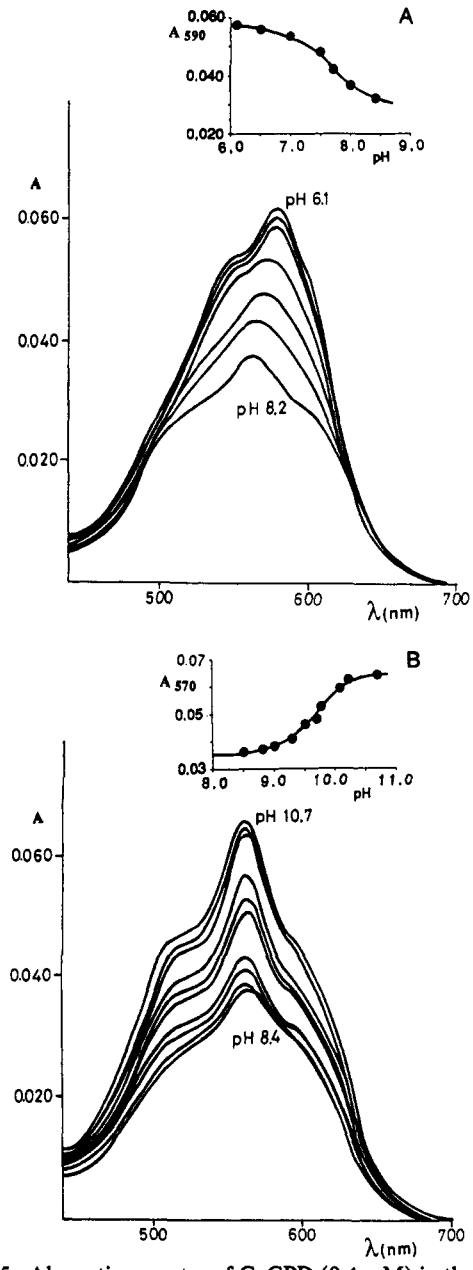

FIGURE 5: Absorption spectra of CoCPD (0.4 mM) in the presence of D-Phe (1.2 mM) over the pH ranges (A) 6.0–8.2 and (B) 8.4–10.7. The inset for (A) shows the pH dependence of the absorbance at 590 nm and the best-fit analysis which yields a pK_a value of 7.7 ± 0.2. The inset for (B) shows the pH dependence of the absorbance at 570 nm and the best-fit analysis which yields a pK_a of 9.75.

depend on pH so that changing the pH causes a change in the relative population of the different species. Thus, at pH 8.2, a K_2 value of 570 M^{-1} is estimated compared to 73 M^{-1} for pH 6.0. The relative amount of the two species can be easily monitored through visible electronic absorption and ^{1}H NMR spectroscopy since the 1:1 and 2:1 adducts yield distinct sets of electronic absorption spectra and of isotropically shifted ^{1}H NMR signals under slow exchange conditions.

Using the conditions of 0.4 mM CoCPD and 1.2 mM D-Phe at pH 6, 1 M sodium chloride, predominant formation of the 1:1 derivative is obtained, characterized by a λ_{max} at 590 nm (ϵ = 190 M^{-1} cm^{-1}) and a shoulder at 530 nm (Figure 5A). The electronic absorption spectrum of this derivative exhibits features which are very similar to those of the CoCPD·L-Phe adduct (Bertini et al., 1990a). At a 1.2 mM D-Phe concentration, the electronic spectrum shows a quite complex pH dependence. It does not change between pH 6 and pH 7 while

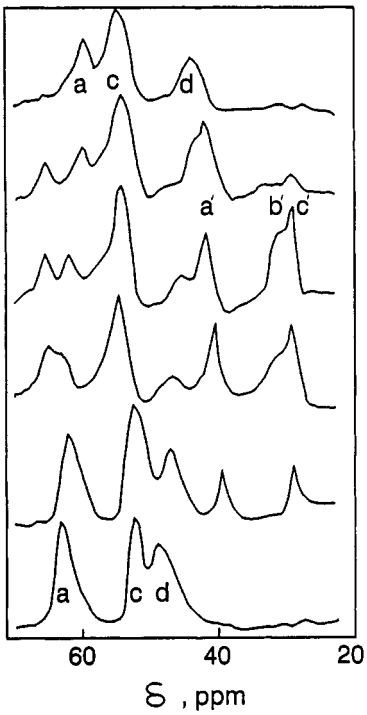

FIGURE 6: ^{1}H NMR spectra of CoCPD (1 mM) in the presence of D-Phe (2.5 mM) at pH values of 6.0 (bottom) and at increasing pH values of 7.0, 7.6, 8.2, 9.0, and 9.9.

from pH 7.0 to pH 8.3 the spectrum blue-shifts and the absorbance of the sample decreases, resulting in a new λ_{max} at 570 nm (ϵ = 110 M^{-1} cm^{-1}) and a shoulder at ~500 nm. The decrease in absorbance to 590 nm can be fit to a titration curve characterized by a pK_a of 7.7 ± 0.2 (Figure 5A). Upon a further increase in pH to 10.7, the 570-nm absorbance of the sample increases again up to a final value of ϵ = 200 M^{-1} cm^{-1} (Figure 5B). This spectral change at 570 nm is characterized by a pK_a of 9.7 ± 0.2. The final spectrum with a λ_{max} of 570 nm and shoulders at ~500 and 605 nm is very similar to that of CoCPD·L-Phe at high pH (Bertini et al., 1990a).

Parallel ^{1}H NMR measurements are of great help in the interpretation of the above complex spectral pattern. The ^{1}H NMR spectra of CoCPD, 1 mM, in the presence of D-Phe, 2.5 mM, recorded over the pH range 6–10 are shown in Figure 6. At pH 6, the predominant species is CoCPD·D-Phe, characterized by three isotropically shifted signals at 63 (a), 52 (c), and 49 (d) ppm, very similar to those observed for the binary CoCPD·L-Phe complex (Figure 3). When the pH is increased from 7.5 to 8.5, new signals, a′, b′, and c′, appear, which characterize the 2:1 species; concomitantly, the signals typical of the 1:1 adduct, a, c, and d, modify their position, while decreasing in intensity. The analysis of the chemical shift variations of signals a, c, and d (Figure 7) yields essentially the same pK_a value, 7.81, obtained in the electronic absorption studies (Figure 5A); this value also closely matches the pK_a value of 7.73 found for the CoCPD·L-Phe complex (Figure 2).

At higher pH values (pH >8.5), there is a progressive decrease in the intensity of the signals of the 2:1 species and a parallel increase of the signals of the 1:1 species (Figure 6); this behavior is rationalized by assuming that, at high pH, an ionization in the enzyme active center occurs which disrupts the binding of the second D-Phe for the metal site so that the CoCPD·(D-Phe)$_2$ species transforms into the alkaline form of a CoCPD-D-Phe species. The final ^{1}H NMR spectrum strongly resembles that of CoCPD·L-Phe at high pH (Figure 3).

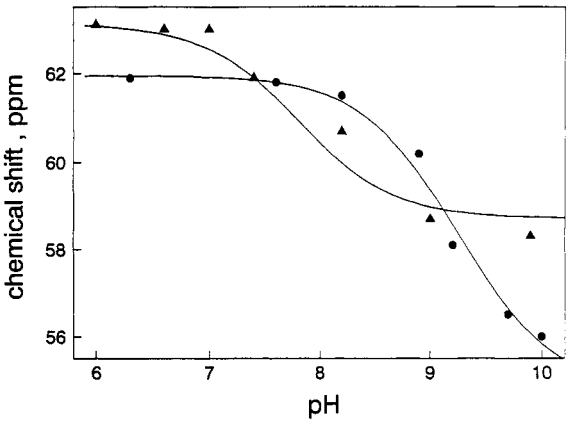

FIGURE 7: Dependence of ^{1}H NMR signal a on pH for the cobalt enzyme in the presence of D-Phe (2.5 mM) (▲) and acetate (10 mM) (●). The lines are theoretical pH titration curves using the parameters for the pK_a and limiting values of the chemical shifts (ppm) for the acid and basic forms of 7.81, 63.13, and 58.70 (D-Phe) and 9.25, 61.95, and 54.74 (acetate).

These ^{1}H NMR results provide an interpretation for the pattern of the electronic spectra. The pK_a of 7.7 for the electronic spectra reflects the progressive formation of CoCPD·(D-Phe)$_2$ from CoCPD·D-Phe, whereas the pK_a of 9.7 reflects the ionization of an active-site group or inhibitor group which leads to conversion of CoCPD·(D-Phe)$_2$ into CoCPD·D-Phe.

Further, ^{1}H NMR experiments were performed in the presence of a 0.1 M D-Phe concentration (data not shown). Under these conditions, at pH 6, quantitative formation of the 2:1 complex is achieved, characterized by the appearance of the signals at 65, 53, 39, and 28 ppm. No signals characteristic of the free enzyme or binary CoCPD·D-Phe complex are observed. The ^{1}H NMR spectrum does not show any major changes upon increasing pH up to 9.3. From pH 9.3 to pH 10.8, a slight variation in the intensity and the shape of signal(s) at 63 ppm occurs while the other signals are essentially not affected.

CoCPD·Acetate. The interaction of acetate with CoCPD has been described at pH 6 through electronic, ^{1}H NMR, and ^{13}C NMR spectroscopy (Bertini et al., 1988a,b). Acetate binds to the protein in both a 1:1 and a 2:1 ratio, the first binding site being a nonmetal site and the second site the metal. The apparent affinity constants for the two sites at pH 6.0 are widely separated, having K_1 and K_2 values for formation of the 1:1 and 2:1 complexes of >500 and 2.5 M^{-1}, respectively (Bertini et al., 1988b). Acetate, also, promotes the binding of pseudohalides to the metal site although to a lesser extent than L-Phe does (Bertini et al., 1988a). In order to understand whether facilitation of azide binding occurs through the same mechanism as L-Phe, i.e., the breaking of the hydrogen bond interaction between the metal-coordinated water and Glu-270, the pH-dependent properties of the 1:1 CoCPD·acetate complex were investigated through ^{1}H NMR.

The ^{1}H NMR spectra of CoCPD in the presence of acetate, 10 mM, at increasing pH values are shown in Figure 8. From the analysis of the spectra, it appears that the chromophore is scarcely perturbed up to pH 8.5. For higher pH values, the ^{1}H NMR signals become broader, and sizable variations in the chemical shift values are observed which closely parallel those observed for CoCPD alone. The pK_a of 9.25 ± 0.2 (Figure 7) for this process is close to that found for CoCPD (Figure 2). This means that, in contrast to L-Phe, occupancy of a nonmetal site by acetate does not increase the acidity of the alkaline group.

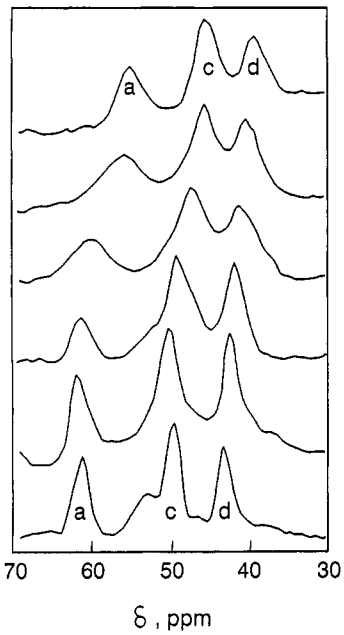

FIGURE 8: ^{1}H NMR spectra of CoCPD (1 mM) in the presence of acetate (10 mM) at pH 6.3 (bottom) and at increasing pH values of 7.4, 8.2, 8.9, 9.6, and 10.0.

Discussion

In the last 2 decades, much interest has been devoted to the identification of those groups which play essential roles in the catalytic mechanism of carboxypeptidase A. X-ray crystallographic analysis of the metal binding site of the free enzyme indicates the metal sits in a distorted tetrahedral environment due to its pentacoordination of the nitrogens of His-69 and His-196, an oxygen of a coordinated water molecule, and the two oxygens of the carboxylate of Glu-72 (Rees et al., 1983). At present, there is evidence that Glu-270, the zinc ion, and Arg-145 are essential for catalysis whereas Tyr-248 has been recognized to be important for substrate recognition but not essential for catalysis (Gardell et al., 1985; Hilvert et al., 1986; Auld et al., 1986, 1989). The role of other groups such as Arg-127, Arg-71, and the metal-coordinated water molecule remains still questionable. Since the catalytic activity of carboxypeptidase displays a well-defined pH-dependent pattern with an acidic pK_{EH_2} ($EH_2 \rightleftharpoons EH$) of 6 for the zinc enzyme and 5.3 for the cobalt enzyme and an alkaline pK_{EH} ($EH \rightleftharpoons E$) of 9 for both enzymes (Auld & Vallee, 1970, 1971), it can be inferred that a specific state of ionization of at least two acid–base groups in the cavity is essential for catalysis.

The lower pK_a, pK_{EH_2}, likely reflects ionization of Glu-270 and its subsequent interaction with the metal-coordinated water molecule to the catalytic zinc ion. Thus, Glu-270 assists in maintaining the enzyme in its catalytically competent EH form by making a H-bond between its carboxylate and the coordinated water molecule. The hydrogen bond would be expected to raise the pK_a of the coordinated water molecule and retard access of monovalent anions to the coordination sphere of the metal, thus explaining the spectral insensitivity of the cobalt enzyme to anions near neutrality (Latt & Vallee, 1971; Geoghegan et al., 1983). The spectroscopically anion insensitive cobalt(II) thermolysin and β-lactamase II also have glutamate residues (Kester & Matthews, 1977; Little et al., 1986) that may have a similar stabilizing role akin to that of Glu-270 in carboxypeptidase A. In contrast, an anion-sensitive enzyme such as carbonic anhydrase does not have a glutamate residue within H-bonding distance of the metal-bound water (Lindskog, 1983).

Table I: ^{1}H NMR Signals for Histidyl Ligands of Carboxypeptidase A Complexes[a]

	acidic region			basic region		
inhibitor ([I], mM)	a	c	d	a	c	d
0	62.0	52.0	45.3	56.1	48.0	42.3
L-Phe (100)	61.9	51.6	45.2	55.2	47.0	39.9
L-Phe (100) + N_3^- (100)	63.8	54.8	42.9	55.7	50.7	41.9
acetate (10)	61.2	49.7	43.1	55.0	45.2	39.3
D-Phe (2.5)	63.3	52.3	48.7	60.7	56.0	44.3
D-Phe (100)[b]	39.2	27.9	26.5	39.2	27.9	25.3

[a] The acidic and basic regions refer to pH values of 6.0 and 10 or 10.8, respectively. [b] These values for the di D-Phe complex are also seen in Figure 7, labeled a′, b′, and c′.

Chemical modifications of Glu-270, lowering the pH to 5.5, or binding of products of ester and peptide hydrolysis such as phenylalanine or phenyllactate all allow access of anions to the metal coordination sphere (Stephens et al., 1974; Geoghegan et al., 1983; Bicknell et al., 1988). Detailed analysis of the free enzyme and binary and ternary complexes by ^{1}H and ^{13}C NMR and electronic and paramagnetic spectroscopy and of the free enzyme and binary complexes by X-ray crystallography is beginning to give a structural picture of the enzyme's metal binding site and the changes that occur during inhibitor binding to the enzyme.

Binding of D-Phe or D-Tyr leads to the formation of a salt link between the α-amino group of the amino acid and O_{E2} of Glu-270 (Christianson et al., 1989). Such a salt link should disrupt any interaction between the Glu-270 carboxylate and the metal–water. In the binary complex, the carboxylate of D-Phe binds to Arg-145, not to the metal, in agreement with Co–^{13}COO distance calculation by ^{13}C NMR (Luchinat et al., 1988). ^{13}C NMR spectroscopy of the ternary complexes of the cobalt enzyme with azide and either L- or D-Phe has further shown the Co–^{13}COO distance, 4.2 Å, is also too great for direct coordination of the carboxylate to the metal in these ternary complexes. Examination of the CoCPD·L-Phe·N_3^- ternary complex by electronic absorption, MCD, and EPR indicates N_3^- is bound to the metal and that the metal is in a more regular tetrahedral environment than is observed for CoCPD or the CoCPD·L-Phe complex (Bicknell et al., 1988). Such a coordination complex could be achieved by displacement of the bound water by N_3^- and simultaneous conversion of the Glu-72 carboxylate from a bidentate to a monodentate ligand, which would result in a tetracoordinated metal complex.

The detection of minor structural changes within the metal coordination sphere is easily accomplished by ^{1}H NMR spectroscopy of isotropically shifted signals in a cobalt-substituted enzyme (Bertini & Luchinat, 1983, 1986). Thus, by examination of NMR spectra in conjunction with the above studies for the free enzyme (E), binary (EI) complexes, and ternary (EI_2 or EIX) complexes, it has been possible to assign signals a and c (Figure 1) to the His NH and C-4 proton of His-69, respectively, signal d to the C-4 proton of His-196, and signal b to the γ-CH_2 of Glu-72 (Bertini et al., 1988a). The conversion of the CoCPD and CoCPD·L-Phe enzymes into the CoCPD·L-Phe·N_3^- adduct is reflected in the ^{1}H NMR spectra (Figures 1, 3, and 4). Thus, histidine signals a, c, and d change by no more than 2 ppm when the free enzyme and the ternary complex (Table I) are compared, but signal b shifts from 56 to 72 ppm, likely reflecting the change in a bidentate to a more monodentate nature of Glu-72.

The most likely groups responsible for the alkaline pK_{EH}, which controls peptide binding (Auld & Vallee, 1970, 1971), are the metal-bound water, a tyrosine, or a coordinated histidine. When the pH is changed from 6 to 10, signals a and c of the free enzyme assigned to His-69 both show an upfield

variation in chemical shift (Figure 1). A pK_a of 8.8 is obtained from the pH dependence of signal a (Figure 2). This pK_a is closely similar to the kinetic pK_{EH} of 9.0 for the zinc and cobalt enzymes (Auld & Vallee 1970) and to the pK_a of 8.9 obtained from titrating the change in absorbance at 620 nm of the cobalt enzyme (Latt & Vallee, 1971). The ^{1}H NMR spectrum clearly shows signal a, reflecting the NH proton of His-69, is still retained even at pH 10. The NH proton of His-196 is in fast exchange in the free enzyme, and no marked change is seen in its C-4 proton, signal d, upon changing the pH from 6 to 10 (Table I). If the NH of His-196 ionized, a negative charge would be placed on the aromatic ring, likely leading to a large chemical shift of the C-4 proton. Since signal d does not undergo a major chemical shift with an increase in pH and signal a is still observed at pH 10, the alkaline pK_{EH} in carboxypeptidase A catalysis likely does not reflect ionization of a ligating histidine as has been suggested (Mock & Tsay, 1988).

A strong case for pK_{EH} reflecting the ionization of the metal-coordinated water molecule can be made. The ^{1}H NMR spectrum of the CoCPD·L-Phe complex is responsive to pH (Figure 3). Signal a undergoes a chemical shift of 7.73 ppm with increasing pH, the curve being characterized by a pK_a of 7.7 (Figure 2). However signals a, c, and d are shifted by $\leq$2 ppm (Table I) when compared to the free enzyme at either pH 6.0 or 10.0, indicating the inner ligation shell of the cobalt coordination sphere is not greatly perturbed by the binding of L-Phe. These results indicate the pK_a of 8.8 in CoCPD and the pK_a of 7.7 in CoCPD·L-Phe reflect ionization of the same group; the principal effect of binding L-Phe is to raise the acidity of the group by about a factor of 20. Electronic absorption studies of CoCPD·L-Phe also show no marked differences in intensity or band pattern shape occur on passing from pH 6 to 10 while a pK_a of 7.6 is observed for the spectral change at 630 nm (Bertini et al., 1990a).

The pH dependence of L-Phe inhibition of ZnCPD is also characterized by a shift in the alkaline pK_a from 9.0 to 7.4 (Auld et al., 1986). While kinetic studies demonstrate that the formation constant for EIH species is 50-fold greater than for the EHIH complex, they cannot unequivocally adjudicate whether the proton comes from the enzyme or the inhibitor. However, in conjunction with the present NMR studies, it can now be ascertained that the proton comes from the enzyme and that it is always the protonated form of L-Phe that binds to the enzyme. The protonated amino group of L-Phe could form a salt bridge to the Glu-270 carboxyl group, thus breaking the latter's interaction with the coordinated water molecule. The resultant metal-bound water might then ionize more readily than in the free enzyme, accounting for a shift in pK_{EH} from 9 to $\sim$7.5 upon binding of the protonated L-Phe. It could also account for the binding of anions to the metal by displacement of the water (Bicknell et al., 1988). Acetate (10 mM), on the other hand, does not raise the acidity of pK_{EH} (Figures 7 and 8). This would be expected since it lacks any positively charged group which could directly disrupt the Glu-270–metal water interaction.

Azide is known to bind to the EHIH species but not to EIH species (Bertini et al., 1990a). If the ionization of EHIH $\rightleftharpoons$ EIH represents the ionization of the metal hydrate to its monohydroxide [M(H_2O) $\rightleftharpoons$ MOH], the decreased binding would be expected since N_3^- displacement of hydroxide should be more difficult than displacement of water. The apparent pK_a of 9.71 for signal a in the presence of 100 mM L-Phe and N_3^- (Figures 2 and 4) would then reflect the competition of hydroxide with N_3^- for the metal. At 100 nM L-Phe, the enzyme would remain in its protonated L-Phe complex even at pH 10.8 (Auld et al., 1986). However, since 100 mM N_3^- is $\sim$100-fold above its apparent dissociation constant at pH 7.5 (Bicknell et al., 1988), the apparent pK_a of 7.7 would be expected to be shifted by about 2 pH units to 9.7. The ^{1}H NMR spectrum of pH 10.8 is quite similar to that of the free cobalt enzyme or its L-Phe complex (Table I), which is also consistent with the pK_a of 9.7 representing release of N_3^- from the metal.

The results on D-Phe interaction with the enzyme appear to be at odds with the assignment of the alkaline pK_a to the metal water ionization. Both the decreased extinctions of the electronic absorption spectrum for the D-Phe complex (Figure 5) and the marked changes seen in the NMR spectrum (Figure 6) indicate the second D-Phe binds to the metal likely through its carboxylate. In this case, ionization of the EHIH species, occurring with a pK_a of 7.7, promotes the binding of the second D-Phe molecule (Figure 5), a result that might not be expected if pK_{EH} represents ionization of the metal-bound water. However, the strong binding of the second D-Phe even at pH 7.5 indicates it likely also binds with a protonated α-amino group. H-bonding of the protonated α-amino group to the ionized zinc water might be a driving force for expansion of the metal coordination shell. The conversion of the di D-Phe into the mono D-Phe enzyme adduct with a pK_a of 9.7 at 2.5 mM D-Phe (Figures 5–7) could reflect ionization of the α-amino group of the inhibitor which has a pK_a of 9.6.

Ionization of Tyr-248 may also play a role in the alkaline pH dependence of anion binding. Both chemical modification and mutagenic studies have shown that a protonated Tyr-248 phenolic group is needed for peptide binding (Auld et al., 1989). Thus, the pH–k_{cat}/K_m profile for peptide hydrolysis by NO_2-Tyr-248 CPD, markedly different from that of the native enzyme, is characterized by an alkaline pK_a of 7.1 which corresponds to a spectrally determined pK_a of 7.0 (Auld et al., 1986). Hydrosulfite reduction of NO_2-Tyr-248 to NH_2-Tyr-248 restores the kinetic alkaline pK_a to 9.2 and shifts the spectral pK_a to 9.4. The near-identity of the alkaline pK_a values derived from the kinetic pH profiles, 7.1 and 9.2, to the corresponding spectrally determined pK_a values of 7.0 and 9.4, respectively, indicates that the alkaline pK_a reflects the ionization of Tyr-248 in the modified enzymes and, by inference, links the native enzyme's alkaline pK_a (8.9) to Tyr-248. The kinetic studies of the native and Tyr-248-modified enzymes further indicate that the consequences of ionization of EH or Tyr-248 result in loss of peptide binding to the enzyme.

The mutant rat carboxypeptidase (Phe-248) exhibits 5- and 9-fold increased K_m values for Z-Gly-Gly-Phe and ClCin-OPhe and a 70-fold-increased K_i value for the potato inhibitor, suggesting that Tyr-248 plays some role in substrate and inhibitor binding (Gardell et al., 1985). The pH dependence of pK_m for the mutant and wild-type enzymes is also in agreement with the results of chemical modification of Tyr-248. Thus, peptide binding, reflected in pK_m, decreases above 9 with a pK_a of 8.9 for the bovine enzyme (Auld & Vallee, 1970) but is pH-independent for the rat Phe-248 mutant up to pH 10.5 (Hilvert et al., 1986; Auld et al., 1989). In the Phe-248 enzyme, there is no phenolic group so no ionization can occur, and a change in pK_m as a function of pH would not be expected and, indeed, none is seen.

^{1}H NMR studies on the Phe-248-mutated carboxypeptidase A should be of help in determining how the ionization of Tyr-248 influences the pH-dependence processes seen in the present study. If Tyr-248 is responsible for the pK_a of 7.7 seen in the binding of the protonated form of L-Phe to the enzyme,

this pK_a should be eliminated. The only pH dependence expected in the process would be due to the conversion of the protonated Phe α-amino group into its neutral form. In addition, N_3^- binding should no longer decrease with a pK_a of 7.7. If, however, pK_{EH} is due to the ionization of the metal-coordinated water, then the pK_a of 7.7 should still be apparent in the binding of L-Phe to CoCPD and N_3^- to the CoCPD·L-Phe complex.

The present NMR studies have demonstrated how structural studies of carboxypeptidase in solution in conjunction with kinetic studies can identify the ionizable groups in the enzyme and the inhibitor that are critical to the formation of the enzyme·inhibitor adduct. These structural and functional approaches combined with mutagenic changes of the protein that alter the chemical properties of amino acids residues in the active site should be capable of determining which groups are essential to the binding and/or hydrolysis of substrates of this enzyme.

Registry No. L-Phe, 63-91-2; D-Phe, 673-06-3; N_3^-, 14343-69-2; His, 71-00-1; Glu, 56-86-0; Tyr, 60-18-4; acetate, 64-19-7.

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