^{35}Cl Nuclear Magnetic Resonance Investigation of Carboxypeptidase A

R. Scott Stephens, Joyce E. Jentoft, and Robert G. Bryant*

Contribution from the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received March 23, 1974*

Abstract: ^{35}Cl nmr line width measurements are used to probe the active site zinc ion chemistry in carboxypeptidase A. A chloride ion displacement reaction occurs at low pH values which is identified with an ionization at glutamic acid-270, and it is suggested that the ionization is coupled to the zinc ion by one or more hydrogen bonded water molecules. The apparent pK of the water molecule coordinated to the zinc ion in the enzyme with a modified glutamic acid-270 residue is approximately 9. The ^{35}Cl line width in carboxypeptidase solutions is dominated by the chemical exchange rate of chloride ion with the metal site. At room temperature, the first-order rate constant is less than or equal to 1×10^6 sec^{-1}. The chloride ion binding constant for the zinc site is 3 M^{-1}.

The metalloenzyme carboxypeptidase A contains a zinc atom located in the active site region. The crystal structure and sequence studies have shown that the active site zinc is bound to the enzyme by coordination with one glutamyl and two histidyl side chains.[1-3] The X-ray data have indicated that an oxygen atom occupies the fourth coordination position of the zinc ion,[2] which implies that the coordination number is brought to four by either a water molecule or a hydroxide ion. Several of the proposed mechanisms for enzymic catalysis by carboxypeptidase A involve specific action by either a water molecule or a hydroxide ion coordinated to zinc.[1] Identification of the oxygen moiety at the zinc atom is therefore a requirement for establishing the catalytic details of the peptidase and esterase activities of the enzyme.

^{35}Cl nmr provides a particularly useful approach to the study of enzymes containing group IIB metals.[4,5] The technique has previously been applied to zinc-containing enzymes such as carboxypeptidase,[6] carbonic anhydrase,[7] and alcohol dehydrogenase.[8] Ward has used the ^{35}Cl nmr line width to suggest that the species most likely to be at the zinc ion of carbonic anhydrase at the pH of optimum activity is hydroxide ion, that only one binding site exists for chloride ions at the zinc site, that enzyme inhibitors such as cyanide ion function by coordination to the zinc ion, and that the ^{35}Cl nmr line width is determined by the rate of chloride ion exchange with the zinc atom.[9] The ^{35}Cl nmr line width is sensitive to the details of zinc ion coordination in carboxypeptidase A,[6] and the present study was undertaken to characterize the zinc–ligand equilibria in the enzyme at various pH values by monitoring the chloride ion interactions.

Experimental Section

Carboxypeptidase A, predominantly γ form, was obtained as an aqueous suspension from Worthington Biochemical Corp. and Miles-Seravac Inc. Enzyme solutions were assayed for peptidase activity using the method of Folk and Schirmer[10] as described in the Worthington catalog. Enzyme concentrations were determined spectrophotometrically at 278 nm using an extinction coefficient of 6.42 M^{-1} cm^{-1}.[11] The sulfide titration was carried out using a 1.91×10^{-2} M Na_2S solution. Microliter quantities of this reagent were added using Hamilton microliter syringes.

β-Phenylpropionic acid (Aldrich) was recrystallized from water–ethanol before use. L-Phenylalanine, D-phenylalanine, and N-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) were purchased from Aldrich Chemical Co., sodium chloride, zinc sulfate, lithium chloride, and sodium sulfide from Mallinckrodt, and EDTA, and 1,10-phenanthroline from J. T. Baker. Diphenylthiocarbazone (dithizone) was purchased from Eastman. These reagents as well as hippuryl-L-phenylalanine (Mann Research Laboratories) and tris(hydroxymethyl)aminomethane (Sigma) were used without further purification.

pH measurements were made using a Corning Model 7 pH meter equipped with a Corning 476060 semimicro combination pH electrode.

Dialysis tubing was freed from metals according to the method of Klotz and Hughes.[12]

Buffers and reagent solutions were adjusted to pH 7 and extracted with 0.001% dithizone in carbon tetrachloride to remove contaminating metal ions. Excess dithizone was removed by repeated extractions with carbon tetrachloride. Solutions were then stored in polyethylene containers.

The pH of the enzyme solutions was adjusted using metal-free 0.1 M H_2SO_4 and 0.1 M NaOH prepared by ion exchange methods.[13,14]

Atomic absorption measurements were performed on a Jarrell-Ash instrument. Standard zinc solutions were prepared by dissolving a weighed amount of metallic zinc (B&A] in dilute metal-free hydrochloric acid. Aliquots of this solution were then diluted with metal-free distilled water to give standards of the desired zinc concentration (0.5–3.5 ppm).

Enzyme solutions were prepared by dissolving the aqueous suspension in metal-free saturated NaCl followed by dialysis against Tris buffer, pH 7.0 or 8.8, and the desired sodium chloride concentration.

The apoenzyme was prepared by dialysis of the native enzyme against 2.0 M NaCl, 2×10^{-3} M o-phenanthroline, 0.1 M or 0.001 M Tris (pH 7.0) followed by changes of the same buffer lacking o-phenanthroline and two changes of 0.5 M NaCl, 0.1 M or 0.001 M Tris (pH 7.0). Atomic absorption measurements showed there to be approximately 8–10% zinc remaining. The apoenzyme was reconstituted by adding 2.32×10^{-2} M zinc sulfate. The addition of 0.9 ± 0.1 equiv was verified by atomic absorption. Peptidase activities of the native and reconstituted enzymes were comparable.

The modifications of glutamic acid-270 were carried out according to the methods outlined by Petra.[15] After reaction with methoxamine at 37° for 14 hr, the solution was centrifuged and the supernatant dialyzed *vs.* 0.5 M NaCl, 0.001 M Tris (pH 7.0). The solution was then concentrated in a Diaflo pressure cell (UM-10 membrane), dialyzed against the same buffer, and centrifuged again; then the supernatant was used for the nmr experiment. [M-CPA] = 8.8×10^{-5} M. The extent of modification was monitored by the change observed in the absorption spectrum.

The ^{35}Cl nmr measurements were made as previously described.[16] Denaturation of the enzyme solutions was noted as the pH was dropped during the course of the nmr experiments. Although some line-width measurements were made on cloudy solutions, this source introduced an error of approximately 5% in enzyme concentration and consequently in line width.

Values for the thermodynamic and kinetic parameters presented were determined using a nonlinear least-squares fit of the data to eq 3.[17]

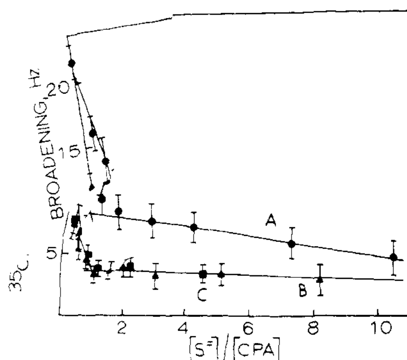


Figure 1. ^{35}Cl line width as a function of $[S^{2-}]/[CPA]$. [NaCl] = 0.5 *M*, [Tris] = 0.1 *M*, pH 7.0, ~27°. (A) -●-, [CPA] = 9.5×10^{-5} *M*. (B) -▲-, [CPA] = 9.7×10^{-5} *M*, [β-phenylpropionic acid] = 9.8×10^{-4} *M*. (C) -■-, [apoCPA] ~ 8.8×10^{-5} *M*.

Theory

The ^{35}Cl line width is usually dominated by quadrupole relaxation so that in the limit of extreme narrowing the line width for a nucleus of spin 3/2 is given by

$$\Delta\nu = (2\pi/5)(e^2qQ)^2\tau_c \qquad (1)$$

where $\Delta\nu$ is the full line width in hertz at half-height, e is the unit charge, q is the electric field gradient at the nucleus of quadrupole moment Q, τ_c is the correlation time for reorientation of the field gradient, and the asymmetry parameter has been neglected.[18]

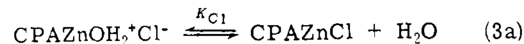
In 1 *M* aqueous sodium chloride solutions, the chloride ion is found in an approximately symmetric environment producing a field gradient at the nucleus approaching zero and a line width of about 14 Hz. If the quadrupolar chlorine nucleus can be found at environmentally different sites in solution, the line width will depend on the relative concentration of each site, the values of $(e^2qQ)^2$ and τ_c associated with each site, as well as the frequency with which the ^{35}Cl nucleus samples the various sites. If the chemical shift on binding of the chloride ion to a site is small compared with the line width at that site, then the rate of exchange may be included in the relaxation equation in a simple way. In the special case of two sites where the concentration of complexed sites is very low compared with the concentration of free or uncomplexed sites, the line broadening is given by the equation

$$\pi\delta\Delta\nu = \pi(\Delta\nu_T - \Delta\nu_f) = P_b/(T_{2b} + \tau_b) \qquad (2)$$

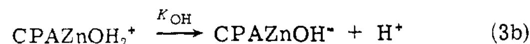
where $\delta\Delta\nu$ is the line broadening, $\Delta\nu_T$ the total observed line width, $\Delta\nu_f$ the line width of the free chloride ion, T_{2b} the transverse relaxation time of the chloride ion at the bound site, τ_b the mean residence time of the chloride nucleus at the bound site, and P_b the probability of finding the chloride ion at the bound site.[19] In the limit where the relaxation time at the metal site is short compared with the residence time, the change in relaxation rate is determined by the exchange rate, $(1/\tau_b)$. However, if the residence time is short compared with the relaxation time at the bound site, the observed relaxation time is independent of the exchange rate, and the observed line width is the weighted average of the sample line widths. Since T_{2b} and τ_b depend differently on temperature, it is possible to determine which dominates the nmr relaxation.

The ^{35}Cl line broadening observed in carboxypeptidase solutions may result principally from two sources: (1) chloride ion exchange with the zinc atom; and (2) chloride ion exchange with nonzinc protein sites. Compared with these, other effects such as those of viscosity are expected to be small. If the nonzinc association of chloride ion may be measured independently, then the broadening due to the zinc site may be determined.

Two chemical equilibria are expected to determine the magnitude of the zinc site line-width contribution.



$$CPAZnOH_2^+Cl^- \xrightleftharpoons{K_{Cl}} CPAZnCl + H_2O \qquad (3a)$$



$$CPAZnOH_2^+ \xrightarrow{K_{OH}} CPAZnOH^- + H^+ \qquad (3b)$$

When these equilibria are included in the relaxation equation, eq 4 is obtained for the line-width contribution of the

$$\pi\delta\Delta\nu = \pi(\Delta\nu_T - \Delta\nu_o) = \frac{K_{Cl}[E_T]\Delta\nu_{EZnCl}}{1 + K_{Cl}[Cl^-] + K_{OH}/[H^+]} \qquad (4)$$

zinc site, where K_{Cl} and K_{OH} are the equilibrium constants defined by eq 3, E_T is the total enzyme concentration, $\Delta\nu_{EZnCl}$ is the relaxation rate, the zinc site, and $\Delta\nu_o$ is the line width of the solution with the zinc site blocked. A study of the pH and chloride ion dependence of the line broadening should therefore provide values of K_{Cl}, K_{OH}, and $\Delta\nu_{EZnCl}$. In general, eq 3b may be taken to represent any pH dependent reaction which results in elimination of chloride ion accessibility to the zinc ion.

Results

The ^{35}Cl nmr line is significantly broadened on the addition of carboxypeptidase A to sodium chloride solutions, as demonstrated by Figure 3; however, it is necessary to determine the source of this broadening before conclusions about the zinc atom are possible. A pH titration of the apoenzyme is the obvious experiment to perform in order to determine the zinc contribution to the observed broadening. However, due to the instability of the apoenzyme at room temperature and the apparent change in the exchange properties below 12° (*vide infra*), a less direct approach is required.

The titration of native CPA with sulfide, a good zinc ligand, is presented in Figure 1A. It is apparent from the sharp endpoint at 1 equiv and the slope of the line after 1 equiv that two sites interact with the sulfide ion. The titration of the native enzyme in the presence of a tenfold excess of β-phenylpropionate, which is known to coordinate to the zinc atom,[20] is plotted as curve B of Figure 1. At this pH of 7, there is 40% decrease in the line broadening, and the weaker interaction is removed, as demonstrated by the increased sharpness of the equivalence point. Although there is some uncertainty in the apoenzyme concentration because of denaturation, essentially the same result is obtained for the sulfide titration of the apoenzyme, Figure 1C.

The interaction of several molecules with the active site zinc ion has been characterized by X-ray analysis in the crystal.[20] The carboxylate ions of both D-phenylalanine and β-phenylpropionate coordinate to the zinc atom and would therefore exclude chloride ion from participation in a four-coordinate zinc complex at the active site. The pH dependence of the ^{35}Cl line width in carboxypeptidase A solutions containing a tenfold excess of these ligands is shown in Figure 2. The broadening apparent at pH values below 7 in curves 2A and 2B is constant and comparable with that of the apoenzyme at pH 7, Figure 1C. L-Phenylalanine, on the other hand, does not eliminate the major low pH contribution to the line width as shown in curve 2C and, in fact, the midpoint of the titration curve is shifted to pH values greater than 7. If both D- and L-phenylalanine are present in tenfold excess over the enzyme concentration, the broadening at pH 6.7 is greater than if only D-phenylalanine were pres-

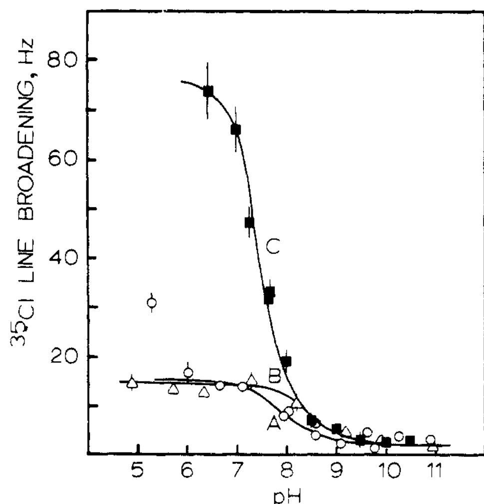


Figure 2. ^{35}Cl line broadening as a function of pH, ~27°. (A) -○-, [CPA] = 8.6 × 10^{-5} *M*, [NaCl] = 0.5 *M*, tenfold excess of D-phenylalanine. (B) -△-, [CPA] = 1.0 × 10^{-4} *M*, [NaCl] = 0.5 *M*, tenfold excess of β-phenylpropionic acid. (C) -■-, [CPA] = 8.4 × 10^{-5} *M*, [NaCl] = 0.5 *M*, 250-fold excess of L-phenylalanine.

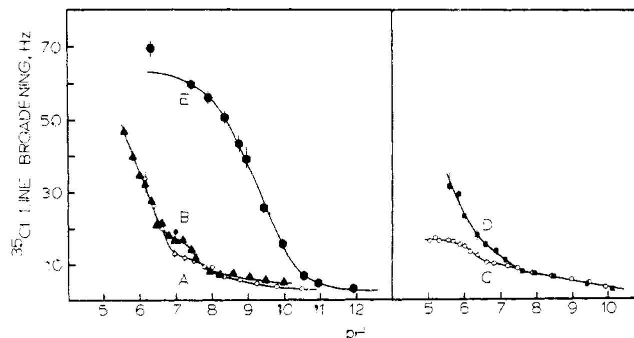


Figure 3. ^{35}Cl line broadening as a function of pH, ~27°. (A) -○-, [CPA] = 9.8 × 10^{-5} *M*, reconstituted with 0.9 ± 0.1 equiv of Zn^{2+}, [NaCl] = 0.5 *M*. (B) -▲-, [CPA] = 9.8 × 10^{-5} *M*, [NaCl] = 0.5 *M*. (C) -○-, [CPA] = 9.6 × 10^{-5} *M*, [NaCl] = 1.5 *M*. (D) -■-, [CPA] = 9.6 × 10^{-5} *M*, [NaCl] = 1.0 *M*. (E) -●-, methoxamine modified glutamic acid-270, [M-CPA] = 8.8 × 10^{-5} *M*, [NaCl] = 0.5 *M*; -●- represents $\delta\Delta\nu$ measurements at pH 7 on solution A several hours later.

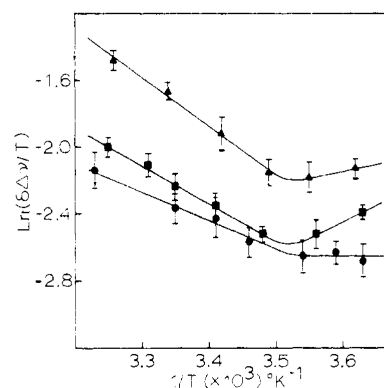


Figure 4. Temperature dependence of ^{35}Cl line broadening. -▲-, [CPA] = 1.2 × 10^{-4} *M*, [NaCl] = 1.0 *M*, pH 5.4. -■-, [CPA] = 9.5 × 10^{-5} *M*, [NaCl] = 1.0 *M*, pH 6.2, 250-fold excess of L-phenylalanine. -●-, [CPA] = 9.7 × 10^{-5} *M*, [NaCl] = 1.5 *M*, pH 5.5.

ent but less than if only L-phenylalanine were in solution, indicating similar equilibrium constants for binding these two isomers.

To determine the extent of the several contributions to the native enzyme line broadening, it is necessary to study the chloride ion concentration dependence of the ^{35}Cl line width as shown in Figure 3. Several points are apparent: (1) the midpoint of the titration is shifted to lower pH values compared with that in Figure 2 taken in the presence of L-phenylalanine; (2) there is a contribution apparent in all solutions as a high pH tail; (3) the experimental errors at low pH are greater than at higher pH.

The proximity of several enzyme groups which are potential zinc ligands in the active site raises the possibility that the decrease in ^{35}Cl line width with increasing pH results from a reaction which involves the displacement of chloride ion by an amino acid side chain functional group rather than hydroxide ion. Examination of the X-ray structure suggests that only glutamic acid-270 and tyrosine-248 could interact with the zinc atom. It has been previously suggested that tyrosine-248 may coordinate to zinc at pH values greater than 8,[21] which are well above the major effects shown in Figure 3. To explore the participation of glutamic acid-270, glutamic acid-270 was converted to an enol ester with Woodward's reagent K and then to the methoxamide. The zinc ion contribution to the line broadening in the K-carboxypeptidase modification of glutamic acid-270 is eliminated but is regained with the removal of the bulky portion of this molecule upon formation of the methoxamide, as shown in curve E. The striking feature of curve 3E is the dramatic shift of the midpoint in the titration curve caused by this modification of glutamic acid-270.

The line widths at the enzyme sites may have significant contributions from the exchange rates of chloride ion as shown by eq 2. The temperature dependence of the chlorine line broadening was measured under several sets of conditions as shown in Figure 4. Although the interpretation of the data is not as simple as could be anticipated, the important features are the increase in line width with increasing temperature (above 12°) and the constancy of slope when chloride concentration is changed or L-phenylalanine added.

Discussion

The sulfide titrations shown in Figure 1 demonstrate the direct interaction of zinc and chloride ion. The decrease in line broadening observed when access of the chloride to zinc is blocked with β-phenylpropionate or when the zinc is removed to form the apoenzyme indicates that zinc contributes to the observed broadening. At pH 7.0 this contribution represents approximately 8 Hz of the broadening. An additional 8 Hz of the broadening is due to a nonzinc site. The nature of this site is not known, but it is highly specific for sulfide ion. This is shown by the sharp endpoint at 1 equiv seen in Figures 1B and 1C. The pH titration of the native enzyme in the presence of β-phenylpropionate shown in Figure 2B points out the constancy of the nonzinc contribution below pH 7 and demonstrates that the majority ofthe broadening seen below pH 7 in the native enzyme is due to the zinc site. The remaining 3 Hz may be attributed to nonspecific interactions of the chloride with the protein. The solutions of native enzyme contained between 1.0 and 1.3 equiv of zinc as determined by atomic absorption measurements. If extraneous metal ion contributions were important in determining the magnitude of the line broadening in the low pH regions of curves 2A and 2B, then a much greater difference between curves A and B in Figure 3 for the native and reconstituted enzyme would be expected. In addition, it will be shown that a zinc ion is not expected to titrate in this pH region unless specific interactions are provided to shift the zinc hydroxide ion equilibrium. Therefore the possibility that extraneous metals are responsible for the broadening observed in Figures 2A and 2B is considered unlikely.

A comparison of the D- and L-phenylalanine results is striking. The X-ray data on the crystalline enzyme have indicated that L-phenylalanine binds in the active site by interaction of the carboxylate anion with the positive charge on arginine-145, leaving the α-ammonium ion close to the zinc ion. This conclusion is consistent with inhibition studies.[22] It is clear from Figure 2C that this mode of binding is confirmed for the solution behavior of the enzyme in that at pH values less than 7, the zinc site is fully accessible to the chloride ion when L-phenylalanine is present in 10- or 250-fold excess over the enzyme concentration. Under the same conditions, the zinc contribution to the chloride ion broadening is eliminated in the presence of a tenfold excess of D-phenylalanine. It has been previously suggested that the difference in the behavior of the two isomers is caused by the very unfavorable interaction of the α-ammonium ion of L-phenylalanine with arginine-145 if the carboxylate ion were to coordinate to the zinc ion.[1,2] It does not seem likely that this difference is important in binding polymeric substrates since the α-amine participates in a peptide bond.

The data shown in Figure 3 for different concentrations of sodium chloride demonstrate that the inflection point for the zinc ion contribution to the ^{35}Cl line width is shifted to lower pH values when L-phenylalanine is not present. Although there is some scatter in the data which could be caused by salt-dependent changes in the details of the enzyme structure, the data are consistent with K_{Cl} of 3 ± 1 M^{-1} which is very similar to other measurements of zinc-chloride ion equilibria reported in the absence of protein donated ligands.[23] The estimate of the equilibrium constant for the chloride ion displacement reaction, K_{OH}, obtained in the presence of L-phenylalanine is 1×10^{-7} M. K_{OH} in the absence of L-phenylalanine is larger by one order of magnitude. Although there are no good low molecular weight analogs of the zinc site in carboxypeptidase A available at present, this result is not consistent with anticipated values for the hydroxide ion displacement since most divalent metals ions hydrolyze in the pH range from 8.5 to 10.[23] Several possible causes for the low value for the low pH titration of chloride ion in carboxypeptidase A are: (1) the reaction which displaces chloride ion from zinc could involve coordination of a protein group in the active site which ionized in this pH range; (2) the equilibrium properties of the zinc ion in the enzyme pocket could be shifted by three orders of magnitude or more, and the observed titration behavior does represent a displacement by hydroxide ion; (3) other factors in the active site region, such as conformation changes, eliminate the possibility of chloride ion exchange with the zinc atom with increasing pH. The data in Figures 2 and 4 permit evaluation of these possibilities.

The enzyme groups near the zinc atom in the active site pocket of carboxypeptidase are glutamic acid-270, arginine-145, and tyrosine-248. Outside the active site pocket, the nearest groups are positively charged and include several arginine and lysine residues.[1,2] It seems unlikely that these groups could interact directly with the zinc atom due to charge repulsions and steric constraints imposed by the tertiary structure of the enzyme. Arginine-145 is not expected to interact with the zinc atom in any of the pH regions studied because it remains protonated and is too far removed from the zinc first coordination sphere, as determined in the X-ray structure. Both glutamic acid-270 and tyrosine-248 have been implicated in the several proposed mechanisms for the peptidase activity. Only tyrosine has been implicated in direct coordination to the zinc atom; however, modification of glutamic acid-270 is reported to decrease activity.[15,21]

Modification of glutamic acid-270 clearly causes a dramatic change in the observed pH dependence of the chloride ion line broadening. The inflection point of curve 3E is at pH 9 which is consistent with ionization of water at the zinc atom.[23] A suggestion that tyrosine-248 displaces chloride ion from zinc ion at pH values close to 6 appears unreasonable. The results in Figure 3 then clearly implicate glutamic acid-270 in the low pH titration behavior of the native enzyme. The X-ray structure, however, indicates that in the solid state the carboxylate ion is too far from the zinc atom to coordinate directly. Therefore, either there are dramatic changes in the pleated sheet portion of the enzyme when the crystal dissolves, allowing the glutamic acid to coordinate, or an alternative mode of interaction is responsible for the observation. If glutamic acid-270 were to coordinate directly to the zinc atom at pH values above 7, it is difficult to understand why other zinc ligands are inhibitors, why tyrosine-248 has been implicated in coordination to the zinc atom in solution at higher pH values, and how the zinc atom could participate in the catalytic mechanism. It sems most likely that in these solutions the glutamic acid does not coordinate to the zinc atom, and there are not dramatic changes in the structure on crystallization in this region of the enzyme.

The chloride ion binding constant for the zinc association is small and corresponds to a free-energy change on coordination on the order of or smaller than that for hydrogen bond formation. The displacement of the chloride ion from the active site zinc atom, then, need not involve a large free-energy change. The distances between glutamic acid-270 and the zinc atom in the X-ray model suggest that it is possible for a water molecule to form a bridge between a water molecule coordinated to the zinc atom and an oxygen atom of glutamic acid-270. All that is required to displace chloride ion from zinc when the glutamic acid residue ionizes is that the free energy of the active site region, including one or more such hydrogen bonded structures, is lower than that of a corresponding structure with a chloride ion coordinated to the zinc and the hydrogen bonding interactions of the displaced water molecule eliminated.

On the basis of the chlorine nmr data, it is not possible to determine whether the water molecule coordinated to the zinc atom actually looses the proton in the low pH region or not. It is possible, however, that a water molecule remains coordinated to the zinc atom throughout the titration region shown in Figure 3 and that its stability with respect to displacement by chloride ion is increased by hydrogen bonding opportunities generated by the ionization of glutamic acid-270.

In contrast, when L-phenylalanine is present in the active site region, such a hydrogen bond system is expected to be altered. As mentioned earlier, the L-phenylalanine is known to bind in such a way that the positive charge on the α-ammonium ion is placed very close to the zinc atom. As shown in curve C of Figure 2, the midpoint of the titration curve is shifted approximately one full pH unit higher compared with the native enzyme. The presence of an additional positive charge in the active site is expected to raise the ionization constant of the glutamic acid-270, if it alters it at all. It therefore seems unlikely that this shift is due to an upward shift in the pK_a and subsequent interaction of glutamic acid-270 with the zinc atom. On the other hand, the proximity of the positive charge to the zinc atom and the water coordinated to it is expected to lower the pK_a of the water molecule on the zinc atom. Therefore it appears reasonable that the ionization in the presence of L-phenylalanine which eliminates the chloride ion from the active site zinc atom is ionization of the zinc coordinated water molecule.

The data presented in curves 3A and 3B for the pH dependence of the line broadening of the native and reconstituted enzyme differ somewhat in the region near pH 7. The additional inflection in curve B between pH 7 and 8 may be due to the nonzinc site discussed previously. However, the same behavior would be expected for the reconstituted enzyme data shown in curve A. Although the effects are close to the experimental errors, it was noted that the broadening of the reconstituted enzyme at pH 7 increased with time. This effect is under further investigation.

As eq 2 demonstrates, the line width associated with the metal site in carboxypeptidase could be determined by the chemical exchange rate of chloride ion with the metal site. If the exchange time, τ_b, in eq 2 is given as a function of temperature by eq 5, then a plot of ln $(\delta\Delta\nu/T)$ *vs.* $1/T$

$$\frac{1}{\tau_b} = \frac{kT}{h} e^{-\left(\frac{\Delta H^*}{RT} - \frac{\Delta S^*}{R}\right)} \quad (5)$$

gives the activation enthalpy, ΔH^*. The temperature dependence shown for the L-phenylalanine and the native forms of the enzyme demonstrate that the exchange rate of the chloride ion does determine the line width of the chlorine resonance at the bound site at temperatures greater than 12°. The data are consistent with a chloride ion exchange rate of 1×10^6 sec^{-1} which is apparently not sensitive to the modification of the active site region of the protein caused by the addition of L-phenylalanine. This value of the rate constant is not inconsistent with other measurements of halide ion-zinc ion exchange rates;[24] however, it may be slower than might be anticipated based on extrapolation of the bromide and iodide ion data obtained for the tetrahalozincate ions. It is important to note that the chloride ion exchange rate is independent of the chloride ion concentration. The details of the behavior of the line width at low temperatures is not entirely understood and is being studied further.

Conclusion

The data reported demonstrate that the zinc ion chemistry in the active site region of carboxypeptidase is very sensitive to the details of the active site structure. The equilibrium properties such as the ionization constant for water coordinated to zinc ion may be shifted considerably by the effects of charges or hydrogen bonding structures generated close to the metal atom by the constraints of the active site geometry. It is clear that when such complicating factors are eliminated by modification of the active site, the hydrolysis of zinc ion occurs in the pH range expected for divalent metals.

Acknowledgment. This work was supported by the National Institutes of Health (GM-18719), the Research Corporation, the Graduate School, The Computing Center, and the Chemistry Department, University of Minnesota. Helpful discussions with Professors Vallee and Lipscomb are gratefully acknowledged.

References and Notes

(1) W. N. Lipscomb, *et al.*, *Brookhaven Symp. Biol.*, **21**, 24 (1968).
(2) W. N. Lipscomb, *Accounts Chem. Res.*, **3**, 81 (1970).
(3) R. A. Bradshaw, *et al.*, *Proc. Nat. Acad. Sci. U.S.*, **64**, 1389 (1969).
(4) T. R. Stengle and J. D. Baldeschwieler, *Proc. Nat. Acad. Sci., U.S.*, **55**, 1020 (1966).
(5) R. G. Bryant, *J. Amer. Chem. Soc.*, **91**, 976 (1969).
(6) R. G. Bryant, H. J. Yeh, and T. R. Stengle, *Biochem. Biophys. Res. Commun.*, **37**, 603 (1969).
(7) R. L. Ward, *Biochemistry*, **8**, 1879 (1969).
(8) R. L. Ward and J. A. Happe, *Biochem. Biophys. Res. Commun.*, **45**, 1444 (1971).
(9) R. L. Ward and M. D. Cull, *Arch. Biochem. Biophys.*, **150**, 436 (1972).
(10) J. E. Folk and E. W. Schirmer, *J. Biol. Chem.*, **238**, 3884 (1963).
(11) R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, **2**, 616 (1963).
(12) I. M. Klotz and T. R. Hughes in "Methods of Biochemical Analysis," Vol. III, D. Glick, Ed., Interscience, New York, N.Y., 1956.
(13) C. J. Keattch, *Lab. Pract.*, **5**, 208 (1956).
(14) J. Steinbach and H. Freiser, *Anal. Chem.*, **24**, 1027 (1952).
(15) P. H. Petra, *Biochemistry*, **10**, 3163 (1971).
(16) R. G. Bryant, Y. Legler, and M. Han, *Biochemistry*, 11, 3846 (1972).
(17) The program CURFIT (P. R. Bevington, "Data Reduction and Error Analysis for the Physical Sciences," McGraw-Hill, New York, N.Y., 1969, p 237) was used for the data analysis.
(18) A. Abragam, "Principles of Nuclear Magnetism," Clarendon Press, Oxford, 1961, p 314.
(19) T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962).
(20) T. A. Steitz, *et al.*, *J. Biol. Chem.*, **242**, 4662 (1967).
(21) J. T. Johansen and B. L. Vallee, *Proc. Nat. Acad. Sci., U.S.*, **70**, 2006 (1973).
(22) J. E. Coleman and B. L. Vallee, *Biochemistry*, **3**, 1874 (1964).
(23) "Stability Constants of Metal Ion Complexes," *Chem. Soc., Spec. Publ.*, **No.** 17 and **25**, 1 (1964).
(24) H. G. Hertz, *Z. Elektrochem.*, **65**, 36 (1961).