Magnetic Resonance Investigation of Ionizable Residues at the Active Site of Thermolysin†

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ABSTRACT: The details of the pH dependence of the thermodynamic and magnetic interactions of the active-site region of thermolysin in which manganese has replaced the active-site zinc atom and the inhibitor *N*-trifluoroacetyl-D-phenylalanine have been examined. These show a number of ionizable groups in the active-site region. A cooperative displacement of manganese at the catalytic site is observed as pH is lowered. This appears to be the result of the protonation of histidine-142 and -146 which act as metal ligands. The metal is 50% displaced at pH 6.0. At higher pH values, the environment of the bound manganese changes as a result of the ionization of at least two groups of approximate pK_a = 8.5 and 9.5. These values are assigned to tyrosine-157 and to the water molecule which acts as a metal ligand at the active site. The binding behavior of the inhibitor strongly suggests that two molecules of inhibitor bind to the enzyme. The weaker site is competitive with the synthetic substrate FAGLA (furylacryloylglycylleucinamide), while the strong site has no effect on FAGLA hydrolysis. This second site is in the vicinity of the active site with a distance of 8 Å or less between the trifluoromethyl group and manganese bound at the active site.

In previously published work, we have demonstrated that the native zinc ion bound at the active site of the endopeptidase, thermolysin, can be reversibly removed and replaced by Mn(II) ion, yielding an enzyme retaining substantial (18%) activity of the native enzyme. Further, nuclear magnetic resonance (NMR) measurements showed that the relaxation rate of the water protons was enhanced in the presence of the enzyme-bound manganese. The relaxation data suggested that there is rapid exchange of one water molecule within the first coordination sphere of the Mn(II) ion when bound to the enzyme (Bigbee and Dahlquist, 1974).

The binding of inhibitors to the manganese derivative of thermolysin was complicated, however. The inhibitors could be placed in three categories. One such category includes those inhibitors which appear to replace the exchangeable water as a manganese ligand. The second category of inhibitors cause no change in the accessibility of the bound manganese ion to bulk water. The third category of inhibitor had an intermediate effect. The most reasonable explanation for this behavior is that two binding orientations for inhibitors exist. One orientation is preferred by the first category of inhibitors, and appears to involve direct interaction of the manganese ion with the bound inhibitor. The second orientation, preferred by the second category of inhibitors, appears not to directly involve interactions of the manganese in the binding of inhibition. The third category of inhibitors are those which have little preference between the two binding orientations and therefore spend a fraction of their bound lifetimes in one or the other orientation. As a result, these inhibitors have characteristics of both the other categories.

In this communication, we present additional ESR and NMR data concerning the interaction of Mn(II) ion with the zinc-free enzyme as a function of pH. These results demonstrate that the manganese bound to the active site of thermolysin can be readily displaced by lowering the pH. The displacement involves cooperative interactions of the metal ion with at least two groups on the enzyme surface of apparent $pK_a \approx 6.0$. These groups are most probably histidine-142 and -146 which are involved in the coordination of the metal at the active site. At higher pH values the environment of the bound manganese ion is perturbed slightly by titration of two ionizable residues of pK_a values in the range 8.5–9.0 and 9.5–10.0. Their identity is unclear, but the metal-bound water and tyrosine-157 are close to the active site and are possible candidates, although a conformational effect acting at a distance cannot be ruled out.

In addition, the pH dependence of the paramagnetic interaction of the bound manganese ion with the inhibitor trifluoromethyl-D-phenylalanine has been examined. These results serve to shed further light on the details of the geometry of the active site of the enzyme, the interaction of the metal ion with the active-site ligands, and the mode of inhibition of the enzyme by inhibitors.

Experimental Section

Materials. Thermolysin, three times crystallized, A grade, and Hepes,[1] *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, A grade, and carboxypeptidase A were obtained from Calbiochem. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Inc. All other chemicals were reagent grade of the highest quality commerically obtainable.

Methods. The zinc-free enzyme was prepared from the native zinc enzyme by chelation of the zinc with 1,10-phenanthroline and purified on Sephadex G-25 columns as previously described (Bigbee and Dahlquist, 1974). The D and L isomers of the inhibitor *N*-trifluoroacetylphenylalanine were prepared from the amino acid by reaction with trifluoroacetic acid and trifluoroacetic anhydride, then subsequent resolution using carboxypeptidase as described by Vine et al. (1973). Kinetic measurements were performed according to the method of Feder (1968) using 1.0×10^{-3} M FAGLA, 10^{-5} M enzyme, 0.01 M $CaCl_2$, 0.01 M Hepes, pH 7.2, and 3×10^{-5} M $ZnCl_2$. These assays, as well as all enzyme concentration determinations, were performed on a Cary Model 14

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid; FAGLA, furylacryloylglycylleucinamide.

spectrophotometer thermostated at 25 ± 1 °C. For determination of the inhibition constant of the fluorinated inhibitor, 5×10^{-3} to 1.0×10^{-1} M concentrations of the inhibitor were added to the assay mixture in the presence of constant enzyme concentration.

ESR measurements of the binding of Mn(II) ion to the zinc-free enzyme were obtained with a Varian E-4 spectrometer equipped with a Varian Model V-4350 variable-temperature accessory which maintained the temperature at 25 ± 1 °C. The quantitative procedure described in our previous paper (Bigbee and Dahlquist, 1974) was used to determine the exact concentration of free MN(II) ion in solution. NMR experiments were performed at 100 MHz for the water and 94.1 MHz for the ^{19}F-substituted inhibitor relaxation studies, respectively, using a Varian XL-100 NMR spectrometer equipped with a variable temperature apparatus. The spectrometer was operated in the Fourier transform mode together with a 16K Varian 620i computer. All solutions contained 10% D_2O which served as an internal lock and reference signal. Spin-lattice (T_1) measurements were made using a $\pi,\tau,\pi/2$ RF pulse sequence and T_2, the spin–spin relaxation time, was determined from line-width measurements with $T_2 = 1/(\pi\Delta\nu_{1/2})$, where $\Delta\nu_{1/2}$ is the width at half-height of the resonance line, or by a modification of the Hahn spin–echo pulse technique suitable for Fourier transform NMR.

Results

The Effect of pH on the Relaxation Rate of Water in the Presence of the Mn(II)–Enzyme. We have recently demonstrated that the relaxation rate of water protons is significantly enhanced in the presence of Mn(II)–thermolysin at pH 7.2. To gain information concerning possible ionizable groups at or near the active site, the pH dependence of the water relaxation rate was examined carefully. These results are shown in Figure 1 as a plot of the paramagnetic contribution to the relaxation rate, $1/T_{1p}$, vs. pH at constant manganese and enzyme concentration. As seen in Figure 1, the relaxation rate is maximal at pH 7 and decreases dramatically at lower pH. There is also a less dramatic but clearly measurable decrease in this rate at pH values between 7 and 8. These relaxation rate changes were seen to be fully reversible. There are a large number of possible explanations for these changes in relaxation rate as a function of pH. These include dissociation of manganese from the active site with pH, changes in the water exchange rate as a function of pH, changes in the correlation time of the interaction between the paramagnetic electrons and water with pH, etc. In the following sections, we present experiments to establish which of these possible explanations are the correct ones.

ESR Measurements of the Interaction of Mn(II) Ion and Zinc-Free Enzyme as a Function of pH. The fact that the room temperature ESR signal of Mn(II) ion broadens beyond detection when it is bound to thermolysin allowed the use of the technique to monitor the binding of Mn(II) ion to the zinc-free enzyme as a function of pH. This was done by carefully measuring the amplitude of the ESR signal (proportional to the concentration free Mn(II) ion) in the presence of constant enzyme concentration at various pH values. The results, which are also shown in Figure 1, demonstrate that the zinc-free enzyme binds 1 equiv of Mn(II) ion at neutral pH and above but the binding decreases as the pH is decreased to values of less than 7. To be certain that the lowering of the pH of the solution resulted in a shift of the binding equlibrium of Mn(II) ion to the enzyme and not merely an irreversible denaturation of the enzyme at low pH, a Mn(II)–enzyme solution was first brought to low pH (pH 4.8) and then returned to neutral pH

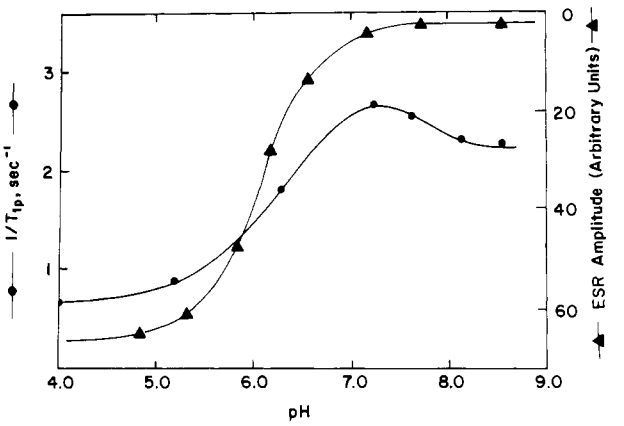

FIGURE 1: The pH dependence of the paramagnetic contribution of the spin–lattice relaxation rate, $1/T_{1p}$, and the binding of Mn(II) to zinc-free thermolysin. The concentration of both the zinc-free enzyme and Mn(II) was 1.0×10^{-4} M. The measurements were performed at 25 °C.

where binding was initially complete. This procedure resulted in the appearance of the free Mn(II) signal at low pH, followed by its dissapearance when the solution was readjusted to pH 7. This demonstrates that lowering of the pH to ~4.8 does not irreversibly denature the enzyme but merely affects the reversible binding of Mn(II) ion. At pH values below 4, however, denaturation of the enzyme is observed as irreversible changes in Mn(II) ion binding followed by precipitation of the protein.

Superficially, the results suggest that the dependence of Mn(II) ion binding is related to the protonation of a residue on the enzyme of $pK_a \approx 6$. However, careful fitting of the data to a theoretical curve derived on the basis of the ionization of a single site of $pK_a = 6$ revealed the binding could not be made to rigorously fit such a scheme. As a result, the following scheme was derived which successfully accounts for the observed binding.

Consider the following equilibria:

$$\begin{array}{ccc} E & \rightleftharpoons & E\text{-}Mn \\ \Updownarrow & & \Updownarrow \\ E\text{-}H_n & \rightleftharpoons & E\text{-}Mn\text{-}H_n \end{array} \qquad (1)$$

Assuming that the enzyme exists with either H^+ or Mn(II) bound (i.e., the species E and E-Mn-H_n in the above scheme exist in negligible concentration), the above equilibria can be reduced to:

$$E\text{-}Mn + nH^+ \rightleftharpoons E\text{-}H_n + Mn(II) \qquad (2)$$

An apparent equilibrium constant, K, may be defined as:

$$K = \frac{[Mn(II)][E\text{-}H_n]}{[E\text{-}Mn(II)][H^+]^n} \qquad (3)$$

Rearranging and taking logarithms gives:

$$\log\left[\frac{[Mn(II)][E\text{-}H_n]}{[E\text{-}Mn(II)]}\right] = \log K + n \log [H^+] \qquad (4)$$

If the amplitude of the ESR signal (which is proportional to the concentration of free Mn(II)) is defined as A and the maximum amplitude (i.e., the value of A when no Mn(II) ion is bound) is taken as A_0, then eq 4 becomes:

$$\log\left[\frac{A^2}{A_0 - A}\right] = -n(pH) + \log K \qquad (5)$$

A plot of the data in Figure 1 according to eq 5 gives a straight line with a slope of −1.9. While the apparent pK_a of the titration is around 6, the binding of Mn(II) ion to the en-

TABLE I.

Solution	$[I]_0$(mM)	I_{oc}	$\frac{1}{T_{1,obsd}}$ (s^{-1})	$\frac{1}{T_{1p}}$ (s^{-1})	T_{1p} (s)	T_{2p} (s)	T_{1p}/T_{2p}
Inhibitor only	30	10	0.73				
	30	20	0.56				
Enzyme + I	5	10	11.53	10.80	0.093		
	5	20	16.36	15.80	0.063		
Enzyme + I	15	10	5.26	4.53	0.221		
	15	20	6.25	5.69	0.176		
Enzyme + I	25	10	3.32	2.59	0.386	0.0984	3.9
	25	20	4.03	3.47	0.288		
	35	10	2.63	1.90	0.526		
	35	20	3.17	2.61	0.383		

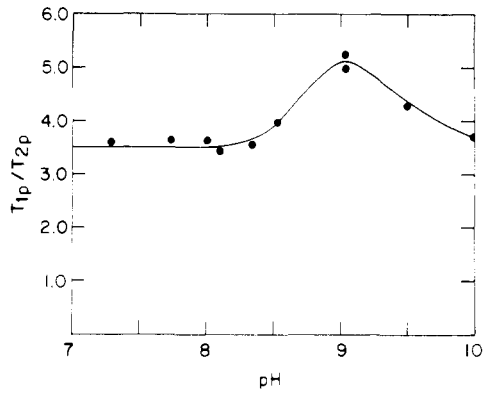

FIGURE 2: A plot of T_{1p}/T_{2p} of water protons at 100 MHz as a function of pH in the presence of Mn(II)-thermolysin at 1.0×10^{-4} M and 25 °C.

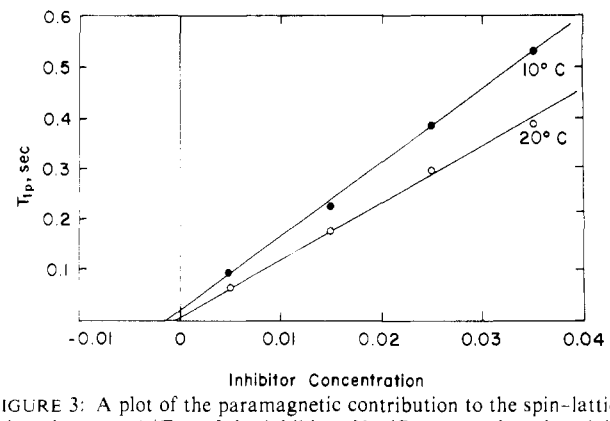

FIGURE 3: A plot of the paramagnetic contribution to the spin-lattice relaxation rate, $1/T_{1p}$, of the inhibitor *N*-trifluoroacetyl-D-phenylalanine at 94.1 MHz vs. inhibitor concentration in the presence of manganese-thermolysin. Temperatures are 10 (●) and 20 °C (○).

zyme is accompanied by the cooperative release of protons from the enzyme. The logarithmic relationship of eq 5 is actually a Hill equation of the displacement data and the parameter n has exactly the same interpretation as a Hill coefficient. Therefore, the fact that the value of the Hill coefficient is 1.9 does not necessarily imply only two residues are involved in the cooperative displacement of manganese by protons, but rather it implies that two or more residues are involved with cooperative interactions between them.

These results establish that the manganese does dissociate from the enzyme as the pH is lowered below 7. This explains the observed decrease in the water relaxation rate under these conditions. However, above pH 7 the manganese is totally bound. Thus, dissociation of the ion from the enzyme will not explain the decrease in water relaxation observed above pH 7. To further examine the source of the change in relaxation rate observed above pH 7, the ratio of the paramagnetic contributions to the bulk water relaxation from the spin-lattice relaxation time and spin-spin relaxation time T_{1p}/T_{2p} was observed as a function of pH. This is shown in Figure 2. This shows a bell-shaped pH dependence. Since all the Mn(II) remains bound to the enzyme at these pH values, there are only two sources of the observed pH dependence. One is the exchange rate of the water between the bound manganese ion and the bulk water. The other possible explanation involves a change in correlation time from the water-bound manganese complex. The reasons for this conclusion are discussed more fully under Discussion.

The Interaction of N-Trifluoroacetylphenylalanine with Mn(II)-Thermolysin. The ^{19}F NMR spectrum of the free *N*-trifluoroacetylphenylalanine inhibitor is a sharp singlet. Typical preparations also exhibited an additional small peak located 12-Hz downfield from the parent peak which was identified as that due to a small amount of the hydrolyzed species trifluoroacetate. Addition of carboxypeptidase A to the L isomer produced this hydrolyzed product but the corresponding D isomer was not cleaved by the enzyme in agreement with the results of Vine et al. (1973). Addition of Zn(II)- or Mn(II)-thermolysin also resulted in specific cleavage of the L isomer, although at a rate much slower than that observed for carboxypeptidase A. The D isomer is not a substrate for thermolysin. For this reason, the D isomer was used in all the following experiments.

The effects of temperature, concentrations of the Mn(II)-enzyme, and the inhibitor on the T_1 and T_2 relaxation times of the ^{19}F-substituted inhibitor were investigated. The dissociation constant of the Mn(II)-enzyme-inhibitor complex was determined at two temperatures by measuring T_{1p} for various concentrations of inhibitor in the presence of a constant enzyme concentration. The data obtained are summarized in Table I. Under these conditions, the observed value of T_{1p} can be related to $T_{1,app}$, the apparent contribution to the observed relaxation time of the trifluoromethyl group when it is bound to the enzyme, by the relationship (Dahlquist and Raftery, 1968)

$$S_0 = E_0 \frac{T_{1p}}{T_{1,app}} - K_s$$

where S_0 and E_0 refer to the total concentrations of substrate and enzyme-manganese complex, respectively. Thus, a plot of T_{1p} vs. S_0 should be linear with a slope equal to E_0/T_{1M} and an intercept on the x axis of $-K_s$. As shown in Figure 3, a plot of T_{1p} vs. [I] was linear for experiments performed at 10 and 20 °C. The value of K_s estimated from these plots is on the order of 1 mM or less.

This result was completely unexpected. Kinetic measurements using trifluoracetyl-D-phenylalanine to inhibit the enzyme-catalyzed hydrolysis of FAGLA showed the fluorinated analogue to behave as a competitive inhibitor of FAGLA hydrolysis. The inhibition constant obtained was 30 mM or at least one order of magnitude weaker than the dissociation constant measured by NMR. This result was the same if either zinc- or manganese-thermolysin was observed. This kinetic result strongly implies that two molecules of the inhibitor can bind. One of these binds tightly but noncompetively with FAGLA. A second molecule of the inhibitor binds at a weaker but competitive site with respect to FAGLA.

The slopes can be used to calculate the values of the apparent bound relaxation rate, $1/T_{1,app}$, at the two temperatures. These values are $1.3 \times 10^3\ s^{-1}$ at 10 °C and $1.75 \times 10^3\ s^{-1}$ at 20 °C, or about a 30% increase in the relaxation rate for a 10 °C temperature increase. This corresponds to a phenomenological activation energy of 4.3 kcal/mol for the relaxation process.

The apparent bound relaxation rate is itself the result of two competing events, relaxation and chemical exchange, such that under the conditions of these experiments

$$\frac{1}{T_{1,app}} = \frac{1}{T_{1B} + \tau}$$

where T_{1B} corresponds to the relaxation time for the trifluoromethyl group of the inhibitor when bound and τ is the lifetime of the inhibitor–enzyme complex. The usual explanation for an increase in the observed relaxation rate with increased temperature is that $1/T_{1,app}$ is largely controlled by the lifetime of the complex, τ. Thus, the expected decrease in τ with increasing temperature gives rise to a larger apparent relaxation rate at higher temperatures. From these arguments it would seem likely that chemical exchange is an important consideration in these experiments. However, if chemical exchange is controlling $1/T_{1,app}$, it will also limit $1/T_{2,app}$, the apparent bound relaxation rate, as well. This should make the ratio of T_{1p}/T_{2p} very nearly equal to unity. As seen in Table I, this ratio is equal to 3.9. Since the T_2 processes are substantially faster than the T_1 processes, chemical exchange cannot completely dominate the observed values of $1/T_{1B}$. In the limit that T_2 measurements reflect only chemical exchange, the ratio T_{1p}/T_{2p} can be seen to give a limiting value to the relative contributions of T_{1B} and τ to the value of $1/T_{1app}$.

Since

$$\frac{T_{1p}}{T_{2p}} = \frac{T_{1B} + \tau}{T_{2B} + \tau} \approx \frac{T_{1B}}{\tau} + 1 \text{ if } T_{2B} \ll \tau$$

so at 10 °C

$$1.3 \times 10^3\ s^{-1} \leq 1/T_{1B} \leq 1.7 \times 10^3\ s^{-1}$$

The relaxation rate of the enzyme-bound trifluoromethyl group consists of a contribution from the paramagnetic interaction with the manganese ion and a contribution from diamagnetic interactions. The diamagnetic contribution can be determined by using the native zinc form of thermolysin and is negligibly small for T_1 measurements.

This relaxation data can be used to estimate the distance between the bound manganese ion and bound trifluoroacetyl group, providing the correlation time of the interaction between them can be evaluated.

If the T_2 measurement is not exchange limited, the T_{1p}/T_{2p} can be used to estimate τ_c directly. Since (Bigbee and Dahlquist, 1974)

$$\tau_c = \frac{1}{\omega_I}\left\{\frac{3}{2}\left[\frac{T_{1p}}{T_{2p}} - \frac{7}{6}\right]\right\}^{1/2}$$

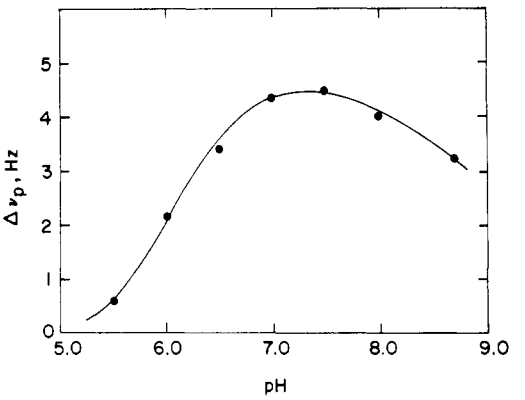

FIGURE 4: A plot of the paramagnetic contribution to the line width, $\Delta \nu p$, of the trifluoromethyl resonance of *N*-trifluoroacetyl-D-phenylalanine vs. pH at a constant concentration of manganese enzyme.

where ω_I is the resonant frequency of the observed nucleus in rad/s. A T_{1p}/T_{2p} ratio of 3.9 corresponds to a τ_c value of 3.4×10^{-9} s. If $T_{2,app}$ is exchange limited, this is the lower limit on the value of τ_c.

The distance can now be estimated using the relationship (Solomon, 1955)

$$r\ (\text{Å}) = 722\left[(T_{1B})\left(\frac{3\tau_c}{1 + \omega_I^2\tau_c^2}\right)\right]^{1/6}$$

or

$$r = 8\ \text{Å}$$

assuming that $T_{2,app}$ is not exchange limited. If the T_2 measurements had exchange contributions, this estimated distance becomes a maximum distance between the fluorine and manganese when bound, since T_{1B} is nearly independent of this assumption and τ_c would be larger than estimated from the T_{1p}/T_{2p} ratio.

The pH dependence of the interaction of the inhibitor with the Mn(II)–enzyme was also examined. As shown in Figure 4, the maximum broadening of the ^{19}F resonance occured at pH 7.2 with a decrease in broadening for the pH values on each side of neutrality. The results between pH 7 and 5 were not unexpected as the Mn(II) ion dissociates from the complex in this range and no significant broadening of the ^{19}F-substituted inhibitor resonance occurs when the Mn(II) ion is free in solution. At pH values above 7, a similar result to that seen for the water proton relaxation is observed with a decrease in the relaxation rate with increasing pH.

Discussion

The results presented here have furnished additional information concerning the active-site region of thermolysin. ESR and NMR investigations of the Mn(II)–enzyme derivative have probed the detailed interaction of the metal ion with the active-site residues of the enzyme. NMR studies of the binding of ^{19}F-substituted inhibitors to the Mn(II)–enzyme have shown that the interaction is more complex than suggested by kinetic inhibition studies in that the NMR results suggest the presence of a tight binding site for trifluoroacetyl-D-phenlalanine located quite close to the active site which does not, however, seem to inhibit the catalytic activity of the enzyme.

The observed pH-dependent binding equilibrium between tle zinc-free enzyme and Mn(II) ion is consistent with a scheme in which the binding of Mn(II) ion to the enzyme is dependent on the cooperative release of at least two protons with an apparent pK_a of 6. While the exact origin of these protons is not known, it is likely that the three metal ligands His-142, His-

146, and Glu-166 (Matthews et al., 1972) are the sources. Initial studies of the Mn(II) form of thermolysin by x-ray crystallographic methods (Kester and Matthews, private communication) suggest that these same ligands are involved in binding the manganese ion. Thus, the binding of Mn(II) ion and by analogy the native Zn(II) ion surely requires that these ligands exist in the unprotonated form. The protonation of one of these ligands, most likely one of the two histidine residues, results in a weakening in the interactions of the ion with the other ligands and the manganese ion is released. The remaining neutral histidine residue is now free to be protonated. Since the glutamic acid residue is most likely to be unprotonated in the metal-ion complex, it may be available for protonation when the metal ion dissociates. However, it is likely that the pK_a of this residue is sufficiently low that the carboxyl group remains dissociated at pH 6.

The environment of the bound manganese ion is also perturbed by the ionization of at least two groups in the pH range 8–10. This perturbation is seen as an increase followed by a decrease in the ratio of the paramagnetic contributions to T_1 and T_2 of the water protons in the presence of manganese-thermolysin. There are two possible explanations for this effect: (1) a pH-dependent change in the exchange rate of water between the bound manganese ion and bulk water, and (2) a pH-dependent change in the correlation time of the water-manganese ion interaction. These alternatives are not easily distinguished from the types of measurements we have made or can make with our instrumentation.

Our earlier work (Bigbee and Dahlquist, 1974) shows that it is very likely that exchange considerations are not important at pH 7.0 for water relaxation. We shall assume that it remains unimportant at all pHs and interpret the T_1/T_2 ratio change as a change in correlation time with pH. However, the arguments which follow could just as easily be applied from a consideration of the pH dependence of the exchange rate. In either case, it is clear that the environment in the vicinity of the bound manganese ion changes with the ionization of groups on the protein in the pH range 8–10. These groups could reside in the immediate environment of the active site and exert their effects directly, or they could reside at positions far removed from the active site and exert their effect through conformational interactions. We have seen no evidence for such pH-induced changes in the NMR spectrum of the protein itself (Bigbee and Dahlquist, unpublished results). It, therefore, seems reasonable that these groups must be active site ones. Examination of the crystal structure of thermolysin shows only four reasonable alternatives for these two ionizable groups. They include Glu-143, Tyr-157, His-231, and the water molecule bound to the active-site metal. The environment of Glu-143 is not sufficiently unusual to suggest a shift in pK_a to 8 or 10. Modification studies (Burstein et al., 1974) suggest a pK_a of 7.6 for His-231 which is outside the range of values which could account for the relaxation data. We, therefore, conclude that the two ionizable groups are most likely Tyr-157 and the bound water itself. In principal, the relaxation data should allow the calculation of the proton–metal distance in the ionized form. Neutron-scattering data (Christensen and Ollivier, 1972) suggest a H–Mn distance of 2.86 Å in $Mn(OH)_2$ which is very nearly the same as the generally accepted value for $Mn(H_2O)_6^{+2}$. However, the sixth power dependence on distance associated with relaxation data means that a small uncertainty in distance implies a large uncertainty in the number of water protons which are bound to the metal. As a result, such a calculation is difficult to justify.

The pH dependence of the T_1/T_2 ratio demonstrates an important consideration for the use of such measurements to determine the correlation time of a particular interaction. Even if one can unambiguously show that exchange is fast, the T_1/T_2 ratio may not reflect the actual correlation time of the interaction. Suppose the pH is such that the T_1/T_2 ratio is changing rapidly as pH varies. Under these conditions, the T_1 and T_2 values reflect averages over at least two protonated forms. The source of the differences in relaxation rates is a pH-dependent correlation time. However, since the relaxation times are not linear functions of the correlation time, an "average" T_1/T_2 ratio gives an incorrect value for the average correlation time. This is not a problem for our fluorine relaxation studies done at a pH range where the T_1/T_2 ratio was constant.

The NMR study of the inhibitor trifluoroacetyl-D-phenylalanine with the Mn(II)–enzyme has shown the inhibitor to be exchanging between free and bound environment at a rate of at least 10^3/s. This rapid exchange condition has allowed the use of the measured paramagnetic contribution to the relaxation time to calculate the correlation time of the dipolar interaction between the fluorine and Mn(II) and also the distance between them. The calculated correlation time of 4×10^{-9} s can be compared with a value of 3×10^{-9} s deduced by Navon et al. (1968) for the interaction of Mn(II)–carboxypeptidase with inhibitors. Although from present data it is not possible to discern the dominant term contributing to τ_c, our earlier work on the interaction of the Mn(II)–enzyme with water protons has suggested that τ_e, the electron relaxation time, is a major contributor (Bigbee and Dahlquist, 1974).

The calculated distance of 8 Å between the trifluoroacetyl group and the Mn(II) ion together with the NMR-determined K_s of 1 mM or less suggest a relatively tight binding of the inhibitor in moderately close proximity to the metal ion bound at the active site. This result is in contrast to the kinetically determined K_I of ~30 mM. A clue to a reason for this discrepancy comes from the effect of near saturating concentrations of the inhibitor on the relaxation rate of the water protons. As we have shown previously, the effect of bound inhibitors on the water relaxation rate is variable depending on the nature of the inhibitor (Bigbee and Dahlquist, 1974). This effect we have interpreted as evidence for mixed modes of inhibitor binding in which one mode, which we have called the productive mode, results in displacement of the bound water molecule from the metal ion by the peptide carbonyl oxygen of the inhibitor. Another binding mode, evidenced by other inhibitors, has no effect on the relaxation rate of the water, suggesting that this inhibitor does not interact with the metal ion and presumably inhibits by binding only in the hydrophobic site on the enzyme. The ^{19}F-substituted inhibitor used in this study had an intermediate effect on the water relaxation rate; i.e., the water relaxation rate in the presence of the inhibitor fell between the rate observed for the Mn(II)–enzyme alone and that seen for Zn(II)–enzyme in the absence of paramagnetic ion. Thus, the tight binding site seen in the NMR experiments (which is fully saturated by bound inhibitor in these experiments) must be one in which the water molecule is not displaced from the Mn(II) ion, and represents what we have previously described as a nonproductive binding mode. Indeed, the calculated distance of 8 Å appears to be inconsistent with the binding of the carbonyl oxygen atom of the trifluoroacetyl group directly to the metal ion.

One might expect that, if even a small fraction of the inhibitor was bound closer to the manganese ion, the $1/r^6$ dependence of the dipolar interaction would result in a clear broadening of the ligand lines. Since we see no such broadening but can infer the second site because it inhibits catalysis, we may only conclude that either the observed nucleus is far from

the manganese or the exchange rate is slow for this inhibitory binding orientation.

From our results we conclude that the active-site region of thermolysin contains two binding sites, or at least two nonoverlapping possible binding orientations, for these small inhibitor molecules. The site seen by NMR observation of the perturbation of the inhibitor resonances is a tight but apparently nonproductive binding. The other site, or orientation, while much weaker is productive in the sense that binding at this site, or in this mode, partially displaces water from the bound metal ion and apparently is bound in an orientation which prevents catalytically effective binding of substrate. Preliminary x-ray crystallgraphic results (Kester and Matthews, unpublished results) are consistent with this interpretation. The inhibitor appears to bind at two positions. One of these suggests that the inhibitor acts as a metal-ion ligand at the active site while the inhibitor occupies the hydrophobic binding pocket in its other orientation.

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Studies on 21-^{3}H-Labeled Corticosteroids: Evidence for Isomerization of the Ketol Side Chain of 11-Deoxycorticosterone by a Hamster Liver Enzyme†

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ABSTRACT: We have previously observed that liver and other tissues catalyze the transfer of tritium from 11-deoxy-[21-^{3}H]corticosterone to water (Willingham, A. K., and Monder, C. (1974), *Endocr. Res. Commun. 1,* 145–153). We now have found that most of the tritium is lost in a reaction that involves exchange of the label with water protons. This exchange is enzyme-catalyzed, yields unlabeled 11-deoxycorticosterone (DOC), and is reversible. The enzyme was purified about 150-fold. It required no added cofactors. The enzyme was yellow, with spectral maxima at 272 and 403 nm, was inhibited by *o*-phenanthroline and EDTA, and was reactivated by Co^{2+}, Fe^{2+}, or Mo^{5+}. Co^{2+} and Fe^{2+} themselves enhanced the activity of the purified enzyme. The apparent molecular weight was 390 000. [21*S*-21-^{3}H]DOC was detritiated faster than the 21*R* epimer. Although 1 equiv of deuterium was quickly incorporated into DOC when the enzymic reaction occurred in D_2O, prolonged incubation resulted in the exchange of both 21-methylene hydrogens. Loss of tritium was not due to a reversible oxidation of the 21-alcohol. A newly synthesized steroid, 20-hydroxy-3-oxo-pregn-4-en-21-al (isoDOC) was converted to DOC by the enzyme. In order to explain these results, we propose that the enzyme is an isomerase that reversibly interconverts both DOC and isoDOC through a common enediol intermediate.

Corticosteroids undergo a wide range of catabolic transformations in man and other animals. Study of the excreted end products of these reactions are valuable in reconstructing metabolic pathways, and in the diagnosis of disease. In most laboratories, it has been the practice to study the "neutral" steroid metabolites of urine, that is, the fraction of the total steroids remaining in the organic extract of urine after the solvent phase has been washed with alkali. It has been pointed out by several investigators (Peterson, 1971; Brooks, 1964; Taylor, 1969; Fukushima et al., 1960) that the neutral and conjugated urinary steroids account for only a part of the total metabolites excreted, and a number of workers have suggested that, among other possibilities, steroid acids may be formed and passed into the urine in addition to the other well-known metabolites (Brooks, 1964; Taylor, 1969; Southcott et al., 1956; Lowy et al., 1969; Gray and Shaw, 1965). It indeed appears that important steroid acids are discarded in the alkali

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