Role of Calcium in the Thermal Stability of Thermolysin†

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ABSTRACT: The effect of calcium ion on the thermal stability of thermolysin has been investigated. The native protein undergoes an irreversible structural change and autolysis at high temperature. Analysis of the calcium ion dependence of the apparent melting temperature observed spectroscopically gives an apparent ΔH of $-x$ (130 kcal/mol) where x is either 1 or 2. Neither zinc ion, when bound at the active site, nor terbium ion, which binds very tightly to the double calcium binding site, shows a stabilizing effect. These sites are therefore presumably not coupled to the transition which leads to autolysis. Removal of calcium ion from the native enzyme at temperatures below 50 °C results in inactivation but not major autolysis. The addition of 1 equiv of terbium before calcium removal results in a protein species which is 40% active and is no longer subject to thermal stabilization by calcium. These results suggest a pathway for the thermal inactivation of the enzyme which involves an irreversible structural change at one or both of the single calcium ion sites. This change propagates to the active site and results in inactivation. The binding of calcium ion to either or both single sites completely inhibits this structural change. The structural change is apparently cooperative and may correspond to a localized denaturation of the native structure.

Thermolysin, an endopeptidase produced by *Bacillus thermoproteolyticus*, has been the subject of much recent research concerned with its heat stability (Ohta et al., 1966; Ohta, 1967). Recent results (Endo, 1962; Feder et al., 1971; Drucker and Borchers, 1971) have shown that the temperature of the onset of thermal denaturation is raised 40 to 50 °C in the presence of calcium ion. The x-ray crystallographic studies of Matthews and co-workers (Matthews et al., 1972) have demonstrated that the native enzyme binds one zinc ion at the active site and four calcium ions in three other distinct sites on the enzyme surface. The two single calcium binding sites are quite exposed. In one case calcium appears to interact with aspartic acid residues 57 and 59 and with a peptide carbonyl group, while at the second site the calcium ligands include aspartic acid residue 200, a threonine hydroxyl, and two peptide carbonyl groups (Matthews et al., 1974). The two calciums bound at the double site are only 3.8 Å apart and neutralize a pocket of five acidic residues. All four calcium sites appear to bind the calcium ions through essentially ionic interactions and do not suggest any unusual interactions to account for the heat stability. The relative locations of these binding sites are shown in Figure 1.

In a previous study, we have employed ESR and NMR[1] techniques to probe the environment of the active site of the enzyme (Bigbee and Dahlquist, 1974). That study placed particular emphasis on the role of the zinc ion in the binding of water and substrates. The object of the present work is to investigate in more detail the roles of metal ions in the mechanism of thermal stabilization of the enzyme.

The problem is complicated by the fact that the denatured form of the protein is itself a good substrate for the remaining native enzyme. As a result, it is very difficult to observe the reversible denaturation of the protein by usual methods. This makes it essentially impossible to directly study the thermodynamic interplay of calcium binding and protein denaturation. This paper presents an approach to obtaining useful information about these types of interactions when observations can be made only under irreversible conditions. Our data demonstrate the following major points. (1) Thermal denaturation of the native enzyme which contains four bound calcium ions and one bound zinc ion invariably results in autolysis. (2) Calcium binding is coupled to an apparently cooperative transition in the protein structure which leads to autolysis at temperatures above 50 °C. (3) Zinc binding to the active site and terbium ion binding to the double calcium ion site are *not* coupled to this cooperative transition in the protein structure. (4) Removal of calcium ion from the enzyme at low temperature (below 50 °C) results in the irreversible loss of catalytic activity. This irreversibly altered material appears to have an organized structure since it undergoes the same spectral transition at 286 nm as the native material. However, this material undergoes the transition at a relatively low temperature and is not subject to thermal stabilization by calcium ion. (5) In the presence of the single tightly bound terbium ion, which is bound at the double calcium ion site, removal of the remaining two calcium ions from their sites results in an irreversibly altered material as well. This altered protein retains 40% of the native catalytic activity. It denatures at low temperature, however, and is not subject to thermal stabilization by calcium ion.

We interpret these results to suggest that the major role of calcium in stabilizing native thermolysin toward thermal denaturation is to protect the region near one or both single calcium ion sites from a cooperative conformation change. The conformation renders that region of the thermolysin molecule susceptible to irreversible modification which leads to autolysis at temperatures above 50 °C.

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; NMR, nuclear magnetic resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. Calcium, terbium, zinc, and manganese refer to Ca(II) ion, Tb(III) ion, Zn(II) ion, and Mn(II) ion, respectively.

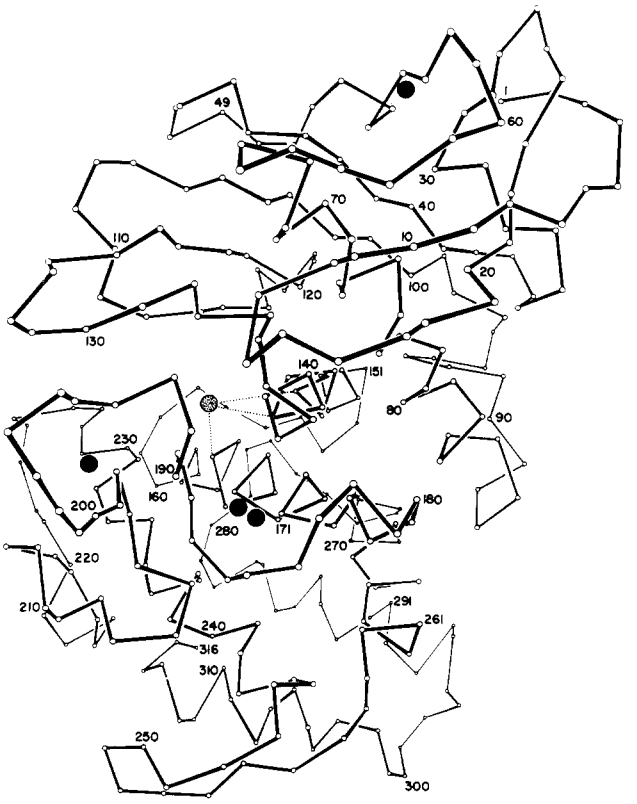

FIGURE 1: A backbone diagram of the thermolysin structure showing the single zinc ion binding site ○ and the four calcium ion binding sites (●). The single calcium sites are located near residues 57 and 229. The double site is near residue 190.

Experimental Section

Materials. Thermolysin, 3× crystallized, A grade, and Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, A grade, were purchased from Calbiochem. Sephadex G-25 and G-100 were obtained from Pharmacia Fine Chemicals, Inc. The lanthanide metal ions used were obtained, as the chlorides, from Alfa Inorganics. Chromatographic standards, insulin, glucagon, and the peptide Trp-Met-Asp-Phe-NH_2 were gifts of Dr. Frank Reithel. All other chemicals were reagent grade of the highest quality commercially available.

Methods. The holoenzyme (native thermolysin containing four calcium atoms and one zinc atom per molecule) was prepared from the commercially available material as previously described (Bigbee and Dahlquist, 1974). All absorption measurements and kinetic determinations using the previously described furylacryloylglycylleucinamide assay (Feder, 1968) were performed on a Cary Model 14 spectrophotometer thermostated at 25 ± 1 °C. Calcium and zinc concentrations were determined with a Techtron Model 5 atomic absorption spectrometer as described (Bigbee and Dahlquist, 1974). For the enzyme melting determinations, a Gilford Model 2000 Multiple Sample Absorbance Recorder utilizing a Beckman DU spectrometer and a Gilford Model 208 Auxilliary Offset Control was used. The temperature was monitored by a Gilford Model 207 Linear Thermosensor and controlled by a Neslab Model TP-1 Temperature Programmer in conjunction with a PMT Type T9/100 and Haake temperature baths. For the fluorescence experiments, a specially designed Schoeffel fluorescence spectrophotometer Model RRS 1000 with a 1000-W xenon arc lamp and dual excitation monochromators was used. The spectrofluorometer was interfaced with a Varian 8K 620i computer for the purpose of ensemble averaging of the data.

The following procedure was used to prepare apothermolysin. The simple addition of a chelator such as EDTA does not produce apoenzyme, but rather results in autolysis. This can be avoided if the apoenzyme was prepared by using the following method. A holoenzyme solution was adjusted to 2 mM in 1,10-phenanthroline. This complexed the Zn(II) but not the Ca(II). Then the solution was adjusted to 10 mM in EDTA and chromatographed on Sephadex G-25 with 10 mM EDTA included in the buffer. The resulting material was seen to have the same molecular weight (as judged by Sephadex chromatography) and showed >90% activity regain when diluted into an assay mixture containing excess Ca(II) and Zn(II). It is important to add phenanthroline first and then add the EDTA. Either the simultaneous addition, or the addition of EDTA before phenanthroline, allows autolysis to take place. Apparently, removal of calcium before inactivation by zinc removal makes the enzyme very susceptible to autolysis. All studies of apothermolysin were performed in the presence of 10 mM EDTA.

EDTA titration experiments were performed in the following way. The holoenzyme was prepared at a concentration of $1-2 \times 10^{-4}$ M. Aliquots of EDTA were added in varying amounts up to final concentrations exceeding four enzyme equivalents. The solutions were allowed to stand at room temperature for 15 min. Then excess calcium was added to quench the reaction and the solutions were assayed using the standard furylacryloylglycylleucinamide assay by removal of an appropriate aliquot of the quenched mixture.

The terbium form of thermolysin was prepared by dialysis of the holoenzyme against terbium ion containing buffer. The holoenzyme solution was dialyzed overnight against a 100-fold volume of 1 mM terbium ion followed by dialysis against a 10^{-5} M terbium solution. Measurement of the calcium by atomic absorption spectroscopy gave routinely approximately 0.2 calcium and 1.0 zinc per enzyme molecule.

All buffers contained 0.01 M Hepes (pH 7.2) and 10% glycerol unless otherwise stated. The glycerol had less than a 10% effect on the catalytic efficiency of the enzyme and appears to do little else than increase the protein solubility.

Results

Nature of the Thermal Transition Observed by Heating Holothermolysin. The x-ray crystallographic results of Matthews and co-workers (Matthews et al., 1972, 1974; Matthews and Weaver, 1974) have demonstrated that one zinc ion and four calcium ions are bound to thermolysin at specific binding sites. This stoichiometry can be demonstrated in solution by gel filtration chromatography of commercial thermolysin preparations. This shows that the protein binds 1 equiv of zinc and 4 equiv of calcium tightly. We shall refer to such a preparation, containing 1 zinc and 4 calcium equivalents, as holothermolysin.

Table I shows a summary of the various forms of thermolysin employed in this work. The activity measurements were performed in the presence of excess zinc and calcium and therefore reflect the activity of reconstituted material in some cases.

Ohta (1967) has shown that an abrupt change occurs in the ultraviolet absorbance of holothermolysin at 286 nm upon heating. This provides a convenient means of monitoring the effects of various ions on the thermal stability of holothermolysin. As shown in Figure 2, a decrease in optical density at 286 nm of ~12% is observed once a critical tem-

Table I: Summary of the Properties of Various Forms of Thermolysin.

Enzyme Species	Ion Stoichiometry	Catalytic Act.[a] (%)	Stabilized by Excess Ca Ion
Holo	1 Zn, 4 Ca	100	Yes
Heated holo	Extensive autolysis	None	No
Apo	None	100	Yes
Heated apo (6 M urea)	None	60–80	Yes
Terbium thermolysin	1 Zn, 3 Tb	100	Yes
Monoterbium thermolysis	1 Zn, 1 Tb, 2 Ca	100	Yes
EDTA-treated holo	1 Zn	None	No
EDTA-treated monoterbium thermolysin	1 Zn, 1 Tb	40	No

[a] Activity measurements are performed in the presence of excess zinc and calcium ion.

perature is reached. This change was seen to be irreversible; i.e., the original value of OD_{286} did not return upon cooling. This suggests an irreversible change in structure associated with the spectral transition. Subsequent Sephadex G-100 gel chromatography showed the heated holoenzyme to be of low molecular weight with apparently no material remaining of the native molecular weight of 34 600. Chromatography of the heated material on Sephadex G-25 showed that the material was a heterogeneous mixture of peptides from molecular weights greater than 10 000 to ~1000 with the majority of the material of apparent molecular weight ~5000.

Heating of the enzyme above the temperature necessary to cause the transition at 286 nm invariably resulted in autolysis. Surprisingly, complete autolysis following heating to the transition temperature could be demonstrated even under conditions where the enzyme is drastically inhibited as judged by assay using the synthetic substrate furylacryloylglycylleucinamide. For example, heating the holoenzyme in the presence of high concentrations of 1,10-phenanthroline, which removes the catalytically essential zinc ion, resulted in complete autolysis. Similarly, pretreatment of the enzyme to remove all but 1–2% of the zinc, followed by heating in the presence of 2 mM 1,10-phenanthroline, also gave complete autolysis. In both cases calcium ion was present as well. The addition of a number of common enzyme modification reagents such as iodoacetate, iodoacetamide, *N*-ethylmaleimide, etc. also had no demonstrable inhibitory effect on the autolysis of the enzyme.

Ion-Free (Apo) Thermolysin Can Be Reversibly Denatured. We have been able to find only one set of conditions which allows thermal denaturation of thermolysin without substantial autolysis. This requires great care in preparing enzyme which is completely devoid of *any* ions which may act as zinc ion replacements.

Heating experiments were performed on the apoenzyme in EDTA containing buffer in the hope that the thermal transition could be observed and that the transition might be reversible. A transition at 286 nm is observed as with the holoenzyme. This transition occurs at a temperature of about 50 °C, much lower than those observed previously in the presence of calcium ion. However, the initial decrease in absorbance is quickly followed by an abrupt increase in optical density which continued to increase with time at constant temperature. Chromatography on Sephadex G-100 showed that while autolysis had not occurred, the apoenzyme had aggregated into units at least as large as tetrameters and probably larger. This aggregated material was nearly completely inactive toward furylacryloylglycylleucinamide when assayed in the presence of zinc and calcium.

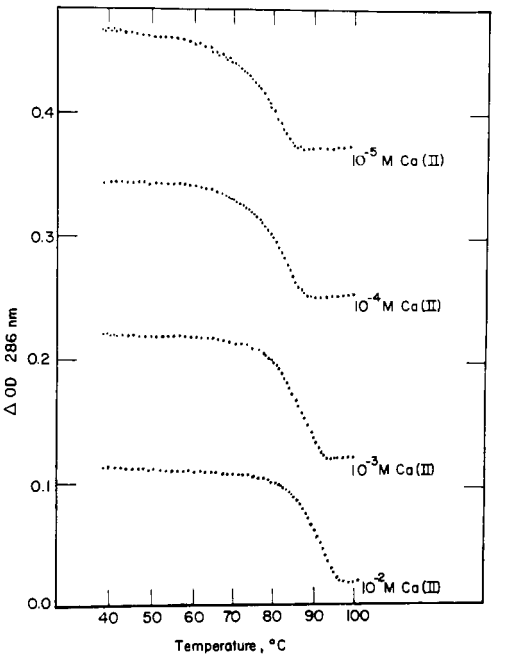

FIGURE 2: Optical transition of thermolysin at 286 nm upon heating. Sample is holoenzyme, 1.8×10^{-5} M, in 0.01 M Hepes buffer (pH 7.2) and 10% glycerol. Solution was heated at a rate of ~0.8 °C/min. The melting temperature, T_m, is defined as the midpoint of the transition.

In order to overcome the difficulty of the aggregation and inactivation of the heated apoenzyme, various concentrations of urea were included in the buffer. Addition of urea in concentrations from 0.5 to 2 M decreased the apparent transition temperature, but also decreased the amount of aggregation. After cooling these solutions, some activity was regained after ion replacement. No optically detectable aggregation occurred in 2 M urea. After cooling, approximately 28% of the original activity was regained. When higher concentrations of urea were used, protection against irreversible aggregation and inactivation was greater. As much as ~80% return of activity after ion replacement was observed after cooling an enzyme solution which had been heated to 55 °C for 15 min in 6.6 M urea.

The thermal transition of the apoenzyme observable at 286 nm was also examined by fluorescence. The fluorescence of the apoenzyme in 2 M urea produced by excitation at 295 nm and observation of the emission at 352 nm was followed as a function of temperature. The transition temperature observed, ~34 °C, agrees quite well with that observed with ΔOD_{286} of ~35 °C. The fluorescence experiments were performed with 10^{-7} M enzyme, about two orders of magnitude lower in protein concentration than the absorbance experiments. Thus, the thermal transition is not strongly dependent on protein concentration.

Measurements performed above the optical transition temperature showed that the enzymatic activity is lost at high temperature. The ultraviolet spectrum of the high temperature form is essentially that of a mixture of peptides with the same relative proportions of chromophoric amino acids as thermolysin. The results suggest that the spectral transition corresponds to the randomization of the thermolysin structure. When no metal ions are present, this ran-

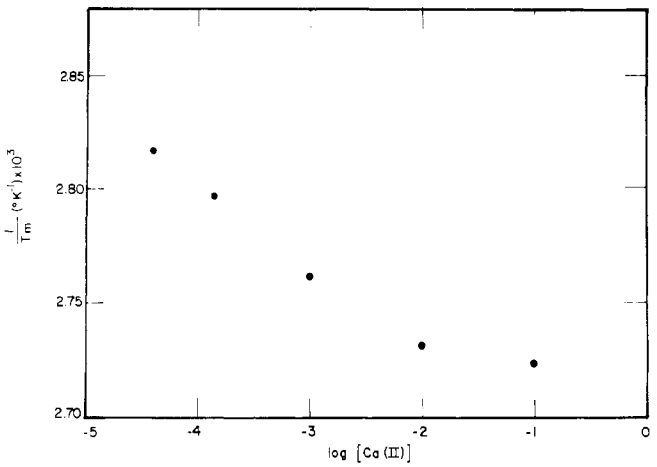

FIGURE 3: Plot of the reciprocal of the extrapolated melting temperature of thermolysin vs. the log of the total Ca(II) ion concentration.

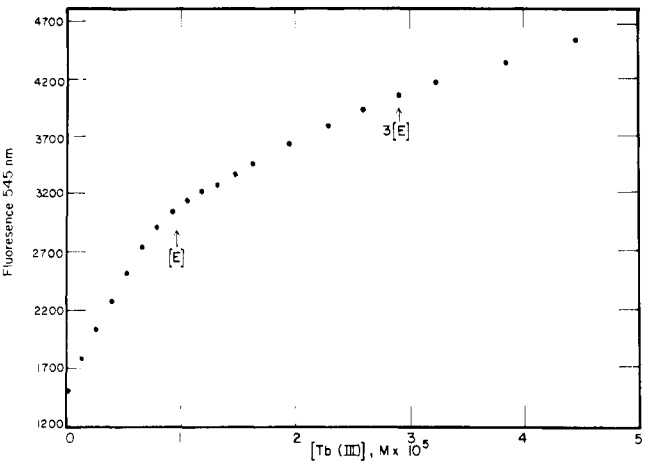

FIGURE 4: Binding of Tb(III) ion to thermolysin by fluorescence at 545 nm. Aliquots of 1 mM Tb(III) were added to a holoenzyme solution, 0.96×10^{-5} M. Excitation wavelength was 290 nm.

domized structure is relatively stable. However, the presence of even low concentrations of free divalent ions renders a fraction of the enzyme active and the denatured form of the enzyme is rapidly hydrolyzed.

Excess Calcium Raises the Observed T_m. Heating of enzyme stored with low concentrations of calcium showed two spectral transitions: one near 50 °C and the other near 80 °C. Addition of high concentrations of calcium increased the melting temperature of only the high transition. This suggests that aging of enzyme solutions containing low amounts of calcium ion results in a species with a relatively low melting temperature and is not capable of stabilization by calcium. When EDTA was added to intact holoenzyme and the solution heated, a ΔOD_{286} was observed as a broad transition in the temperature range of ~40–50 °C. Addition of large amounts of calcium (enough to completely overwhelm the EDTA present) had little or no effect on the temperature of this transition. The temperature of this transition was very similar to that observed for the first transition seen in the biphasic "melting" of the aged holoenzyme. These results suggest that the intact enzyme is susceptible to aging only when the calcium ions are not bound to the enzyme.

Freshly prepared samples of the holoenzyme were heated in the presence of various concentrations of calcium and the change in absorbance as a function of temperature was observed. The results of these experiments are shown in Figure 2. A dramatic increase in the observed transition temperature, T_m, with increasing calcium ion concentration is observed. Thus, 0.01 M $CaCl_2$ causes about a 40 °C increase in the transition temperature as compared to that of apoenzyme. Again, gel filtration chromatography of the material obtained after melting showed that complete autolysis had occurred. There was no indication that any cleavage had occurred in solutions heated to temperatures 5 °C below the T_m observed at that particular calcium ion concentration.

The shape of the temperature transition appears to be the result of a kinetic effect, since it depends on the heating rate. The effect of heating rate on the observed transition temperature at several calcium ion concentrations was determined. This allows an empirical extrapolation of the observed T_m to infinite heating rate. Under these conditions the kinetic effects associated with autolysis should be minimized. The values of T_m are not strongly heating rate dependent, showing about a 4 °C change for a tenfold change in heating rate. Most importantly, however, the change in T_m which is induced by the presence of excess calcium is not a function of the heating rate. These results suggest that the increase in T_m associated with the presence of calcium ion may be treated as an equilibrium phenomenon, followed by an irreversible step. Thus, T_m appears to reflect the real thermodynamic melting temperature for the reversible step.

As shown in the Appendix, the melting temperature should vary with calcium ion concentration according to eq 5, where x is the number of calcium ions directly coupled to the cooperative transition and where ΔS_{eff} and ΔH_{eff} are the effective enthalpy and entropy changes associated with the transition.

$$\frac{1}{T_m} = \frac{\Delta S_{eff}}{\Delta H_{eff}} + \frac{xR}{\Delta H_{eff}} \ln [Ca] \qquad (5)$$

Figure 3 shows a plot of the apparent $1/T_m$ vs. the logarithm of the free calcium ion. At low calcium ion concentrations, there is quite a linear relationship which is maintained over about three orders of magnitude in calcium ion concentration. The stabilization shows saturation at about 0.1 M calcium ion, suggesting that binding to the conformationally changed (denatured) enzyme occurs at these high concentrations. The slope of the line at low concentration, together with eq 5, gives a value for the quantity $\Delta H/x$ equal to −130 kcal/mol. This large value for ΔH_{eff} suggests a cooperative transition in the structure of the enzyme which leads to autolysis.

One Terbium Ion Binds Very Tightly to the Double Calcium Ion Site. Matthews and Weaver (1974) have shown by x-ray crystallographic methods that one lanthanide ion binds quite specifically to the double calcium ion binding sites of thermolysin, displacing both calcium ions. This binding occurs even in the presence of high concentrations of calcium ion and suggests a very high affinity of the double calcium ion site for lanthanide ions. The work of Luk (1971) had demonstrated that the intrinsic fluorescence of terbium ion is greatly enhanced when the ion is bound to the protein transferrin. We observed a similar enhancement of terbium fluorescence in the presence of thermolysin. In particular, one can observe an approximately 100-fold enhancement of terbium fluorescence in the presence of the enzyme when excited at 290 nm and observed at 545 nm.

As shown in Figure 4, one can titrate the holoenzyme with terbium by monitoring the increase in fluorescence at

545 nm as terbium is added. One observes a linear increase in fluorescence as terbium is added until 1 equiv of terbium has been added. Above this point, the fluorescence continues to increase but to a lesser degree and does not saturate until quite high concentrations of terbium have been added. To test if terbium was binding to the calcium sites, or other sites for which both calcium and terbium compete, calcium was added to solutions of the holoenzyme containing 3 to 5 equiv of terbium. Upon addition of high concentrations of calcium (1 mM), the fluorescence intensity is reduced to the value obtained when only 1 equiv of terbium was originally present. Addition of concentrations of calcium as high as 0.1 M did not decrease the intensity further. Careful titration with calcium showed a smooth decrease of fluorescence intensity to this limiting level.

The final fluorescence intensity corresponded to that obtained in the direct titration of the enzyme with one terbium ion per enzyme molecule. These results agree with those obtained by Matthews and Weaver (1974) and suggest that a single terbium ion is bound very tightly, indeed. The gradual increase in terbium fluorescence obtained above 1 equiv suggests that terbium ion binds to other calcium sites but with a lower affinity.

Addition of calcium ion to terbium thermolysin in which all calcium ions had been replaced by terbium reduced the terbium fluorescence to the same level obtained when a single equivalent of terbium was added to the holoenzyme. The competition between terbium and calcium for these weaker sites suggests an approximately equal affinity for terbium and calcium ion at the sites.

Neither Zinc Bound to the Active Site nor Terbium Ion Bound at the Double Calcium Ion Site Causes Stabilization. The addition of 10^{-3} M zinc to a solution containing 1×10^{-5} M enzyme with 1 equiv of zinc and 10^{-3} M calcium ion results in no further stabilization of the native form. The affinity constant of zinc for the active site of the enzyme has been estimated to be in the range of 10^{13} M. In the absence of any additional zinc ions, the enzyme acts as a zinc buffer, holding the free concentration at 10^{-11} M or below. Our previous studies (Bigbee and Dahlquist, 1974) show no evidence that calcium ion competes effectively for the zinc binding site. If any competition does occur, the relative affinities of the zinc site for calcium ion are at least six orders of magnitude weaker than the affinity for zinc. Thus, the highest concentration of zinc available in the absence of additional zinc ion is about 10^{-9} M. The addition of zinc to the enzyme may be treated in an analogous way to the approach used for calcium ion binding. The change in transition temperature ΔT_m associated with a change in zinc ion concentration from Zn^0 to Zn will be given by eq 7 of the Appendix:

$$\Delta T_m = -\frac{(T_m^0)^2 R}{\Delta H_{eff}} \ln \frac{[Zn]}{[Zn^0]}$$

for relatively small values of ΔT_m. Substituting the values $[Zn]/[Zn^0] = 10^6$ and $\Delta H_{eff} = -x(130$ kcal/mol):

$$\Delta T_m = 26\ ^\circ\text{C}/x \qquad x = 1\text{-}4$$

or a change of between 6 and 26 °C would be expected if zinc binding were coupled to the cooperative step in the temperature-induced autolysis of the enzyme. Since no stabilization is observed, it appears that zinc binding is not directly coupled to the cooperative transition.

The observation of extremely strong binding of terbium to the double calcium site suggests a method to further delineate the region of the thermolysin molecule which is stabilized toward autolysis by calcium ion binding. As shown in Table II the addition of 1 equv of terbium to thermolysin in the presence of 10 mM calcium resulted in essentially no change in the observed transition temperature. Since the single tightly bound terbium is not displaced by even 0.1 M calcium ion, one would expect a substantial stabilization by terbium if its binding site were involved in the transition. The fact that there is no further stabilization argues strongly that the double calcium ion binding site region is not coupled to the cooperative transition. Therefore, neither the zinc site nor the double calcium site is associated with that part of the thermolysin molecule which undergoes the cooperative transition which leads to autolysis.

Table II: Effects of Various Ions on the Apparent Melting Temperature of Thermolysin.

	T_m (°C)
A. Holothermolysin	
Alone	78
+ 10 mM KCl	78
+ 10 mM $CaCl_2$	92
+ 1 equiv of $TbCl_3$	79
+ 1 mM $CaCl_2$	86
+ 1 equiv of $TbCl_3$ + 1 mM $CaCl_2$	86
B. Apothermolysin	
Alone	~50
+ 4 equiv of $CaCl_2$	78
+ 4 equiv of $CaCl_2$ + 1 equiv of $ZnCl_2$	78
+ 10 mM $TbCl_3$	78

In contrast to these results, terbium stabilizes thermolysin when it can bind to the single calcium sites. Thus, thermolysin which has had its four calcium ions replaced by three terbium ions shows the same high melting temperature as the native holoenzyme. This terbium substituted enzyme can be further stabilized by terbium concentrations as high as 1 mM. At these concentrations the enzyme precipitates.

These results imply that terbium binding at a site other than the double site gives rise to stabilization while terbium binding directly to the double site has little or no effect.

Removal of Calcium at Low Temperatures Results in Loss of Catalytic Activity, but Not Substantial Autolysis; This Inactive Material Is Not Subject to Stabilization by Calcium. The addition of EDTA to native thermolysin results in loss of activity of the enzyme. The loss of activity coincides with the appearance of a protein which is not capable of being stabilized by calcium and which shows a low temperature thermal transition at 286 nm. In order to better understand this inactivation process, quantitative additions of EDTA to thermolysin were performed. The results of these experiments are summarized as a plot of the activity remaining vs. EDTA added and are shown in Figure 5. Two important observations are apparent from the figure: (1) that 4 equiv of EDTA is required before the enzyme is inactivated; (2) that apparently one calcium ion can be removed from the enzyme with little change in activity. We interpret these results to mean that the calcium ion which is least tightly bound to the enzyme can be removed with little effect on the activity of the enzyme. Measurements of the thermal denaturation of EDTA-treated protein were performed. After 15 min of incubation with EDTA, calcium was added to make the final solution 10 mM in calcium. Then the usual optical melting measurements were made on these solutions. As shown in Figure 6 the result of the addi-

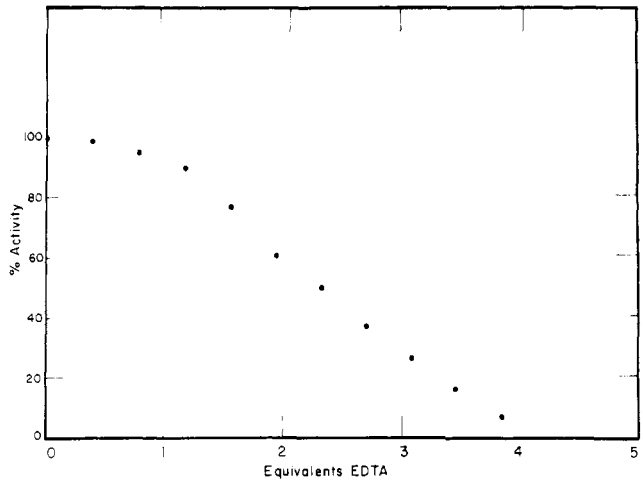

FIGURE 5: Titration of holothermolysin with EDTA. Activity was determined by the furylacryloylglycylleucinamide assay. Enzyme concentration was 1.0×10^{-4} M.

tion of successively greater amounts of EDTA was to produce a corresponding greater amount of the material which displayed the lower temperature thermal transition. Thus, there is a one-to-one correspondence between the loss of activity and the appearance of low melting temperature material upon addition of EDTA.

Sephadex chromatography of EDTA treated enzyme which had not been heated showed that the majority of the material chromatographed at the same elution volume as the native enzyme with the remaining material of lower molecular weight. The material which was eluted at the same position as the native enzyme was not fully active however. For example, enzyme which had been treated with 2.5 equiv of EDTA showed only half the specific activity of the untreated enzyme in the high molecular weight peak. Thus, it appears that the result of treatment of the enzyme with EDTA is to produce, at least initially, material of essentially the same molecular weight as native enzyme but having little or no activity. Heating of this EDTA-treated enzyme solution to 60 °C (above the lower temperature transition but well below the high temperature transition) produced material which showed an increase of low molecular weight products at the expense of the parent peak. The remaining material, eluting at the position corresponding to native enzyme, now was *fully* active. As usual, heating above the temperature necessary to cause autolysis of the native enzyme resulted in only low molecular weight material. Electrophoresis using sodium dodecyl sulfate-acrylamide gels shows the EDTA treated material to contain some material of nearly the same molecular weight as the native material. In addition, substantial quantities of three peptides of smaller molecular weight are observed.

The EDTA titration results suggest that one of the four calcium ions which bind to the native enzyme may be removed with a relatively slight effect on the activity. However, removal of the second calcium ion results in the production of a high molecular weight, inactive protein. This species is susceptible to autolysis at temperatures above 50 °C and this susceptibility is not modified by the presence of calcium ion. It also appears that after the removal of the second calcium ion, the protein loses its ability to bind calcium as tightly as the native enzyme. This follows since the enzyme is not inactivated completely after the removal of the average of two calcium ions but requires the removal of an average of four. When the enzyme is an average of half

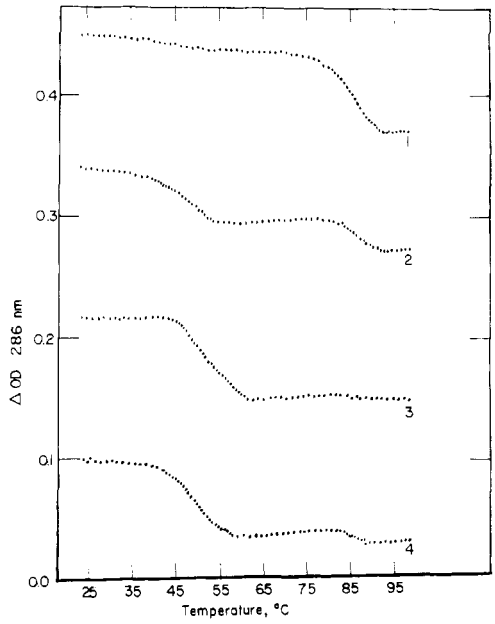

FIGURE 6: Melting behavior of EDTA-treated holothermolysin. Curves 1, 2, and 3 are the results for solutions of holothermolysin to which have been added 1.0, 2.5, and 4.0 equiv of EDTA, respectively. Sample 4 was holoenzyme containing 1 equiv of Tb(III) followed by the addition of 6.0 equiv of EDTA. All solutions were made 5×10^{-3} M in Ca(II) before heating.

inactivated, the solution is an equal mixture of fully native enzyme and the inactivated protein.

EDTA Titration of Monoterbium-Dicalcium Thermolysin Results in ~60% Loss in Catalytic Activity and Complete Loss of Thermal Stabilization by Calcium Ion. To further distinguish which calcium ion binding sites were responsible for the inactivation, we made use of the high affinity of the double calcium ion binding site for terbium. Since terbium is bound tightly at the double calcium site, displacing the two calcium ions in the process, the addition of terbium to the enzyme serves as a probe of that specific part of the structure. As shown in Figure 7, only 2 equiv of EDTA can be added to the terbium treated enzyme before irreversible loss of activity occurs. These 2 equiv of EDTA account for the two calcium ions which are displaced by the addition of terbium. This implies that one of the two calciums bound in the double site is the one which is most easily removed by EDTA in the holoenzyme.

The striking and unexpected result of these experiments is that addition of more than 6 equiv of EDTA does not cause the activity of the terbium enzyme to go to zero. In fact, the activity of the enzyme falls to about 40% of the native after 4 equiv of EDTA has been added and remains at that level even after the addition of up to 10 equiv of EDTA. These results suggest that the terbium bound at the double site calcium confers to the molecule some local structure in the region of the active site (recall that the double calcium site is quite close to the zinc at the active site) which results in an enzyme conformation which retains about 38–40% activity. Melting experiments performed on the monoterbium enzyme treated with 6 equiv of EDTA, and which are still 40% active, showed only the lower temperature transition. Thus, the role of terbium and by analogy the two calcium ions bound at the double site appears to be that of conferring local stability to the active region of the molecule. Thus, the single calcium ion binding sites 3 and/or 4 are likely to be responsible for the gain in thermal

stability, and addition of EDTA sufficient to cause loss of calcium ions from these sites appears to cause a conformational change which is irreversible in the presence of active enzyme. This structure is more susceptible to autolysis and has lost the ability to be greatly stabilized by addition of calcium.

Discussion

Table I summarizes our observations concerning the thermal stability of various forms of thermolysin. We have shown that excess calcium ion stabilizes native holothermolysin against an apparently cooperative structural transition which results in autolysis at temperatures above 50 °C. However, neither zinc, which binds at a site distinct from the four calcium sites, nor terbium, bound at the double calcium site, shows this stabilizing effect. Interestingly, the binding of terbium to sites other than the double calcium site also gives rise to this stabilization. These results suggest that the transition which leads to autolysis involves only an isolated region of the thermolysin molecule and that region is distinct from the active site and the double calcium ion site.

At temperatures below 50 °C, the binding of calcium also protects the enzyme from loss of catalytic activity. At these low temperatures the majority of the inactive material has essentially the same molecular weight as native enzyme as judged by gel chromatography or sodium dodecyl sulfate gel electrophoresis. This could mean that the simple removal of calcium from its sites on the enzyme surface results in an irreversible denaturation of the enzyme. However, this almost surely is not the correct explanation. If both zinc and calcium ions are removed, the enzyme is completely active over long times when diluted back into zinc and calcium ion buffer for the assay. Thus the absence of calcium does not necessarily result in inactivation. These results imply that *both* the presence of zinc and the absence of calcium ion are necessary for inactivation at low temperature. This suggests that inactivation at low temperatures requires the catalytic activity of the enzyme. There was no evidence of a substantial change in molecular weight by gel chromatography under nondenaturing conditions. However, under denaturing conditions evidence of autolysis was found. Therefore, it appears that an autolytic cleavage occurs. This cleavage results in catalytic inactivation of the protein. Under native conditions the autolyzed protein retains structure such that dissociation of the peptides does not occur unless denaturing conditions are employed. The inactive material also appears to have structure since an ultraviolet spectral change is observed when the material is heated above 50 °C. The details of the position of the autolytic cleavage and structure of the autolyzed material are under investigation in this laboratory.

The observation of ion specificity in the stabilization demonstrates that the stabilization is not the result of a bulk effect, such as ionic strength, but rather depends on the interaction of calcium ion at a specific site or sites on the enzyme. The possible positions for the stabilizing effect of calcium ion are either or both single calcium sites seen by x-ray methods, or some weaker calcium ion site which is not observed crystallographically. This latter possibility seems remote for several reasons. The holoenzyme, with only four bound calcium ions, shows a dramatic stabilization compared with the apoenzyme. The EDTA titration of holoenzyme suggests that the second most tightly bound calcium is intimately involved with the stabilization process. The removal of this calcium in the monoterbium, dicalcium form of the enzyme results in an irreversible conversion to an enzyme species which is no longer subject to stabilization by calcium, although it retains a relatively high (40%) activity against furylacryloylglycylleucinamide. All these data suggest that the single calcium ion sites observed crystallographically are the most likely candidates to explain the calcium stabilization. It is not possible to decide which single site or indeed if both sites are involved in the stabilization from our data.

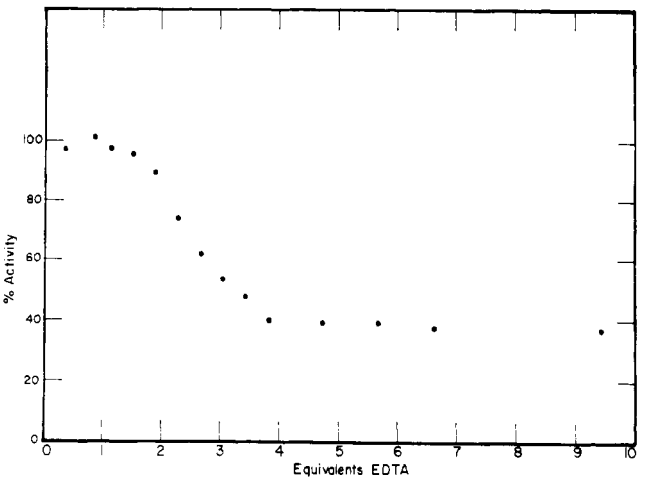

FIGURE 7: EDTA titration of holothermolysin containing 1 equiv of Tb(III).

From the coupling of the apparent melting temperature and calcium ion binding we can estimate the change in enthalpy (ΔH_{eff}) associated with the conformational change. This ΔH_{eff} includes ΔH for the conformational change as well as ΔH for the binding of the important calcium ions. Our data suggest $\Delta H_{eff} = -x(130$ kcal/mol) where x is the number of calcium ions which stabilize the enzyme. From the arguments given above, the value of x is either 1 or 2. For either value of x, the large apparent value of ΔH suggests that a highly cooperative change occurs.

One possible source of such a cooperative change would be the complete thermal denaturation of the molecule. Other possible sources include a local cooperative conformational change or localized denaturation which render a portion of the thermolysin molecules susceptible to hydrolysis, rapidly followed by complete autolysis of the enzyme. If the cooperative transition were the complete denaturation of the entire enzyme structure, the binding of both zinc to the active site and calcium to their sites should stabilize the native structure and should shift the apparent T_m to considerably higher temperature. However, if a localized conformational change were the source, additional zinc ions would stabilize the structure *only* if the zinc binding site were coupled to the cooperative transition. Again, since neither zinc nor terbium bound at the double calcium site confers this stability, the cooperative transition cannot correspond to complete denaturation but rather it must involve only a localized area of the thermolysin structure.

The EDTA titration experiments of holothermolysin show that the most weakly bound calcium ion can be removed with little effect on activity. This weakly bound ion almost surely resides at the double calcium ion site since EDTA treatment of holoenzyme in the presence of 1 equiv of terbium shows that in addition 2 equiv of EDTA (corresponding to the two calciums displaced by terbium) may be

added with no effect. However, addition of the third equivalent of EDTA results in inactivation. Thus, the weakly bound calcium is displaced by terbium, implying that it is bound at the double site.

When EDTA is added to native holothermolysin, the enzyme species present are either inactive or fully active; This suggests a cooperative interaction such that when the second calcium is removed, the remaining calciums are more weakly bound. This change in affinity may be the result of some autolytic event, or possibly be the result of some conformational change. Voordouw and Roche (1974) have also shown that there is cooperatively in calcium binding to thermolysin which has been catalytically inhibited by removal of zinc by a zinc specific chelating agent. Apparently, terbium bound to the double calcium site cannot be removed by EDTA. Thus, it is possible to work with an enzyme solution which consists of exclusively monozinc (at the active site) monoterbium thermolysin. This species is very interesting. It retains 40% catalytic activity but is no longer subject to thermal stabilization by excess calcium. This species can only be made by removal of calcium from the holoenzyme to which 1 equiv of terbium has been added. Therefore, it seems that some irreversible change occurs when calcium is removed from one or both of the single calcium sites.

Using our data, we can propose a pathway for the thermal inactivation of the holoenzyme and explain the role of calcium. As seen in Figure 1, the two single calcium ion binding sites are quite distant from the active site or double calcium ion binding site. The dissociation of calcium from one or both of the single calcium sites (either near aspartic acid residue 57 or near aspartic acid residue 200) results in a conformation change of the protein in that region. This conformationally changed material undergoes complete thermal denaturation, with a T_m of about 50 °C, corresponding to the value for apoenzyme or for enzyme which has lost its ability to be stabilized by calcium. Apparently the conformational change destroys the calcium ion binding site. The role of calcium in the native enzyme is to stabilize this localized region against undergoing the conformation change. At some stage the process is irreversible when zinc or a catalytically active zinc replacement is bound at the active site. Presumably, there is hydrolysis or rearrangement of some covalent bond. Following the irreversible step, it appears that a conformation change is propagated to the active site in a way which involves the double calcium site. When calcium is bound at that double site, the propagation to the active site occurs, apparently displacing calcium, and the enzyme is inactivated. When a single terbium ion is bound at the double site with its higher binding energy, the conformation change is blocked or at least modified so that a stable protein retaining 40% activity is produced. This modified protein cannot be stabilized by calcium since the single calcium site or sites are destroyed by the irreversible structure change.

In this model, we have tacitly assumed that removal of calcium by EDTA and removal by raising the temperature are similar processes and results of the two experiments are directly comparable. We cannot rigorously show that this assumption is justified. Until more experiments are performed, however, the application of Occam's razor seems to justify this assumption.

Acknowledgment

We wish to thank Brian Matthews and his research group for helpful discussions and for providing us with Figure 1.

Appendix

A Model for Stabilization of Thermolysin by Calcium and Other Ions. Consider the thermodynamic and kineic scheme shown in eq 1:

$$E(Ca)_x \overset{K_x}{\rightleftharpoons} E + xCa \qquad E \overset{K_c}{\rightleftharpoons} E' \longrightarrow \text{autolysis} \tag{1}$$

Here we consider the conformational change between two forms of the enzyme E and E′ to be defined by the equilibrium constant K_c. This equilibrium is coupled to the binding of several (x) calcium ions. It is known that four calcium ions bind specifically to the native form of the thermolysin molecule. If the proposed conformational change were actually the complete thermal denaturation of the thermolysin molecule, the value of x would be 4. If the conformational change involved a region containing only one calcium ion binding site, the value of x would be unity. We see that x is actually the excess number of calcium ions bound to E as compared to E′. Under saturating conditions, all E will exist in the bound form and the coupling of conformation change and calcium ion binding may be considered as:

$$E(Ca)_x \rightleftharpoons E' + x(Ca) \tag{2}$$

The equilibrium between native enzyme and E′ may be defined by an apparent equilibrium constant:

$$K_{app} = [E][Ca]_x/[E'] \tag{3}$$

By defining an apparent free energy change for the transition as

$$\Delta G_{app} = -RT \ln K_{app}$$

the dependence of the apparent value of the transition temperature, T_m, on calcium ion may be obtained as the temperature at which $\Delta G_{app} = 0$

$$0 = -RT_m \ln K_{app}$$

$$0 = -RT_m \ln (K_x K_c) - xRT_m \ln [Ca]$$

where K_x is the phenomenological constant for the dissociation of x calcium ions to the native protein. Then

$$0 = \Delta H_{eff} - T_m \Delta S_{eff} - xRT_m \ln [Ca] \tag{4}$$

where $\Delta H_{eff} = \Delta H_c + \Delta H_1 + \Delta H_2 + \ldots \Delta H_x$, $\Delta S_{eff} = \Delta S_c + \Delta S_1 + \Delta S_2 - \ldots \Delta S_x$, and $\Delta G_{eff} = \Delta G_c + \Delta G_1 + \Delta G_2 - \ldots \Delta G_x$, and the subscripts c, 1, 2, and x refer to the conformational change, first, second, and final calcium binding steps, respectively. Rearranging and solving for $1/T_m$ gives:

$$\frac{1}{T_m} = \frac{\Delta S_{eff}}{\Delta H_{eff}} + \frac{xR}{\Delta H_{eff}} \ln [Ca] \tag{5}$$

Thus, a plot of $1/T_m$ vs. the logarithm of free calcium ion concentration should give a straight line of slope $xR/\Delta H_{eff}$. Our experimental results show that the value of T_m obtained at a particular calcium ion concentration depends to some extent on the heating rate used in the experiment, but that the change in T_m induced by a change in calcium ion concentration at a particular heating rate did not depend upon the heating rate. Thus, the slope of a $1/T_m$ vs. logarithm of calcium ion concentration will be independent of the heating rate. The value of the intercept will change

slightly with heating rate, however. This relationship will hold for calcium ion concentrations which are sufficiently low so there is not much binding of calcium ion to the E′ form.

This analysis assumes that ΔH_{eff} and ΔS_{eff} are not strongly temperature dependent. This implies that there are not large heat capacity changes associated with the conformational change and ion binding steps.

For changes in melting temperature with ion concentration, the differentiated form of eq 5 can be used (eq 6). For small changes in melting temperature from an initial melting temperature, T_m^0, caused by a change in calcium ion concentration from Ca^0 to Ca, eq 7 applies. These arguments can be easily expanded to include the thermal stabilization induced by the binding of any other ions by replacing x in these equations with the number of other ions which are coupled to the transition. Similarly ΔH_{eff} and ΔS_{eff} would become modified to include the ΔH and ΔS of binding of the ion.

$$-(dT_m/T_m^2) = (xR/\Delta H_{eff})d \ln [Ca] \quad (6)$$

$$T_m - T_m^0 = \Delta T_m = \frac{-xT_m^2R}{\Delta H_{eff}} \ln \frac{[Ca]}{[Ca^0]} \quad (7)$$

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^{1}H Nuclear Magnetic Resonance Double Resonance Study of Oxytocin in Aqueous Solution†

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ABSTRACT: Peptide NH resonances in the 250 MHz ^{1}H nuclear magnetic resonance (NMR) spectrum of oxytocin in H_2O were assigned to specific amino acid residues by the "underwater decoupling" technique (i.e., decoupling from corresponding $C^\alpha H$ resonances, which are buried beneath the intense water peak). These experiments confirm previous assignments of A. I. Brewster and V. J. Hruby ((1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3806) and A. F. Bradbury et al. ((1974), *FEBS Lett. 42,* 179). Three methods of assigning NH resonances of peptides—solvent titration, underwater decoupling, and isotopic labeling—are compared. As the solvent composition is gradually changed from dimethyl sulfoxide to H_2O, oxytocin undergoes a conformational change at 70–90 mol % of H_2O. Exposure to solvent of specific hydrogens of oxytocin in H_2O was studied by monitoring intensity changes of solute resonances when the solvent peak was saturated. Positive nuclear Overhauser effects (NOE's) of 14 ± 5 and 9 ± 5 were observed for the Tyr ortho CH and meta CH resonances, respectively. Comparative studies with deamino-oxytocin indicate that these effects result predominantly from intermolecular dipole–dipole interaction between aromatic side chain CH protons and protons of the solvent. The NOE's therefore indicate intimate contact between water and the aromatic CH hydrogens of the Tyr side chain. The extent of saturation transferred by proton exchange between water and NH groups varies with pH in a manner which appears to reflect the acid–base catalysis of the protolysis reaction. There is no indication that any NH protons are substantially shielded from the solvent.

Oxytocin [*cyclo*(Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6)-Pro^7-Leu^8-Gly$(NH_2)^9$], a neurohypophyseal hormone, has, in addition to its physiological activity of stimulating the ejection of milk from mammary tissue, a broad range of well-defined biological properties (Sawyer and Knobil, 1974). Determination of the free solution conformation of oxytocin in water and in less polar solvents serves as a starting point for elucidation of the molecular mechanism of interaction of this hormone with its intracellular carrier protein neurophysin (for a recent symposium on this subject see *N.Y. Acad. Sci. 248,* 1975) and, eventually, also the mechanism of its interaction with oxytocin receptors, which are believed to be located on the outer surface of cell membranes (Schwartz and Walter, 1973). ^{1}H nuclear magnetic resonance (NMR) studies of peptides are most readily performed in solvents such as $(CD_3)_2SO$, in which identifica-

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