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Fluorescence Energy Transfer between Metal Ions in Thermolysin. Thermal Denaturation Studies†

Shakoor M. Khan, Edward R. Birnbaum, and Dennis W. Darnall*

ABSTRACT: Thermolysin derivatives have been prepared with one zinc ion, two calcium ions, and one terbium ion. Excitation of this derivative at 280 nm results in emission of visible Tb^{3+} fluorescence at 545 nm. When Co^{2+} is substituted for the Zn^{2+}, the Tb^{3+} emission is quenched due to energy transfer between the Co^{2+} and Tb^{3+}. Distances have been calculated between the two metal ion binding sites assuming a dipole–dipole mechanism for energy transfer (Berner, V. G., et al. (1975) *Biochem. Biophys. Res. Commun. 66,* 763; Horrocks, W. D., Jr., et al. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 4764). We have extended these studies by following the Co^{2+}–Tb^{3+} distances as a function of temperature. We have found a gradual increase in the distance between the two metal ions that ranges from nearly 14 Å at 25 °C to nearly 22 Å at temperatures above 80 °C. Conventional techniques for measuring conformational changes in proteins (tryptophan–tyrosine fluorescence, optical rotation or enzyme activities) show little or no change in the protein structure up to 70 °C. At higher temperature drastic changes in thermolysin properties indicate an extensive unfolding of the protein. In contrast to the optical rotation, fluorescence, and activity measurements, viscosity measurements indicate changes in the protein structure at temperatures well below 70 °C, and these changes correspond rather well with the distance changes between the Co^{2+} and Tb^{3+}. This indicates that both the viscosity and fluorescence energy transfer experiments are detecting changes in the protein structure which are not detectable by the other techniques.

Thermolysin is a neutral metalloendopeptidase isolated from the thermophilic organism *Bacillus thermoproteolyticus* Rokko which has an unusual stability toward thermal denaturation (Endo, 1962; Matsumara, 1967). The amino acid sequence (Titani et al., 1972) and three-dimensional structure have been determined (Matthews et al., 1974, and references therein). The protein consists of two distinct lobes with the active site of the enzyme lying in the cleft formed by these two lobes. A zinc ion, essential for enzyme activity, lies at the base of the cleft while 4 calcium ions presumably provide structural stability.

In recent years, paramagnetic, chromophoric metal ions such as trivalent lanthanide ions have proven useful as a structural probe in the study of structure–function relationships in proteins and enzymes (Darnall & Birnbaum 1970). In the particular case of thermolysin, the calcium ions at sites 1 and 2 have been replaced by a single lanthanide ion while still maintaining calcium ions at sites 3 and 4 (Matthews & Weaver, 1974). Similarly, the zinc ion can be replaced by a cobalt ion without affecting the conformation of the protein (Holmquist & Vallee, 1974). Making use of these substitutions, fluorescence spectroscopy has been used to determine a distance of 13.6 Å between the calcium sites 1 and 2 and the zinc binding site (Berner et al., 1975; Horrocks et al., 1975). These measurements agree well with the distance obtained from X-ray crystallographic measurements. In this present study we have used the same method to monitor the increase in the distance between these two metal sites as a function of temperature. To our knowledge this is the first study where the distance between two sites on a protein can be quantified as the protein is thermally unfolded. Activity, ORD, and viscosity measurements have been carried out to compare the results from fluorescence energy transfer measurements with the more typical measures of conformational changes in proteins.

Methods

Activity. The activity of thermolysin was measured by monitoring the decrease of 3-(2-furylacryloyl)glycyl-L-leucinamide (FAGLA)[1] absorbance at 322 nm at constant temperature (Khan & Darnall, 1978). To obtain a temperature profile, the following procedure was used: 3 mL of FAGLA (5.0×10^{-5} M) in 0.01 M Hepes buffer, 0.01 M $CaCl_2$, 1.0 M NaCl at pH 7.5 was placed in the 1-cm pathlength sample cell at the desired temperature. A similar concentration of FAGLA was placed in the reference cell to partially balance the high initial absorbance of FAGLA at these concentrations. After 10 min, a 50-μL aliquot of a 2×10^{-5} M thermolysin solution which had been incubated at the same temperature for the same length of time was added to the FAGLA solution in the sample compartment. Within 10 s after mixing, the decrease in absorbance at 322 nm was followed as a function of time. A fresh sample of enzyme was allowed to equilibrate for a 10-min period at each particular temperature.

Fluorescence. An Aminco-Bowman ratio spectrophotofluorimeter with a thermostated cell compartment was used for all fluorescence measurements. In a typical experiment, 200 μL of the enzyme solution was placed in a 3-mm fluores-

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[1] Abbreviations used: FAGLA, 3-(2-furylacryloyl)glycyl-L-leucinamide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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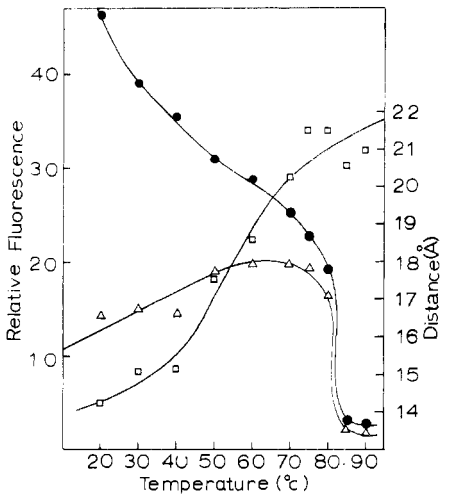


FIGURE 1: Temperature dependence of Tb^{3+} fluorescence in Zn^{2+}-Tb^{3+} thermolysin (●--●), and in Co^{2+}-Tb^{3+} thermolysin (△—△). Distances (□—□) were calculated between the cobalt and terbium binding sites in thermolysin as a function of temperature from the terbium fluorescence data using eq 1 and 2. Protein concentrations in both samples were 5.0×10^{-6} M. Unless otherwise indicated, the protein in this and all following figures is dissolved in 0.01 M Hepes buffer containing 0.01 M $CaCl_2$, 1.0 M NaCl at pH 7.5. The protein was excited at 280 nm and the Tb^{3+} emission was followed at 545 nm.

cence cell and allowed to equilibrate in the spectrophotometer for 10 min at a given temperature, after which time the spectrum was recorded.

ORD. A Cary 60 spectropolarimeter was used with thermostated 1-cm pathlength cells. The protein sample was equilibrated for 10 min at the chosen temperature after which the rotation was measured. The cell was maintained in the same position for all experiments to minimize artifacts.

Viscosity. Cannon-Funske viscometers were used to measure viscosity as a function of both concentration and temperature. In the temperature study, two viscometers were used, both immersed in the same temperature bath. The ratio of flow of 4 mL of buffer in one viscometer vs. 4 mL of an enzyme solution in the other viscometer was used to determine specific viscosity as a function of temperature. All solutions were equilibrated for 10 min before measuring the flow rate. The ratio of the flow rates of the two viscometers with the same buffer solution was determined at 25 °C to correct for differences between viscometers. Flow times were measured using a VWR Vanlab timer with an accuracy of 0.1 s. The times used in the calculations were an average of at least three runs. No deterioration of the sample after several runs was observed.

Temperature Control. Lauda circulating baths were used to control the temperature of all solutions in this study. The temperature in the particular cell was measured using a Tele-thermometer YSI Model 4256. Effective temperature control was within ±0.1 °C.

Preparation of Different Metallo Derivatives of Thermolysin. Different metallo derivatives of thermolysin were prepared basically as previously described (Berner et al., 1975; Horrocks et al., 1975). Thermolysin, obtained from Sigma, was recrystallized according to Holmquist & Vallee (1974). The recrystallized thermolysin was dissolved in a buffer system consisting of 5 M NaBr, 0.01 M Hepes buffer, 0.01 M $CaCl_2$ at pH 7.5. The zinc-free enzyme was prepared by dialysis against three changes of a solution of 0.01 M Hepes, 0.01 M $CaCl_2$, 1.0 M NaCl, 2×10^{-3} M *o*-phenanthroline, pH 7.5, 4 °C. This was followed by dialysis against three changes of the same buffer solution without the *o*-phenanthroline. This results in a thermolysin preparation which has no zinc but still retains the full complement of Ca^{2+}. The Zn^{2+}-Tb^{3+}-thermolysin (i.e., thermolysin with one zinc ion, two calcium ions at sites 3 and 4, and one terbium ion located at calcium sites 1 and 2) and the Co^{2+}-Tb^{3+}-thermolysin were prepared from the above preparation by adding stoichiometric amounts of Zn^{2+} or Co^{2+} to the zinc free enzyme followed by the addition of a 15-fold excess of Tb^{3+}. The solutions were then allowed to equilibrate for at least 12 h. The Zn^{2+}-Tb^{3+} derivative can also be prepared by adding a 15-fold excess of Tb^{3+} directly to zinc-containing thermolysin.

Theory

Energy transfer between a fluorescent lanthanide ion as a donor and an absorbing cobalt ion as an acceptor has been shown to proceed via a dipole-dipole mechanism described by the Förster theory (Förster, 1966; Berner et al., 1975). Under conditions where the Co^{2+} ion quenches the Tb^{3+} ion fluorescence of Co^{2+}-Tb^{3+}-thermolysin compared with the Tb^{3+} fluorescence of the analogous Zn^{2+}-Tb^{3+} enzyme, the amount of quenching is related to the distance between the two ions, R, by the following equation:

$$\frac{F_{Co^{2+}\text{-}Tb^{3+}}}{F_{Zn^{2+}\text{-}Tb^{3+}}} = 1 - T = \frac{1}{1 + \left[\frac{R_0}{R}\right]^6} \quad (1)$$

where $F_{Co^{2+}\text{-}Tb^{3+}}$ is the terbium fluorescence intensity of Co^{2+}-Tb^{3+}-thermolysin, $F_{Zn^{2+}\text{-}Tb^{3+}}$ is the terbium fluorescence intensity of Zn^{2+}-Tb^{3+}-thermolysin, T is the fraction of energy transfer between the donor-acceptor pair, and R_0 is the distance (in Å) at which the energy transfer is 50% efficient. R_0 is defined by eq 2:

$$R_0 = 9.79 \times 10^3 (Jn^{-4}k^2Q)^{1/6} \quad (2)$$

The critical transfer distance, R_0, is dependent on: J, the integrated spectral overlap between the fluorescence spectrum of the donor, Tb^{3+}, and the absorption spectrum of the acceptor, Co^{2+}; n, the refractive index; k, the relative orientation of the two dipoles; Q, the quantum efficiency of the donor in the absence of energy transfer. The effect of temperature on each of these parameters and the consequent effect on the calculated distance R between the Co^{2+} and Tb^{3+} ions bound to thermolysin will be considered later. A more detailed description of the Förster theory and the parameters contained in eq 1 and 2 can be found in Berner et al. (1975), Horrocks et al. (1975), and references therein.

Results

Tb^{3+} Fluorescence. The intensity of the Tb^{3+} fluorescence at 545 nm in Co^{2+}-Tb^{3+}-thermolysin is quenched compared with Zn^{2+}-Tb^{3+}-thermolysin. As has been discussed previously, by making use of the Förster theory (eq 1 and 2), the distance between the two metal ions when bound to thermolysin was determined to be ~14 Å at room temperature (Berner et al., 1975; Horrocks et al., 1975). In the present work we have measured the change in the quenching of the Tb^{3+} fluorescence as a function of temperature and using the same relationships calculated the change in the distance between the two metal ions as a function of temperature. Figure 1 shows how the relative terbium fluorescence of Zn^{2+}-Tb^{3+}-thermolysin and Co^{2+}-Tb^{3+}-thermolysin changes as a function of temperature. Taking the ratio of these fluorescent intensities at each temperature and utilizing eq 1 and 2, the distance between the metal ions was calculated, assuming no temperature dependence for R_0 (vide infra). These results, showing an increase in distance with an increase in temperature, are plotted

in Figure 1. Above ~70 °C the difference between the Tb^{3+} fluorescence of the Zn^{2+}-Tb^{3+}- and Co^{2+}-Tb^{3+}-thermolysins is very small, and the error in the distance calculation can be correspondingly large.

The distance measurements calculated in this manner are apt to be in error if (1) any of the parameters in eq 2 has a large temperature dependence, or (2) if the binding of Tb^{3+} or Co^{2+} is seriously affected by increasing temperature, so that the protein–metal ion complex is no longer stoichiometric at the higher temperatures. In order to discount the latter possibility, thermolysin was titrated with Tb^{3+} at 25 and 80 °C (Figure 2). Both plots show that the Tb^{3+} fluorescence increases until the Tb^{3+}-thermolysin ratio reaches 1:1, after which there is no further increase. This is consistent with the premise that at least under the conditions of these experiments, the thermolysin is fully bound with Tb^{3+} at the higher temperatures. Likewise Figure 2 shows that titration of Tb^{3+}-thermolysin at 25 and 80 °C with Co^{2+} results in a quenching of Tb^{3+} fluorescence and that the maximum quenching occurs at a Co^{2+}-thermolysin ratio of 1:1, after which Tb^{3+} fluorescence is unaffected by further cobalt additions. This also indicates that the protein has a fully bound complement of cobalt at 80 °C. These data are consistent with previous results (Dahlquist et al., 1975; Fontana et al., 1977) which indicate that the protein still maintains these two metal ion binding sites, even at elevated temperatures where the protein begins to unfold.

The four parameters used to calculate R_0 in eq 2 which may have a significant temperature dependence are: n, the refractive index; J, the spectral overlap integral; Q, the quantum yield of Tb^{3+}; and k, the orientation factor. The refractive index of water only changes from 1.33 at 20 °C to 1.32 at 100 °C (Weast, 1974) and the temperature effect is negligible in the calculation of R_0. The quantum yield of the Tb^{3+}-protein complex could not be measured directly (Berner et al., 1975; Horrocks et al., 1975); however, quantum yield measurements of Tb^{3+} complexes of citrate and EDTA showed no significant changes between 25 and 90 °C and therefore the quantum yield of the Tb^{3+} when bound to the protein would not be expected to change either. The orientation factor, k^2, was taken to be 2/3 since the Co^{2+} absorption band in the region of overlap with the terbium emission results from slightly split components of a triply degenerate transition corresponding effectively to an isotropically distributed acceptor moment (Berner et al., 1975; Horrocks et al., 1975) at 25 °C. The justification for using this value at higher temperatures is also valid. The value of J, the spectral overlap integral, can be affected by either the Tb^{3+} fluorescence or Co^{2+} absorption spectrum. Since the shape of the Tb^{3+} thermolysin fluorescence and quantum yield of the model compounds show no change with temperature, there is no affect on the value of J. However, there is a change in the extinction coefficient of the cobalt-thermolysin visible absorption with temperature. At 25 °C we measured the absorptivity at 555 nm of the Co^{2+}-thermolysin complex to be 58 M^{-1} cm^{-1} in close agreement with the values of Berner et al. (1975). This value is somewhat lower than that obtained by Holmquist & Vallee (1974). At 80 °C the molar extinction coefficient drops to 22 M^{-1} cm^{-1}. However, even this relatively large change in the extinction coefficient results in only a 10% error in the calculation of the distances. As a result, the data plotted in Figure 2 were plotted without compensating for the change in the Co^{2+}-thermolysin extinction coefficient with temperature.

An additional complicating factor in the distance calculations would occur if the protein was aggregating at the higher temperatures. In order to investigate the possibilities of aggregation, gel filtration of the native enzyme on Sephadex G-75 (0.01 M Hepes, 0.01 M $CaCl_2$, 1.0 M NaCl, pH 7.5, 82 °C) was carried out, and no detectable species with a molecular weight higher than native monomeric thermolysin was observed. In fact initial experiments at low flow rates through the Sephadex G-75 column indicated the presence of lower molecular weight fragments, due presumably to autolysis of the enzyme at the high temperature. Supporting evidence for this came from the fact that the amount of thermolysin fragments of low molecular weight decreased dramatically when the column flow rate was increased in a later experiment.

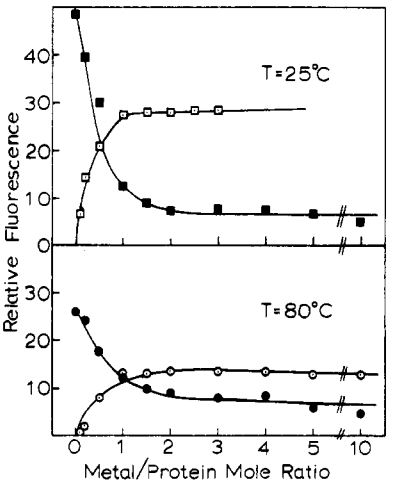


FIGURE 2: Titration of Zn^{2+}-thermolysin with Tb^{3+} at 25 °C (□—□) and at 80 °C (○—○) and titration of Tb^{3+}-thermolysin (no zinc) with Co^{2+} at 25 °C (■—■) and at 80 °C (●—●). Protein concentration was 5.0×10^{-6} M. The spectrofluorimeter sensitivity settings and slit widths were different at the two temperatures, so the relative fluorescence intensities between the two temperatures cannot be compared directly. Excitation wavelength was at 280 nm and emission was followed at 545 nm. The decrease in fluorescence at 545 nm as the cobalt concentration is increased reflects the quenching of Tb^{3+} emission by Co^{2+}.

The above considerations tend to indicate that the data in Figure 1 reflect a real distance change between the metal sites in the protein as a function of temperature. We have therefore tried to correlate this distance change with more conventional techniques of following conformation changes in proteins, e.g., protein fluorescence, ORD, and viscosity.

Intrinsic Fluorescence. Since the fluorescence we observe for Tb^{3+} bound to thermolysin is enhanced by energy transfer from one or more of the protein tryptophans to the Tb^{3+} (allowing us to excite at 280 nm), it was of interest to see if the difference in the effect of temperature on the Tb^{3+} fluorescence of the Zn^{2+}- and Co^{2+}-substituted enzymes was due to changes in the tryptophan fluorescence of these enzymes, resulting in different relative amounts of energy transfer (i.e., enhancement) at different temperatures, rather than due to changes in the quenching efficiency of the Co^{2+} ion. The intrinsic fluorescence of the protein occurs at 338 nm when excited at 280 nm. Figure 3 shows the temperature dependence of the intrinsic fluorescence of the two different metal ion bound proteins from 20 to 90 °C. The monotonic decrease of the protein fluorescence from 20 to 70 °C for both the Zn^{2+}-Tb^{3+} and Co^{2+}-Tb^{3+} enzymes is consistent with the normal temperature-dependent collisional quenching of tryptophan moieties exposed to solvent (Guilbault, 1973). The much more rapid decrease in fluorescence intensity after 70 °C is suggestive of an unfolding of the enzyme structure causing additional tryptophans to be exposed to the solvent and is similar to that observed by Fontana et al. (1977) for thermolysin. The fact that the two plots are essentially identical suggests that the amount of energy transfer from tryptophan to Tb^{3+} in the Zn^{2+}-Tb^{3+}-

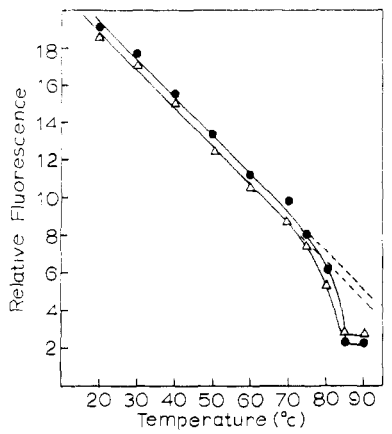


FIGURE 3: Intrinsic fluorescence intensity of Zn^{2+}-Tb^{3+} (●—●) and Co^{2+}-Tb^{3+} (△—△) thermolysin as a function of temperature. Excitation wavelength was at 280 nm and the emission was followed at 338 nm.

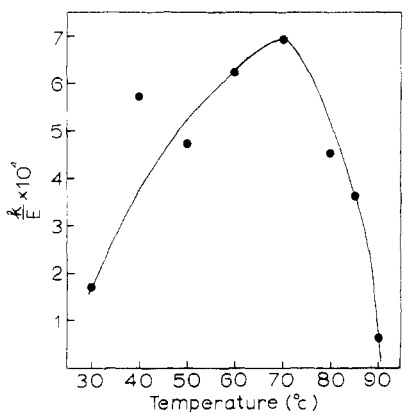


FIGURE 4: Activity of thermolysin as a function of temperature. The activity is expressed as k/E where k is the first-order rate constant and E is the enzyme concentration.

and Co^{2+}-Tb^{3+}-thermolysins is the same at any given temperature. However, since there is no observable quenching of the tryptophan fluorescence by Tb^{3+} in thermolysin compared with Ca^{2+}, the possibility that there may be different amounts of energy transfer in the two enzyme systems cannot be completely ruled out.

ORD Measurements. The temperature dependence of the circular dichroic spectra of thermolysin and apothermolysin has been measured previously (Fontana et al., 1975). It was shown in that study that Ca^{2+} at 10 mM levels protects thermolysin from heat denaturation and/or autolysis up to ~75 °C. However, these studies were carried out by continuously increasing the temperature on a single sample from 30 to 85 °C, rather than a 10-min incubation period of a fresh sample at a given temperature. In order to more directly compare our fluorescence work, we have measured the ORD spectra of these various metallo thermolysin derivatives as a function of temperature using the same conditions as the fluorescence work. The ORD spectrum of Zn^{2+}-Ca^{2+}-thermolysin has a negative peak at 233 nm and the UV spectrum in this region is essentially unchanged for the Zn^{2+}-Tb^{3+} and Co^{2+}-Tb^{3+} enzymes. Since this peak is expected to be sensitive to conformational changes, we followed the optical rotation at 233 nm as a function of temperature for the following three metallo thermolysin derivatives: Zn^{2+}-Ca^{2+}-thermolysin, Zn^{2+}-Tb^{3+}-thermolysin, and Co^{2+}-Tb^{3+}-thermolysin. All three

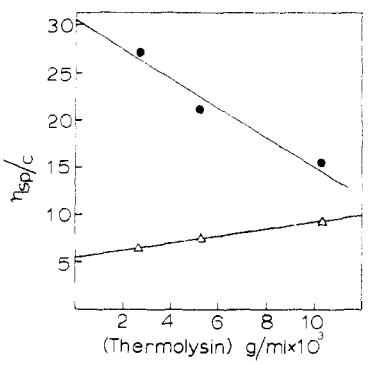


FIGURE 5: Determination of intrinsic viscosity at 25 °C (△—△) and at 93 °C (●—●). Ordinate is reduced viscosity and abscissa is thermolysin concentration in g/mL. Extrapolation to zero concentration gives intrinsic viscosity.

temperature curves are essentially the same, and it is clear that at least for a 10-min incubation period all three enzymes are stable up to 70 °C after which a substantial conformational change begins to occur.

Activity Measurements. The activity of thermolysin was measured using the modified procedure of Khan & Darnall (1978). First-order rate constants were determined by the Kezdy method (Kezdy et al., 1958). Activity is expressed as $k_{obsd}/[E]$ in units of M^{-1} min^{-1} where k is the first-order rate constant and [E] is the enzyme concentration. The plot of activity vs. temperature is shown in Figure 4 for Zn^{2+}-Tb^{3+}-thermolysin. The gradual increase in activity of the enzyme up to 70 °C is consistent with the endothermic ΔH for the hydrolysis of FAGLA. At temperatures higher than 70 °C where the enzyme begins to unfold, the catalytic activity is lost at a rate that exceeds the enthalpy effect.

Viscosity Measurements. Viscosity is a hydrodynamic property which is sensitive to the shape of the macromolecule. The intrinsic viscosity was determined as well as the temperature dependence of the reduced viscosity. Intrinsic viscosity, $[\eta]$, was determined at two temperatures, 25 and 93 °C, using Huggin's equation shown below (Huggins, 1942):

$$\frac{\eta_{sp}}{c} = [\eta] + k[\eta]^2 c \qquad (3)$$

where η_{sp} is the specific viscosity, c is the protein concentration, and k is the Huggin's constant. A plot of η_{sp}/c vs. c should be a straight line whose slope is a measure of the interaction between macromolecule and solvent and whose y intercept is the intrinsic viscosity. Figure 5 shows plots of η_{sp}/c vs. c at 25 and 93 °C. The value $[\eta]$ = 5.3 mL/g at 25 °C, although a bit high, is typical for a roughly globular protein while the value $[\eta]$ = 30.5 mL/g at 93 °C is more typical of an unfolded, random coil protein. The fact that the slope of the line is positive at 25 °C and negative at 93 °C is of interest. Negative slopes of η_{sp}/c vs. c have been observed before and explanations for such behavior have included simple aggregation, or protein-protein interactions which cause the protein to become more globular in nature (Oosawa, 1971; Fuoss & Eldelson, 1951). Neither of these explanations can account for the negative slopes we observed, since gel-filtration experiments have ruled out the possibility of aggregation at high temperatures.

Reversibility. Reversibility of the thermal unfolding of thermolysin was examined using activity, intrinsic protein fluorescence, ORD, and viscosity measurements. Solutions were heated to the desired temperature for 10 min and cooled to 25 °C and physical parameters were measured. Heating for

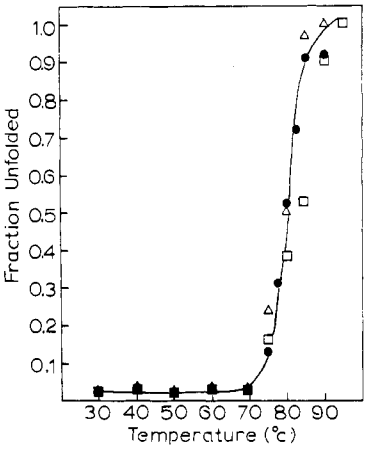


FIGURE 6: Temperature dependence of fraction of thermolysin molecules unfolded as a function of temperature. (●—●) Represent fraction obtained from ORD. (△—△) Represent fraction obtained from intrinsic fluorescence. (□—□) Represent fraction obtained from activity.

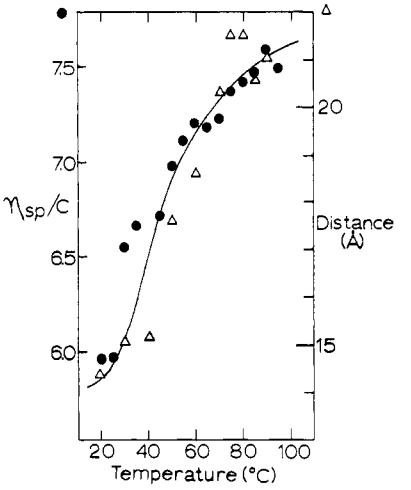


FIGURE 7: Temperature dependence of reduced viscosity and Co^{2+}-Tb^{3+} distance in thermolysin. Left-hand scale is the reduced viscosity which was calculated from the relative viscosity using the method of Holocomb & Van Holde (1962). Right-hand scale is the distance in angstroms which is replot of Figure 2. (●—●) Represent reduced viscosity; (△—△) represent cobalt-terbium distances.

10 min at 70 °C or lower, followed by cooling, gave complete reversal of the physical parameter changes as judged by all four techniques. Heating at 80 °C for 10 min followed by cooling showed 87–88% reversal as judged by ORD and fluorescence measurements. However heating at 90 °C followed by cooling showed only a 35–45% reversal of the ORD, activity, and fluorescence parameters. It is of interest to note that viscosity measurements indicated a nearly 100% reversal over the entire temperature range.

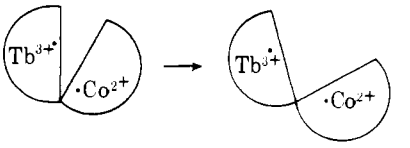
Discussion

Thermal denaturation curves were constructed following the equation below, where F_u is the fraction unfolded (Pace, 1975).

$$F_u = \frac{Y - Y_n}{Y_u - Y_n}$$

Y is the value of the property at temperature T, Y_n is the value for the property of the native protein at the lowest temperature measured (25 °C), and Y_u is the value of the property after heating for 20 min at the highest temperature measured which in most cases was 95 °C. In order to construct these curves using the intrinsic protein fluorescence and enzyme activities as parameters, corrections were made for the temperature dependence of the fluorescence and activities by extrapolating the data taken from the 25–60 °C range. Deviations from this normal temperature dependence above 60 °C were then used to calculate the unfolding curves. The resulting data are shown in Figure 6. The enzyme activity, tryptophan fluorescence, and protein ORD all show very little change with temperature up to 70 °C, followed by a rapid unfolding of the protein with a transition temperature near 80 °C. This corresponds well to UV absorption measurements (Dahlquist et al., 1976) as well as CD measurements observed by others (Fontana et al., 1975). On the other hand, both the Co^{2+}-Tb^{3+} distances and viscosity show very similar variations with temperature in the range from 25 to 70 °C (Figure 7).

The different temperature behavior observed for the different experimental techniques can be explained by a consideration of the three-dimensional structure of thermolysin. The enzyme is comprised of two major lobes with the zinc located toward the bottom of one lobe near the hinge point between the two halves of the molecule. The double calcium site (in which Tb^{3+} is bound) is located in the half of the molecule not containing zinc and toward the upper side of that lobe (Matthews & Weaver, 1974). From 25 to 70 °C our measurements show that the Co^{2+}-Tb^{3+} distance and the viscosity gradually increases, whereas intrinsic protein fluorescence, ORD, and activity measurement show no changes. The distance measurement suggests that the two lobes of the protein are separating with increasing temperature as shown below, resulting in an increase in the Co^{2+}-Tb^{3+} distance. This change need not be a large one, since Figure 1 shows that up to 60 °C the Co^{2+}-Tb^{3+} distance changes by only 3 Å.



Corresponding to the distance change detected by Tb^{3+} fluorescence is the change monitored by viscosity. Since viscosity is sensitive to shape changes, the viscosity changes observed below 60 °C may correspond to a change in the asymmetry of the molecule as indicated above. On the other hand it is not possible to separate shape changes and hydration changes from these data, so that the viscosity measurements may also reflect changes in hydration as a function of temperature. In either case, both the viscosity and distance measurements would be sensitive to this "hinge-like" action of thermolysin.

If the two lobes of the protein partially separate as the temperature is raised toward 70 °C, it is possible that changes in the intrinsic protein fluorescence, UV absorption, ORD, and activity may not be sensitive to this protein structural alteration. The first two properties depend primarily on the environment of the tryptophan moieties. There is only a single tryptophan close to the cleft of thermolysin and inspection of the model of thermolysin derived from X-ray coordinates (Colman et al., 1972; Matthews et al., 1974), while not conclusive, suggests that this tryptophan is sufficiently buried so that its environment will not undergo a significant change for relatively small movements of the hinge. Thus both the intrinsic protein fluorescence and the UV absorption may not be sensitive to small changes in the separations of the two lobes of thermolysin. Similarly since only a very small peptide region of the protein is involved in the hinge, the ORD would also not

be expected to be sensitive to the hinge action, so long as the conformation of the backbone in each lobe remains relatively constant.

It would appear from examination of the model that, if the two parts of the molecule are indeed opening as the temperature is raised to 70 °C, then the activity of the enzyme should be severely affected. Our activity measurements show no such effect; however, since these measurements are necessarily carried out in the presence of substrate, it may be that binding of a substrate or inhibitor will oppose any change in Co^{2+}-Tb^{3+} distances. Experiments are presently in progress to determine the effect that inhibitors have on the measured distance between the two metal ions as the temperature is increased.

Above 70 °C all techniques, i.e., intrinsic protein fluorescence, ORD, activity, metal distances, and viscosity, show drastic conformational changes are occurring in the protein. This follows in agreement with Fontana et al. (1977) and Dahlquist et al. (1976) who observed transitions in protein absorbance and fluorescence at these same temperatures. These changes can undoubtedly be ascribed to an overall general unfolding of the protein.

The fact that we observe changes in viscosity and distance measurements at temperatures where no changes are observed in protein fluorescence or ORD indicates at the very least that the unfolding of thermolysin cannot be described by a simple two-state theory, i.e., $N \rightleftarrows D$.

Our distance measurements showed a change of only ~3 Å in the Co^{2+}-Tb^{3+} distance as the temperature was increased from 25 to 70 °C. However, this rather small distance change resulted from rather large quenching changes of Tb^{3+} fluorescence. Since the distance has a sixth order dependence on the quenching, the large change in quenching corresponds to a small change in distance which points out the sensitivity of the method for following small distance changes.

The maximum distance that we obtained between Tb^{3+} and Co^{2+} was 20–21 Å at 90 °C. It should be mentioned that there is considerable error in the measurements at the higher temperatures. Because the intrinsic protein fluorescence, i.e., tryptophan fluorescence, decreases rapidly at the higher temperatures, the Tb^{3+} fluorescence also becomes quite small at the high temperatures. This makes the measurement of any Tb^{3+} quenching by cobalt difficult and hence the distances above 80 °C may be even larger than what we report. It should also be noted that, if more than one species exists in solution with different Co^{2+}-Tb^{3+} distances, the distance calculated between the metal ions by the fluorescence method will be an average which is very heavily weighted toward the species with the shortest distance. Again this is because of the sixth order dependence of the quenching on the donor-acceptor distances.

Acknowledgment

We would like to thank Molybdenum Corporation of America for providing samples of rare earth oxides.

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