A SPECTROSCOPIC ANALYSIS OF THE THERMALLY INDUCED FOLDING-UNFOLDING TRANSITION OF β-TRYPSIN

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ABSTRACT Absorption and fluorescence changes were used to monitor the thermally induced folding-unfolding transition of β-trypsin. These parameters reflect changes in the microenvironment of different subsets of the four tryptophanyl residues of this protein. The thermal transition was found to be sequential.

INTRODUCTION

It is well known that the red edge of the trypsin difference absorption spectrum (thermally unfolded vs. native) at acid pH exhibits a maximum at 293 nm (1, 2). This absorbance decrease must reflect the exposure of tryptophanyl residues, since no other aromatic chromophore contributes to the protein absorbance at wavelengths >290 nm (3). In contrast, the indole fluorescence excitation spectrum of heat-denatured trypsin was recently reported to be identical to that obtained for the native enzyme, indicating that heat denaturation does not perturb the absorption properties of the *fluorescent* tryptophanyl residue(s) (4). These apparently conflicting observations can be rationalized by the following postulate: the observed 293-nm absorbance decrease is due to a weakly fluorescent or nonfluorescent fraction of the four tryptophanyl residues of trypsin, whereas the fluorescent fraction does not contribute significantly to the observed absorption change.

If these two fractions were located in different portions of the protein, the concurrent measurement of indole absorbance and fluorescence changes might permit the characterization of the thermally induced folding-unfolding transition of β-trypsin. This is because, for a sequential conformation change, spectroscopic changes reflecting the exposure or burial of indole side chains in different parts of the molecule should occur in tandem. By contrast, they would be superimposed if the conformation change were solely an equilibrium between a folded and an unfolded form (i.e., a two-state process) (5, 6).

For the comparison to have validity, two sources of error must be avoided. Fluorescence is roughly three orders of magnitude more sensitive than absorption. Because of this, previous comparisons of this sort have often been made on separate samples that differed greatly in protein concentration (7). Since melting curves are known to be sensitive to small differences in pH (8), any observed divergence in the shape or position of the melting curves (observable vs. temperature) could be attributable either to differences in the state of aggregation of the protein or to poor pH matching.

The nature of the thermally induced folding-unfolding transition of β-trypsin is reinvestigated here, in light of the proposed postulate. The sources of error enumerated above were

avoided by monitoring both absorbance and fluorescence changes on the same sample solution.

EXPERIMENTAL PROCEDURES

Materials

β-Trypsin was prepared by equilibrium column chromatography on sulfoethyl-Sephadex from a sample obtained from Worthington Biochemical Corp., Freehold, N.J., and was characterized as described previously (9). The purity of the preparation was estimated by *p*-nitrophenyl-*p*-guanidinobenzoate titration to be 95% (10). The same pH 3 stock solution of trypsin (41 μM), also containing 0.1 M NaCl, was used in all the melting experiments. The model compound *N*-acetyl-L-tryptophanamide (*N*-AcTrpNH), purchased from Vega-Fox Biochemicals, Tucson, Ariz. was also studied for comparative purposes.

Methods

The decrease in 293-nm absorbance (ΔA^{293}) vs. temperature (T) plot was determined discontinuously on a single 3-ml trypsin sample by a procedure similar to that described by Pohl (11). Two constant-temperature circulators were employed: one was set at a reference temperature (21–24°C) well below that of the transition region; the other was set to any desired value in the 10–80°C range. The absorbance (A) at 293 nm at the reference temperature was first recorded. Then, flow through the water-jacketed, 1-cm path-length spectrophotometric cuvette was diverted to the other circulator. The A^{293} was again recorded once thermal equilibrium at the new temperature was reached. The temperature of the cuvette was returned to that of the reference circulator, and the A^{293} was again recorded to ensure that the observed absorbance change was completely reversible (a typical experiment is shown in Fig. 1). A copper-constantan thermocouple immersed in the solution was employed to continuously monitor the sample temperature (DANA Digital Multimeter, model 5800, Irvine, Calif.). Because the thermocouple was mounted in the center of a Teflon stopper, it was possible to tightly seal the cuvette and thereby avoid significant sample evaporation. This procedure was advantageous because it minimized the time (<3 min) needed to maintain the sample at temperatures at which the trypsin autolysis rate is high. The reversibility of the transition was confirmed after each experiment by an activity measurement. Enzyme activity was determined using benzoyl-L-arginine ethyl ester as the substrate (12). A Cary model 16K spectrophotometer (Monrovia, Calif.) was used to measure the change in A with a precision of ± 0.001. Since a decrease in A at 293 nm is observed on heating trypsin, the placement of the reference and the water-jacketed experimental cuvettes was reversed, so that the strip-chart recorder would yield a positive deflection with increasing temperature. The reference cuvette was maintained at ambient temperature (~24°C) and was unaffected by temperature changes in the adjacent sample compartment.

A Farrand Optical Co. (Valhalla, N.Y.) Mark I spectrofluorometer was used to measure the temperature dependence of the trypsin fluorescence intensity. The sample was illuminated in a standard 1-cm path-length fluorometric cuvette with 300 ± 2.5-nm light. This excitation wavelength was employed because the 300-nm trypsin absorbance is almost temperature independent, a partial photoselection of "red" absorbing indole side chains is achieved (3, 13), and tyrosinyl residue excitation and screening effects are avoided. The sample temperature was controlled by water circulating through the cuvette holder and was monitored as described above. Poor thermal contact with the cuvette precluded the possibility of performing this experiment discontinuously. Thus, the fluorescence intensity at the emission maximum for the native enzyme (335 nm) was continuously recorded as the temperature was raised from 22° to 75°C at an approximate rate of 2°C/min. The temperature was then lowered back to 22°C in ~5 min, to ensure that the intensity change was reversible and that no instrument drift had occurred. Activity measurements confirmed that no trypsin autolysis had occurred.

Data Analysis

Melting curves can be analyzed if the native (n) and unfolded (u) conformations of a protein exist as well-defined initial and final states and if the observable Y (for example, the fluorescence intensity at

335 nm) is either constant or a weakly varying monotonic function of temperature. The characteristic values Y_u and Y_n (defined in the *inset* of Fig. 3) can be obtained, by extrapolation, for any stage of the transition. For a two-state unfolding process, the fraction of molecules in the unfolded state (f_u) may be determined for any temperature within the transition range, using the relation $f_u = (Y - Y_n)/(Y_u - Y_n)$ (14).

RESULTS

Temperature Dependence of the A^{293} of β-Trypsin

A typical experimental run is shown in Fig. 1. By repeating this experiment at various temperatures in the 10–80°C range, the melting curve shown in Fig. 2 (solid triangles) was obtained. Characteristically, this melting curve is not symmetrical. Above and below the transition region the ΔA^{293} values are essentially constant. Assuming the two-state model, this observation permits the scaling of the ordinate in terms of the fraction of unfolded molecules (f_u). The temperature at which $f_u = 0.5$ (T_m) is 52.5°C. These data are only qualitatively similar to those reported previously because of numerous differences in experimental

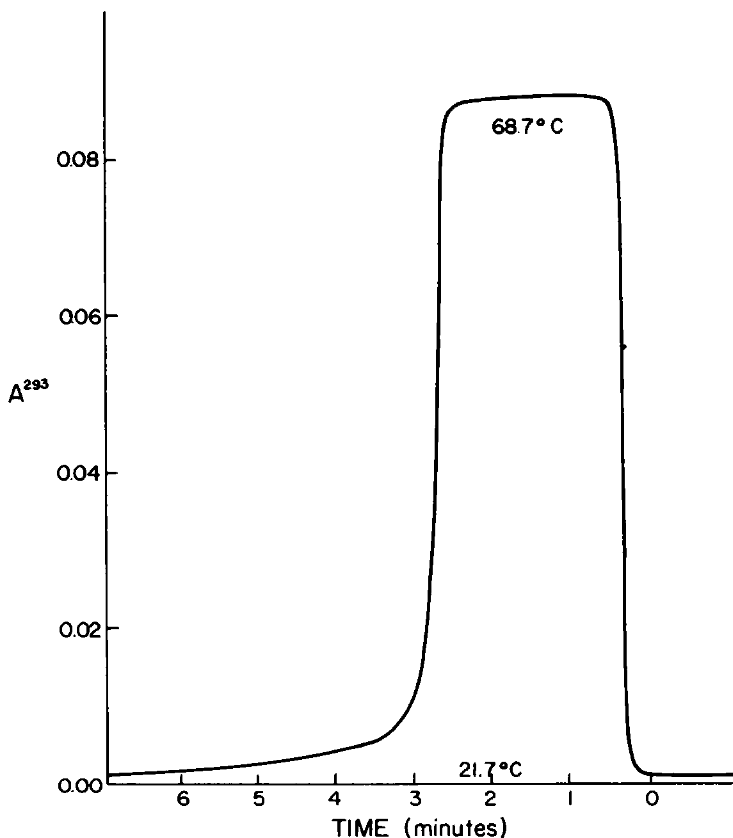

FIGURE 1 Variation of the absorbance of β-trypsin at 293 nm with temperature. The temperature of a 41 μM trypsin solution (pH 3, 0.1 M NaCl) was first raised from 21.7° to 68.7°C and then returned to 21.7°C while an indentical reference solution was maintained at room temperature.

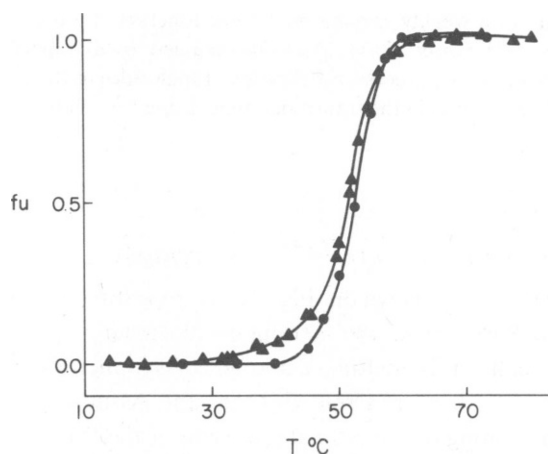

FIGURE 2 Trypsin melting curves as measured by changes in absorbance and fluorescence. The data are plotted as ΔA^{293} (▲) and f_u^{335} (●) vs. T.

conditions and design, but especially because of the way in which the sample temperature was measured (1, 2, 8, 15, 16).

Temperature Dependence of the β-Trypsin Fluorescence Intensity at 335 nm

The relative indole fluorescence intensity of trypsin is observed to decrease with increasing temperature. As can be seen in Fig. 3, a progressive decrease in intensity is observed in the temperature range 22°–44°C; between 44° and 60°C a much larger decrease is obtained, while a smaller one is seen at temperatures >60°C.

The activation energy for the temperature dependence of the indole fluorescence yield was calculated for native and unfolded trypsin and compared to that of *N*-AcTrpNH in water, using the relation

$$\ln\left[\Phi^{-1} - \alpha\right] = \ln\frac{A}{k_i} - \frac{E_a}{RT}, \quad (1)$$

where Φ is the fluorescence quantum yield; k_i is the rate constant that describes the emission of fluorescent photons from the excited singlet; E_a and A are the Arrhenius activation energy and frequency factors, respectively; R is the gas constant; T is the temperature in degrees kelvin; and α is a fitting parameter used to linearize the plot of $\ln[\Phi^{-1} - \alpha]$ vs. T^{-1}. (A detailed discussion of the derivation and use of this relation can be found on pp. 445–446 of reference 3). An E_a value of 3.6 kcal/mol ($\alpha = 2.5$, correlation coefficient = 0.9945), assuming a Φ of 0.1 at 25°C (3), is obtained for native trypsin in the temperature range 24°–43°C. The unfolded enzyme has an E_a of 6.6 kcal/mol ($\alpha = 1.0$, correlation coefficient = 0.9976) in the temperature range 61°–73°C. The latter value is the same as that determined for *N*-AcTrpNH ($\alpha = 3$) over the temperature range 8°–65°C. These data are similar to those reported previously (3, 17, 18).

The fluorescence maximum of β-trypsin is observed to shift from 335 to 348 nm, while the full width half maximum of the emission spectrum increases from 52 to 65 nm when the temperature is raised from 22° to 80°C. The *shape* and wavelength maximum of the emission

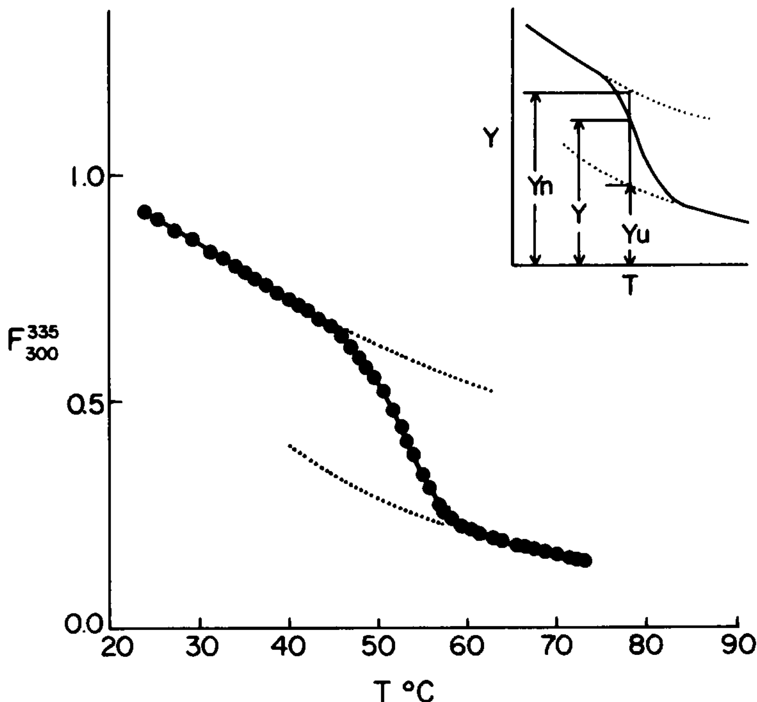

FIGURE 3 Relative trypsin fluorescence intensity (335 nm) as a function of temperature. The 41 μM trypsin sample (pH 3, 0.1 M NaCl) was excited at 300 nm. *Inset:* defines the parameters Y, Y_u, and Y_n to be used in the calculation of the fraction of trypsin molecules unfolded (f_u).

spectrum are temperature independent above and below the transition region, confirming Barenboim's observations (17).

Thus, a melting curve can be constructed, since the native and unfolded conformations of trypsin exist as well-defined initial and final states. An f_u^{335} vs. T plot (closed circles in Fig. 2) was constructed, assuming the two-state model, by extrapolating the Φ vs. T plots for native and unfolded trypsin into the melting region. The Φ values at each T were calculated using Eq. 1 and the appropriate A/k_i, E_a and α parameters. The f_u^{335} values were then determined as explained in the data analysis section and are schematically shown in the *inset* of Fig. 3.

As can be seen by comparing this melting curve to the ΔA^{293} vs. T plot, the former is more symmetrical and has a higher T_m (55.5°C). It has a slightly preceeding termination and, most importantly, its onset lags the other by >10°C. Thus, the melting temperature range of the fluorescence change is narrower than, and is encompassed by, that of the absorbance change. Similar results were obtained with less pure trypsin preparations.

DISCUSSION

The main uncertainty in analyzing melting curves is that the dependence of both the native and unfolded states on temperature must be accurately and precisely known for a valid extrapolation within the transition region to be made. However, this is rarely the case, since factors determining the functional dependence of parameter Y on temperature are usually not

fully understood. For example, Cooper (19) reported that the A^{293} of N-acetyl-L-tryptophan ethyl ester in water increases with increasing temperature (5°–65°C) due to a temperature-induced absorption spectrum red shift. This observation was confirmed in our laboratory using N-AcTrpNH as the model compound. Since the ΔA^{293} above the thermal transition remains constant, additional factors must be operative to overcome this effect (reference 1 and present work). Similarly, since the derivation of Eq. 1 assumes a single homogeneous emitter, its use in the analysis of the heterogeneous emission of a multitryptophan-containing protein becomes ambiguous (3, 17).

In spite of these uncertainties, the following qualitative interpretation is still possible. The two melting curves shown in Fig. 2 were calculated under the assumption that the thermal transition is a two-state process. If this were in fact the case, the two curves should be superimposable within experimental error. Because they are not we conclude that the thermally induced folding-unfolding transition of β-trypsin is a sequential process. This confirms the postulate that fluorescence and absorption changes reflect microenvironmental modifications of different subsets of the four indole side chains of the protein. Further, the >10°C lag in the onset of the f_u^{335} curve suggests that a portion of the weakly fluorescent or nonfluorescent indole groups, which undergo a loss of 293 nm absorbance, is located in a more thermally labile part of the molecule than the fluorescent one(s).

This work illustrates how the absorption and fluorescent properties of intrinsic chromophores may, in combination, be profitably used to characterize conformation changes of selected proteins.

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