pH Dependence of the Thermal Unfolding of Ribonuclease A†

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ABSTRACT: Rates of unfolding and refolding have been measured as a function of temperature and pH for the reversible thermal unfolding of ribonuclease A. At 40°, the rate of unfolding increases 3000-fold as the pH is changed from 7.5 to 1.2, while the rate of refolding decreases 75-fold over the same pH interval. Two models to explain the pH dependence are discussed. The first model, in which titration of a few critical groups triggers unfolding, seems to be incompatible with the known titration curve of the protein. The second model postulates that the pH dependence of the rate constants arises mainly from differences in the titration curves of the native and unfolded protein, caused by changes in the Linderstrom-Lang electrostatic interaction parameter, w, which is known to be a function of the conformation and hydration of globular proteins.

The pH dependence of the rates of protein denaturation has frequently been analyzed in terms of the "trigger group" model, which postulates that titration of a few proton binding groups alters the properties of the native protein in such a way that the rate of denaturation is greatly increased (Steinhardt, 1937; Levy and Warner, 1954). The model has recently been extended by Pohl (1968, 1969) to draw parallels between the thermal denaturation of trypsin and the cooperative binding of ligands to subunit enzymes.

An alternative hypothesis was proposed by Hermans and Scheraga (1961) who deduced from the theory of linked functions that the pH dependence of the equilibrium constant for thermal denaturation of ribonuclease indicated a difference in proton binding between the native and unfolded forms of the molecule. In their model, most of this difference was assumed to arise, not from large differences in the intrinsic ionization constants of a few groups, but from differences in w, the electrostatic interaction parameter of Linderstrom-Lang (1924), which affects the titration of all ionizing groups in the protein. A similar explanation had also been advanced by Tanford (1957) to account for the large uptake of protons which accompanies the acid denaturation of hemoglobin.

A determination of the rates of unfolding and refolding of ribonuclease makes it possible to test the validity of these hypotheses by using the principle of microscopic reversibility.

Experimental Procedure

Materials and Methods. Slow T-jump experiments were performed in the apparatus described previously (Tsong *et al.*, 1972). Kinetic experiments and difference spectra were recorded in a Cary 15 spectrophotometer. Equilibrium melting curves were performed in a Beckman DU spectrophotometer, equipped with thermospacers. "pH-jump" experiments were carried out using an "adder-mixer" of the type described by Crothers (1964). pH values were measured with a Radiometer pH meter 26, using a GK-2321-C combined electrode. Ribonuclease A (RASE) was obtained from the Worthington Biochemical Corp. Concentrations of protein were measured using a molar absorbance of 9.8×10^3 at 278 nm. All other reagents were analytical grade.

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Results

Slow temperature-jump experiments were performed on solutions of ribonuclease A at temperatures between 20 and 70° and pH values between 7.5 and 1. The solutions were adjusted to the required pH and to an ionic strength of 0.1 M by the addition of sodium perchlorate and perchloric acid. Kinetic parameters outside the equilibrium melting range of the protein were obtained in pH-jump experiments. Under all conditions, most of the absorbance change at 287.5 nm resulting from either type of perturbation followed a single slow exponential process. The results were analyzed in terms of the nucleation-dependent sequential folding model described previously (Tsong *et al.*, 1972)

$$A_0 \underset{\sigma b}{\overset{\sigma f}{\rightleftharpoons}} B_1 \underset{kb}{\overset{kf}{\rightleftharpoons}} B_2 \underset{kb}{\overset{kf}{\rightleftharpoons}} B_3 \cdots\cdots \underset{kb}{\overset{kf}{\rightleftharpoons}} B_n \qquad (1)$$

where A_0 and B_n are the fully unfolded and fully folded forms of the protein, and B_1, B_2, etc., are intermediates in the reaction. σ ($= \sigma_f/\sigma_b$) and S ($= k_f/k_b$) are the equilibrium constants for nucleation and for propagation of folding.

This model has been proposed as an attempt to explain a number of paradoxes which have arisen from studies of the reversible unfolding of a number of proteins. Model calculations using reasonable values of s, σ, and n show that this model can mimic two-state behavior by a number of tests, even when sizeable concentrations of partially folded protein are present. Thus, calculated melting curves showing the variation of fully folded protein (B_n), of all nucleated species (B_1, B_2,... B_n) or of other more complex functions, which can be related to experimentally observed quantities, are almost indistinguishable. The enthalpy of unfolding measured by a van't Hoff analysis of these curves is close to the total enthalpy of unfolding which would be measured calorimetrically. The model is an oversimplification of the real situation. The position of the nucleation step and the equality of the equilibrium constants for the folding steps are approximations which aid in calculations. At present it is not possible to use the model to derive actual values for the equilibrium and rate constants or the number of steps in the reaction. Rather, the model is intended as a base from which to interpret our observations and to plan further experiments.

From a kinetic point of view, the nucleation reaction approximates a rate-limiting step and under steady-state condi-

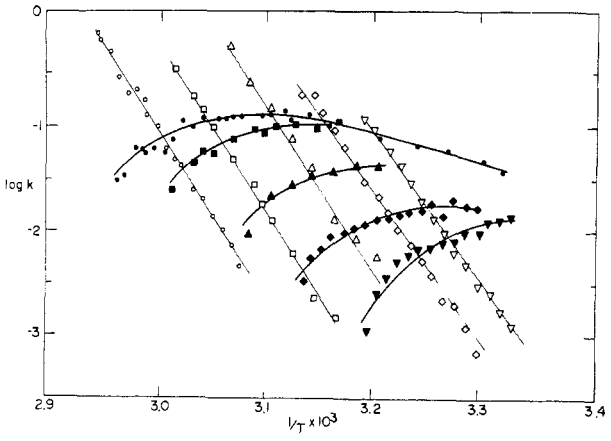


FIGURE 1: Representative Arrhenius plots showing the temperature dependence of the rates of unfolding (k_{21}, open symbols) and of refolding (k_{12}, filled symbols) at a number of pH values. The rate constants are expressed in terms of sec^{-1}: ○, ●, 7.55; □, ■, 3.95; △, ▲, 2.94; ◊, ◆, 2.24; ▽, ▼, 1.24.

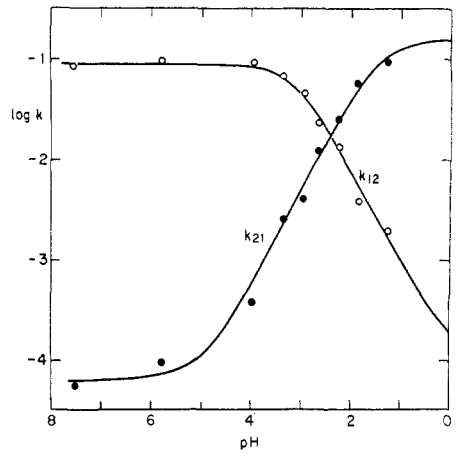


FIGURE 2: The pH dependence of the rates of unfolding (k_{21}) and of refolding (k_{12}) at 40°. The lines passing through the points were fitted to the data using the trigger group model, as explained in the text.

tions, this reaction scheme will show a single relaxation time τ_s, given by

$$\tau_s^{-1} = k_{12} + k_{21} \tag{2}$$

$$k_{12} = \frac{\sigma \cdot k_f \left(\frac{S-1}{S}\right)}{1 + (S-1)\frac{k_b}{\sigma_b}} \tag{3}$$

$$k_{21} = \frac{k_b \left(\frac{S-1}{S}\right)^2 \left(\frac{1}{S}\right)^{n-2}}{1 + (S-1)\frac{k_b}{\sigma_b}} \tag{4}$$

where k_{12} and k_{21} are the rate constants for refolding and unfolding, respectively (Baldwin, 1968). If the measured change in absorbance is assumed to occur in the first step of refolding, then the apparent equilibrium constant for the reaction is

$$K = \sigma S^{n-1}\left(\frac{S}{S-1}\right) = \frac{k_{12}}{k_{21}} \tag{5}$$

Hence we may write

$$k_{21} = \frac{\tau_s^{-1}}{1 + K} \tag{6}$$

$$k_{12} = \frac{K \cdot \tau_s^{-1}}{1 + K} \tag{7}$$

Equations 6 and 7 are directly analogous to those used to analyze the results assuming only a two-state process (Pohl, 1968). τ_s was taken to be the relaxation time of the slow process. The equilibrium absorbance of the protein as a function of temperature and pH was obtained from the kinetic experiments and also from control equilibrium melting curves. The equilibrium constant, K, was calculated as outlined by Brandts and Hunt (1967).

At present, the location of the change of absorbance in the first step is purely arbitrary. However, as stated above, calculated melting curves for the model showing the variation of a number of parameters fall very close together. Apparent two-state equilibrium constants derived from these curves are very similar and, consequently, the errors arising from using the experimentally determined equilibrium constant in eq 6 and 7 will not be large. This conclusion seems to be vindicated by the results obtained at pH 7.55. pH-jump experiments performed below 50° measure only the rate of refolding, *i.e.*, $\tau_s^{-1} = k_{12}$, which may be evaluated directly. Temperature-jump experiments above 50° measure $\tau_s^{-1} = k_{12} + k_{21}$ and the rate of refolding must be evaluated by the use of eq 7. Nevertheless, both sets of data points fall on the same smooth curve.

Figure 1 shows values of the derived rate constants at a number of pH values, as functions of temperature. The curves shown are derived by least-squares analysis of the data. The variation of the rate constants with temperature and pH seems similar to that reported for other pancreatic proteins (Pohl, 1969; Zimmerman and Coleman, 1971).

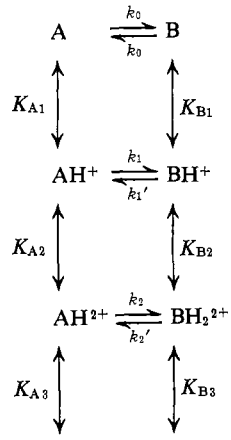
Discussion

The temperature dependence of the rate constants shown in Figure 1 can be interpreted in terms of the sequential folding model proposed earlier. The large temperature dependence of the rate of unfolding ($\Delta H^{\ddagger} \sim 75$ kcal/mole) arises from the fact that unfolding of the protein requires reversal of a large number of steps before nucleation can be reversed. The model also predicts that the rate of refolding should be essentially independent of temperature, since nucleation reactions are generally characterized by large, unfavorable entropy changes. The temperature dependence of the activation energy for refolding has been interpreted by Lumry and Biltonen (1969) in terms of specific heat changes resulting from the exclusion of water from the vicinity of hydrophobic protein side chains in the activated complex for refolding, which we equate with the nucleated species. No attempt has been made to include this kind of variation in the model.

The basic postulate of the trigger group theory is that a few of the prototropic groups of the native protein are involved in specific interactions (hydrogen bonded or electrostatic in nature) which are important in maintaining the structure of the molecule. Titration of these groups alters the properties of the native molecule and changes it into a form which can

unfold more rapidly. Back titration of the same groups in the unfolded protein may accelerate the rate of refolding in like manner (Steinhardt and Zaiser, 1955).

Figure 2 shows the variation of the rates of refolding and unfolding at 40° ($1/T = 3.19 \times 10^{-3}$), a temperature at which values can be estimated at all pH values without large extrapolations. The curves drawn through the points are derived from the trigger group model in which the A's and B's are



the various protonated forms of the unfolded and folded protein, K_{Ai} and K_{Bi} are the equilibrium constants for the protonation of the various trigger groups, and k_i and k_i' are the rate constants for refolding and unfolding of the various forms of the native and unfolded protein. If the protonations are assumed to be fast, then the system will show a single steady-state relaxation time and

$$k_{21} = \frac{k_0' + \frac{k_1'(H^+)}{K_{B1}} + \frac{k_2'(H^+)^2}{K_{B1}K_{B2}} + \cdots}{1 + \frac{(H^+)}{K_{B1}} + \frac{(H^+)^2}{K_{B1}K_{B2}} + \cdots} \quad (8)$$

$$k_{12} = \frac{k_0 + \frac{k_1(H^+)}{K_{A1}} + \frac{k_2(H^+)^2}{K_{A1}K_{A2}} + \cdots}{1 + \frac{(H^+)}{K_{A1}} + \frac{(H^+)^2}{K_{A1}K_{A2}} + \cdots} \quad (9)$$

The variation of the rate of unfolding, k_{21}, can be accounted for by one group of $pK_{B1} = 1.5$, $k_0' = 6.25 \times 10^{-5}$ sec^{-1}, and $k_1' = 0.157$ sec^{-1}. The rate of refolding, k_{12}, also follows the ionization of one group, $pK_{A1} = 3.0$, $k_0 = 0.089$ sec^{-1}, and $k_1 = 10^{-4}$ sec^{-1}. The absorbance of the native protein is known to be a function of pH at low temperature following the ionization of one group of $pK_{app} = 1.5$, while the absorbance of the unfolded protein at 75° is a weak function of pH, following the ionization of one group of $pK_{app} = 3.6$, when the pH is measured at 25° (Tsong *et al.*, 1972). Taken together, these observations suggest the presence of one group of abnormally low pK, which is involved in maintaining the native structure of the protein.

The principle of microscopic reversibility demands that the total free-energy change around the reaction scheme, written above, be equal to zero. This is mathematically equivalent to saying that the product of all the equilibrium constants must be equal to 1 or

$$\frac{(k_1'/k_1)}{(k_0'/k_0)} = \frac{K_{B1}}{K_{A1}} \quad (10)$$

From the values given above it can be seen that the term on the left-hand side is equal to 2.25×10^6, while that on the right is 31.6. This discrepancy could be resolved by assuming that there are, in fact, four trigger groups, all of $pK_{app} = 1.5$ in each molecule. Nozaki and Tanford (1967) have stated that the acid branch of the titration curve of ribonuclease is essentially normal, and it is difficult to see how four carboxyl groups of such low pK's could be missed in their analysis. On the other hand, Salahuddin and Tanford (1970) have analyzed the pH dependence of the unfolding of ribonuclease by concentrated guanidine hydrochloride in terms of two carboxyl groups of $pK_{app} = 3.0$. Any physical interpretation of their thermodynamic analysis must be rather tenuous, in view of the very high and varying ionic strengths in their experiments. These and other considerations discussed below lead one to question the validity of the trigger group model as usually applied.

To explain the variation of the rate constants by the alternative hypothesis, it can be assumed that unfolding of the molecule will cause a decrease in the electrostatic interaction parameter, w, by expansion of the molecule or by increased permeation to solvent and that any abnormal groups in the molecule will be normalized. If the transition to the nucleated species B_1 is regarded as a simple equilibrium

$$B_n \rightleftharpoons B_1 + \Delta\nu H^+ \quad (11)$$

Where $\Delta\nu$ is the increase in the number of protons bound, caused by the changes in w and in the pK's, then the apparent equilibrium constant

$$K_{app} = \left(\frac{1}{S}\right)^{n-1} \quad (12)$$

and K_{app} will vary with pH according to the relationship

$$\frac{d \log K_{app}}{dpH} = \Delta\nu \quad (13)$$

(Wyman, 1964; Tanford, 1970).

To fit the variation of k_{21} with pH, it is assumed that the variation of $(1/S)^{n-1}$ is the dominant term in eq 4. To determine values of $\Delta\nu$ as a function of pH, the titration of the native protein was calculated using intrinsic pK's for the ionizing groups of the protein given by Nozaki and Tanford (1967).

The value of $w = 0.079$ for the native protein was interpolated from the data of Tanford and Hauenstein (1956). One group, arbitrarily taken as an aspartate residue, was assigned a $pK_{app} = 1.5$ (intrinsic $pK = 2.8$). The titration curve for the nucleated species, B_1, was calculated assuming that all groups titrated normally and that w lay between 0.079 and 0. $\Delta\nu$ was defined as the difference between these two curves. Curves of $\Delta\nu$ against pH were integrated using Simpson's routine and plots of the integral against pH fitted to the variation of log k_{21} with pH. Curve "a" in Figure 3 shows the predicted variation of k_{21} with pH, assuming the nucleated species to have $w = 0.065$. Equally good fits could be obtained if it was assumed that the native protein had zero or two abnormal aspartate residues, though the value of w assigned to the nucleated species was different, in each case, 0.056 and 0.069, respectively. Curve "b" in Figure 3 is the predicted variation of k_{21}, assuming that the only difference between the two forms of the protein is the presence of one abnormal group, $pK = 1.5$, in the native protein.

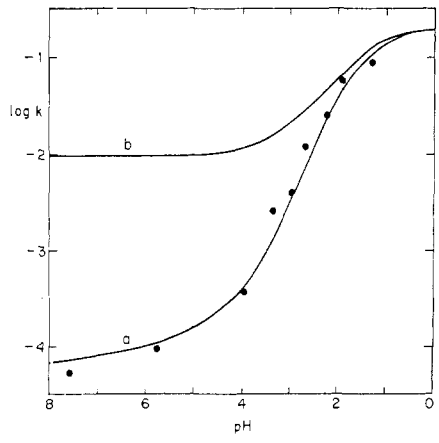


FIGURE 3: The pH dependence of the rate of unfolding (k_{21}) at 40°, predicted by eq 13. Curve a: the fully folded protein has one abnormal aspartate group, $pK_{app} = 1.5$ and $w = 0.079$, while the nucleated species has no abnormal groups and $w = 0.065$. Curve b: the fully folded protein has one abormal group, $pK_{app} = 1.5$, which titrates normally in the nucleated species. Both forms have $w = 0.079$.

Attempts to fit the variation of k_{12} with pH to a difference between two titration curves were unsuccessful. The reason for this has been discussed by Lumry and Rajender (1970). Some of the variation of k_{12} is caused by changes in specific heat which occur on forming the nucleated species, B_1. Since these changes may vary with temperature and pH, no calculation which involves only ionizing groups on the protein can successfully predict the variation of k_{12} with pH. That this variation is a function of temperature is clear from Figure 1 at 40° ($1/T = 3.19 \times 10^{-3}$), k_{21} increases 75-fold between pH 1.24 and pH 7.55, whereas at 27° ($1/T = 3.32 \times 10^{-3}$) the increase is only 3-fold over the same pH difference.

There is an important difference between this interpretation of the pH dependence of k_{12} and the trigger group model as it is normally applied. To explain data by equations similar to eq 8 and 9 the characteristic rate constants of the protonated and unprotonated forms of the protein are assumed to be equal to the observed rate constants at extremes of pH. The number and the pK's of the trigger groups are then adjusted to give curves which best follow the variation of the rate constant. The concept of the trigger group may be valid for some proteins, but both the extent and the form of the variation of the rate or the equilibrium constants in question must follow eq 13. Under these circumstances, $\Delta\nu$ will depend on the differences of the pK's of the trigger groups in the different forms of the protein. These differences in turn will be determined by the free energy necessary to overcome the specific interactions in which the groups are involved. The increase in rate constant caused by the titration of any trigger group will be a direct consequence of this extra free energy and can be calculated directly from it. Consequently, the ratio of the rate constants at extremes of pH is not arbitrary and cannot be used as another adjustable parameter in fitting the data to this model, either.

A superficial analysis of the pH dependence of the rate of unfolding of ribonuclease, which neglected the implications of eq 13, suggested the presence of a trigger group, with a pK of 1.5, involved in the organized structure of the molecule in such a way that protonation of this group caused the rate of unfolding to increase more than 1000-fold. One group of this pK was demonstrated by spectrophotometric titration (Hermans and Scheraga, 1961). Application of eq 13 showed that normalization of this group could only increase the rate of unfolding tenfold and that most of the observed pH dependence must reflect some other aspect of the molecular structure. An example of a conformational change in a protein for which the trigger group concept may be valid is the alkaline transition of acetylated δ-chymotrypsin, recently studied by Garel and Labouesse (1971).

In the alternative interpretation presented above there is only one adjustable parameter, w for the species B_1 (or in a two-state treatment of the data, for the activated complex), as long as the intrinsic pK's of the titratable groups of the protein and w for the native protein are known. The similarity between these results and Pohl's (1968) results for trypsin suggests that this mechanism may also apply in his case.

Two other theories have occasionally been used to explain the pH dependence of a number of protein-unfolding reactions. Since they bear some similarity to the two theories considered above, it is worthwhile to consider them briefly here. The first of these theories involves the creation or unmasking of titratable groups on unfolding of the protein structure. If such groups are buried within the molecule in an un-ionized form and can only be titrated by unfolding the protein, there will be a growing decrease in free energy in favor of the unfolded protein as the pH of the solution is taken through the normal titration range of these groups and beyond. This model was proposed by Beychok and Steinhardt (1959) to explain their results with horse hemoglobin and by Acampora and Hermans (1967) in a study of the thermal unfolding of sperm whale myoglobin. It is a well-documented fact that three of the tyrosine residues in ribonuclease are buried within the molecule and cannot be titrated without irreversible denaturation of the molecule. Indeed, the experimental parameter studied in these experiments is the absorbance change resulting from the unmasking of one or more of these tyrosines. However, in the pH range with which we are concerned, these residues are un-ionized in both folded and unfolded forms of the protein and hence should make no contribution to the pH dependence of the reaction. These residues would become extremely important in any studies of the reaction at higher pH values. Tanford and Haunstein (1956) were able to account for all other ionizing groups in the molecule; consequently this type of mechanism cannot be involved in the reaction.

As ribonuclease is taken to lower pH values and more carboxyl groups are protonated, the molecule becomes increasingly positively charged and there is a large increase in its electrostatic free energy. As stated previously, unfolding of the molecule will cause a decrease in the electrostatic interaction parameter, w, and a concomitant decrease in the electrostatic free energy. Tanford (1970) has recently shown that it is an oversimplification to attempt to explain the pH dependence of protein unfolding in terms of this decrease alone.

Consider a protein molecule carrying a charge, Z, and having an electrostatic interaction parameter, w. The electrostatic free energy of this molecule is

$$W_{el} = kTwZ^2 \tag{14}$$

The molecule undergoes a conformational change, such that the electrostatic interaction parameter decreases by an amount dw. The resulting loss in electrostatic free energy is

$$dW_{el} = kTZ^2dw + 2kTwZdZ \tag{15}$$

The meaning of eq 15 is clear. The first term is the change in free energy to be expected from such a change in w. However, w and Z are related by the Linderstrom-Lang equation. At constant pH, a decrease in w will also reduce the interaction between groups in the molecule and allow further titration of these groups with an increase in the charge of the molecule. The second term in eq 15 represents the work which must be done to produce the increase in charge, dZ, resulting from dw. The second term will disappear outside the titration range of the ionizing groups in the protein, but in the pH range we are concerned with it will be important. If w is independent of pH, then

$$\frac{dW_{el}}{dpH} = 2kTwZ\frac{dZ}{dpH} \quad (16)$$

Let the native and unfolded proteins have electrostatic interaction parameters w and $w - \Delta w$ and electrostatic free energies $W_{el,n}$ and $W_{el,u}$, respectively.

From the Linderstrom-Lang equation, it will be seen that the effect of w on the titration curve of the protein is to move each point on the curve, at charge Z, to a lower pH by an amount $0.868wZ$, compared to a calculated curve following the titration of the same number of groups with the same intrinsic pK's. At constant pH, the difference in charge, ΔZ, resulting from such a shift will be

$$\Delta Z = 0.868wZ\frac{dZ}{dpH} \quad (17)$$

From eq 16 and 17

$$-\frac{dW_{el}}{dpH} = 2.303kT\Delta Z \quad (18)$$

$$-\frac{d}{dpH}(W_{el'n} - W_{el'u}) = 2.303kT(\Delta Z_n - \Delta Z_u) \quad (19)$$

If changes in the charge of the protein are caused only by changes in w and by the binding of protons to the molecule, this equation becomes

$$\frac{d\log K}{dpH} = \Delta\nu \quad (20)$$

where K is the equilibrium constant of unfolding. The pH dependence of the reaction arises from a difference in charge between the two species involved.

The difference in charge results from differences in the Linderstrom-Lang interaction parameters of the two forms of the molecule. This is precisely the model used above to explain the pH dependence of the rate of unfolding of ribonuclease.

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