THE MECHANISM OF UNFOLDING OF GLOBULAR PROTEINS

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Received April 17, 1972

SUMMARY: Kinetic studies on the unfolding of ribonuclease A and horse heart metmyoglobin show that under certain conditions, most of the absorbance change accompanying unfolding can occur in a fast phase. The temperature and pH dependence of the size of this fast phase are interpreted in terms of a recently proposed sequential model, to indicate a fast equilibrium between nucleated forms, which is followed by a slow reversal of the nucleation reaction.

In a recent publication[1], a simple sequential model of protein folding was proposed, in which unfolding and refolding of the molecule occurred through a number of rapid steps and one slow, rate limiting step. Theoretical equilibrium melting curves calculated from the model showed good approximation to two-state behavior by a number of tests, even when appreciable concentrations of partially folded intermediates were present.

One important prediction from the model is that kinetic studies of unfolding reactions should show a rapid initial phase, followed by a slow, steady state process. A fast absorbance change has now been detected in temperature jump studies on the thermal unfolding of ribonuclease A[2]. Although the absolute size of the fast change was always small, it became more evident as the protein became more unfolded, contrary to the original prediction[1]. We wish to report experiments on two proteins, ribonuclease A and horse heart metmyoglobin. By subjecting these proteins to pH-jumps, in a stopped flow device, it is possible to produce such a large perturbation of the system that most of the absorbance change accompanying unfolding occurs in the fast phase. The implications of these observations with regard to the mechanism of unfolding are discussed.

Materials and Methods. Stopped-flow experiments were performed in an

Aminco-Morrow Stopped Flow Apparatus, which was mounted on a Beckman DU monochromator. Absorbance changes were measured with a R 136 photomultiplier and displayed on a Tektronic RM564 oscilloscope. Slit widths and photomultiplier settings were such that Beer's law was obeyed throughout the wavelength range used. Melting curves and spectrophotometric titrations were performed in a Beckman DU, fitted with thermospacers. pH's were measured with a Radiometer pH meter 26 with a GK 2321 C combined electrode.

Results and Discussion. At pH 1.13, the absorbance unfolding transition of ribonuclease A occurs between 20-40°C, whereas at neutral pH unfolding occurs between 50-70°C. Thus there is a temperature zone, 40-50°C, where it is possible to proceed from fully folded to fully unfolded protein (at least as judged by absorbance). This will be particularly helpful in the interpretation of our results. A 2 mg/ml solution of ribonuclease A in 2 mM

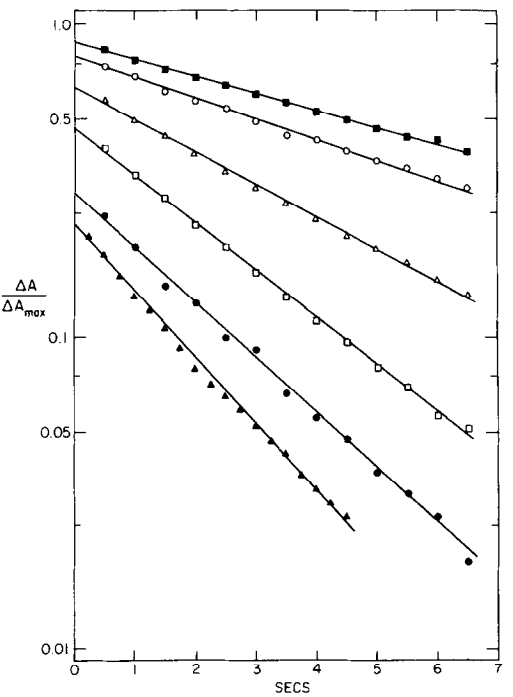

Figure 1. Semilogarithmic plots, showing results of stopped flow experiments on ribonuclease A. At each temperature, ΔA_{max} is the calculated difference in absorbance between the native and fully unfolded forms of the protein. Temperatures; (■) 39°, (○) 41°, (△) 43°, (□)45°, (●) 49°. Protein concentration 1 mg/ml, 1 mM Tris, 0.1 M perchloric acid, pH = 1.13, at 25°C.

Tris perchlorate, pH 7.2 was mixed with an equal volume of 0.2 M perchloric acid, to give a pH of 1.13 and the resulting absorbance change at 287.5 nm followed. Below 20°, a large change in absorbance occurred within the mixing time (25 msecs). This was due to the acid-transition of ribonuclease, a property of the native molecule[3], which seems to be complete within a few microseconds[4]. We will concern ourselves only with the difference in absorbance between the native and unfolded forms of the protein at this pH.

Fig. 1 is a semilogarithmic plot showing the results of kinetic experiments at higher temperatures. At 39°C, 85% of the total absorbance change accompanying unfolding occurred in a slow process, the other 15% being complete within the mixing time. The size of the fast process is a marked function of temperature, in this range. At 49°C, less than 25% of the total absorbance change could be accounted for by the slow contribution. Refolding experiments, performed by jumping from pH 1.13 to pH 7.55, over the same temperature range, showed only a small absorbance change within the mixing time. This could be accounted for by the pH dependence of the absorbance of the unfolded protein[1]. Such behavior is consistent with the sequential model and suggests the absence of complicating side reactions, such as those observed by Ikai and Tanford[5].

The stability of horse heart metmyoglobin is a very marked function of pH; between pH 4.5 and pH 3.5 there is a highly cooperative decrease in the intensity of the Soret band at 408 nm. Other investigators have shown that this change is accompanied by extensive unfolding of the molecule[6, 7]. For this system, pH jumps were made at constant temperature, mixing a neutral solution of horse heart metmyoglobin with glycine buffer of decreasing pH. The disappearance of the Soret band was followed as a function of time. Typical results, shown in Fig. 2 are similar to those obtained with ribonuclease. At pH 3.32, close to the transition zone, all the expected absorbance change occurs at a slow rate. With increasing perturbation of the system, an early fast change becomes increasingly evident.

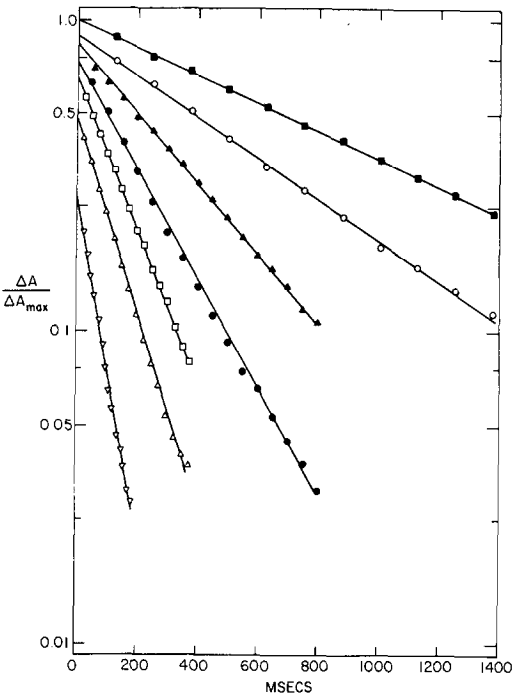

Figure 2. Semilogarithmic plots, showing results of stopped flow experiments on horse heart myoglobin, at 18°C. Final pH of solution; (■) 3.32, (○) 3.10, (▲) 2.88, (●) 2.72, (□) 2.56, (△) 2.30, (▽) 2.01. Protein concentration 0.08 mg/ml, glycine-HCl buffer, 0.025 M.

Fast processes of such magnitude are not predicted by the model used by Tsong et al.[2] to explain their observation. This is not too surprising, considering the simplicity of the model and the complexity of the problem. Nevertheless, it seems worthwhile to look for some way to reconcile our observations and perhaps gain some qualitative information about the mechanism of unfolding. In the interpretation of experiments such as these, it is important to consider the significance of the experimental parameter being measured. Tsong et al.[2] analyzed their kinetic curves in terms of θ, a function to which each species along the reaction chain makes a weighted contribution, depending on the amount of structure it possesses. Such a function is a good representation of the melting of a nucleic acid or an α-helix. However, one may question its validity as a description of the behavior of specific chromophores in proteins.

Bigelow has suggested that the unfolding of ribonuclease at acid pH involves the exposure of only one tyrosine residue to solvent[8]. The Soret band in heme proteins results from specific interactions between the heme and binding groups in the protein. It seems more likely that changes in these parameters will be of the "all or none" form, occurring at some specific step in the reaction, rather than diminishing gradually as the α-helix content of the proteins might. It is from this postulate that we have tried to explain our results.

Consider the sequential unfolding reaction shown in equation (1)

$$B_n \underset{s}{\rightleftarrows} B_{n-1} \underset{s}{\rightleftarrows} B_{n-2} \underset{s}{\rightleftarrows} \cdots\cdots \underset{s}{\rightleftarrows} B_o \underset{\sigma}{\rightleftarrows} A_o \qquad (1)$$

A_o and B_n are the fully unfolded and folded forms of the protein, $B_o \cdots B_{n-1}$, partially folded intermediates, σ and s are the equilibrium constants for nucleation and propagation of folding, respectively. We arbitrarily assume that the absorbance change occurs in the first step of unfolding, which implies that we are following the disappearance of the fully folded protein, B_n. Initially all the protein is present in the fully folded form, at concentration $[B_n]$. At the end of the experiment all the protein is in the fully unfolded form, A_o, and $[B_n] = 0$. If the nucleation reaction is slow and the propagation steps are fast, we may regard the reaction as occurring in two stages. Immediately following a pH or temperature jump the protein will quickly redistribute itself through the species $B_n \cdots\cdots B_o$. Only when the concentrations of all these are at the equilibrium ratio, s, to each other will the slow, steady state unfolding reaction predominate. Let the concentration of the fully folded protein at the end of the fast phase be $[B_n']$, then

$$[B_n'] = s\,[B'_{n-1}] = s^2\,[B'_{n-2}] = s^n\,[B'_o] \qquad (2)$$

$$[B_n] = [B_n'] + [B'_{n-1}] + [B'_{n-2}] + \cdots\cdots + [B'_o]$$

$$= [B_n']\,(1 + 1/s + 1/s^2 + \cdots\cdots + 1/s^n)$$

$$= [B_n']\,\frac{(1 - 1/s^{n+1})}{(1 - 1/s)} \qquad (3)$$

The ratio of the fast change to the total change

$$= \frac{([B_n] - [B_n'])}{[B_n]} = \frac{1/S - 1/S^{n+1}}{1 - 1/S^{n+1}} \simeq 1/S \qquad (4)$$

This implies that the size of the fast change will show a temperature and pH dependence inverse to that of the equilibrium constant, increasing with temperature and hydrogen ion concentration, as observed[2]. In the more general case, where the absorbance change occurs between species B_{n-m+1} and B_{n-m}, the ratio would be $1/S^m$ and the temperature and pH dependence would increase accordingly. If the unfolding of proteins is found to bear a more than superficial resemblance to the idealized sequential model presented here, these two parameters may give some information about the position of the absorbance change in the reaction sequence.

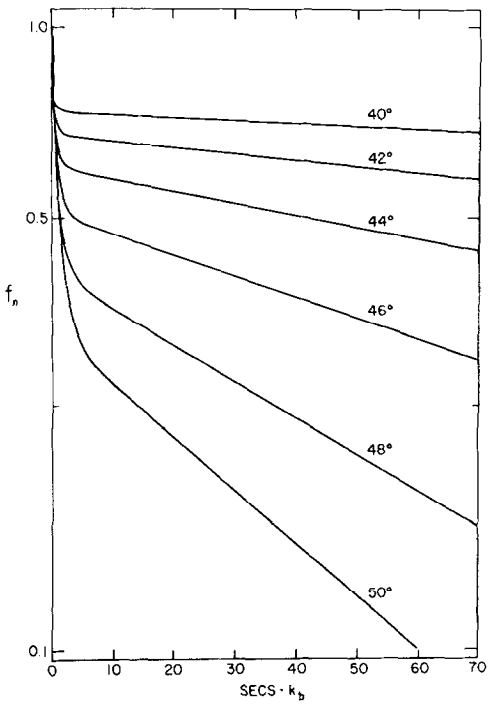

Figure 3. Kinetic curves calculated for the hypothetical model described in the text. The rate constants of the nucleation reaction $\sigma_f = 0.0001 \cdot k_b$ and $\sigma_b = 0.1 \cdot k_b$. All other steps have identical rate constants for folding $k_f = S \cdot k_b$ and for unfolding, k_b. The initial and final states are defined by the temperature dependence of S, ($\Delta H = -25$ kcals/mole) and by setting $S^3 \cdot \sigma = 1$ at the midpoint of the transition. At neutral pH, $T_m = 60°$ and at pH 1.13, $T_m = 32.5°C$. F_n is the fraction of fully folded protein present.

To check the validity of our analysis, kinetic curves were calculated for a number of models, as described previously[1,9]. A reasonable approximation to the ribonuclease results was obtained with a model in which folding occurs through an initial nucleation step, whose equilibrium constant is independent of temperature and three propagation steps, whose equilibrium constants are equal and functions of temperature, each having $\Delta H = -25$ kcals/mole. The absorbance change is assumed to occur in the last step of folding, i.e., the first step of unfolding (Fig. 3). The results of Tsong *et al.*[2] could also be represented fairly well by this model. No calculations were made for the metmyoglobin results, since the pH dependence of the reaction has not yet been elucidated.

We conclude that our observations are compatible with the sequential model of protein folding, if we assume that the absorbance changes occur at some fast step in the unfolding process, before the nucleation step has been reversed. Consequently, they can give no information concerning the position of the nucleation step in the reaction sequence.[2]

We would like to thank Dr. E. Elson of Cornell University for making available to us his computer program used in calculating kinetic curves for model systems and for a number of discussions concerning our results.

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