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DYNAMIC LIGHT SCATTERING STUDIES OF RIBONUCLEASE

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Dynamic light scattering has been used to measure the translational diffusion coefficients of bovine pancreatic ribonuclease A as functions of temperature and concentration in the presence of 1 M Guanidine-HCl. Data was collected throughout a temperature range including the folding-unfolding transitions. Evidence of a pretransition "swelling" of the protein was observed. Entropy and enthalpy changes upon unfolding were obtained using a two-state model.

1. Introduction

Dynamic light scattering is now a standard technique for measuring the translational diffusion coefficients of macromolecules and particles in solution [1]. The technique has the advantage of being rapid. After proper sample preparation most measurements can be completed in less than 30 seconds. For dust-free samples exhibiting strong signals, the precision of the results is usually within ±1%. Experiments can also be performed with relatively small amounts of sample. For example, experiments have been performed on samples with volumes ≈0.01 ml [2]. In addition, the method is non-destructive; the sample can be used for other purposes after completion of the light scattering measurements.

It is well known that translational diffusion coefficients can be related to macromolecule and particle size. For instance, for rigid spherical molecules, the translational diffusion coefficient D, is related to the particle radius R by the well-known Stokes–Einstein relation

$$D = k_B T / 6\pi\eta R , \qquad (1)$$

where η is the solvent viscosity, k_B is Boltzmann's constant and T is the absolute temperature. Even if the molecule is non-spherical, one may use eq. (1) and call R simply the "hydrodynamic radius" and take it as a rough measure of the particle size in solution. This relation is particularly useful in estimating size changes of particles in solution.

Globular proteins in solution have been the objects of much study in recent years. Extensive measurements of, for instance, thermal and chemical unfolding of these proteins have been followed by spectroscopic probes which study local structure of the proteins [3,4]. Ribonuclease A is such a small protein whose folding and unfolding has been intensively studied [5]. The heat-induced unfolding transition has been studied by many techniques and been found to be highly cooperative [6–11] and the two-state approximation has been applied to this transition with some success [8].

The dynamic light scattering technique gives a method for following gross changes in particle size during denaturation. In fact, since large deviations from spherical shape are necessary to invalidate eq. (1), the Stokes–Einstein relation probably provides a good measure of protein size throughout the denaturation transition. Eq. (1) applies to these electrolyte solutions as long as one has fairly high salt concentration to screen the long range Coulomb forces which can also cause deviations from it. Extensive studies of the denaturation of lysozyme have been performed by this technique [12, 13] while less data has been obtained on ribonuclease [13,14]. In this article we present dynamic light scattering studies of the thermal denaturation of ribonuclease in the presence of 1 M Guanidine HCl. The Guanidine HCl was added to ensure that aggregation of the protein would be minimized.

2. Experiment

The dynamic light scattering apparatus used in this work is shown in fig. 1. Light from a Spectra Physics

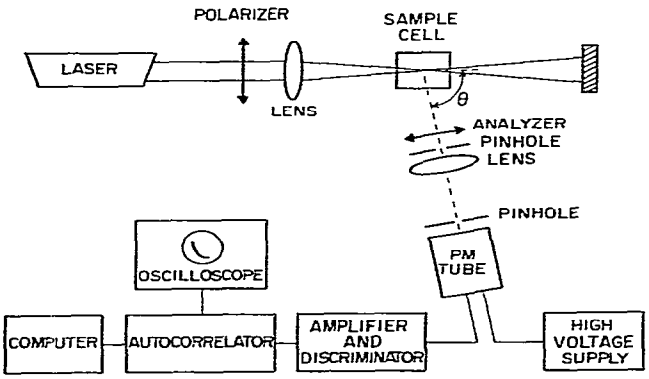

Fig. 1. Dynamic light scattering apparatus used in this work.

Model 165 Argon ion laser was, after passing some optics, incident upon the sample. The light scattered at 90° was collected and focused on the photocathode of an EMI 9502-RF photomultiplier tube. The output of the photomultiplier tube was then processed successively by an SSR Model 1120 amplifier-discriminator system, a Malvern 48 channel autocorrelator, and finally a Data General Nova 3 computer.

The output of the autocorrelator is fit by the computer to the equation

$$C(t) = A + B\exp(-2q^2Dt), \qquad (2)$$

where A and B are constants; $q\,(=(4\pi/\lambda)\sin\theta/2)$ and t is the time. Typical laser power was 1.5 watts and data acquisition time was 20 seconds to a few minutes depending upon the concentration.

Bovine pancreatic ribonuclease A was purchased from Sigma (Type XII A, Lot No. 16C-8056) and was further purified by chromatography on CM-Sephadex 5D. Guanidine-HCl was Schwarz–Mann ultrapure. All samples were run in the presence of 50 mM Cacodylate which was purchased from Fisher.

3. Results

Diffusion coefficients were obtained for solutions of ribonuclease at 1 M Guanidine-HCl, 50 mM Cacodylate and pH 7.2. The RNAse concentration was varied from 1.0 to 11 mg/ml and measurements were performed at each concentration at 10 different temperatures ranging from 4.5°C to 79.8°C. Except for measurements at the highest three temperatures (60.8, 70.2 and 79.8°C)

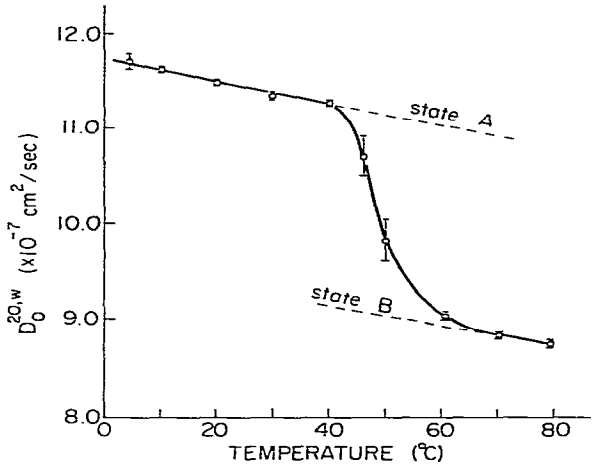

Fig. 2. Diffusion coefficients extrapolated to zero ribonuclease concentration corrected to the viscosity of water and 20°C versus temperature. All measurements were performed in the presence of 1 M Guanidine-HCl and 50 mM cacodylate at a pH of 7.2.

there was no observable concentration dependence of the translational diffusion coefficient. At the three temperatures noted, there was a slight decrease of the diffusion coefficient with increasing concentration. This may be due to some aggregation of the unfolded enzyme.

Values of D extrapolated to infinite dilution and corrected to the viscosity of water and 20°C are shown in fig. 3 as a function of temperature. The error limits are the standard deviation of the results of the least-square fit to a straight line of the data at a given temperature. The viscosity of the solution with 1 M Guanidine is considered to be the same as that of pure water at the same temperature [15].

Previous measurements [7] have indicated that thermal unfolding takes place over the range from about 40 to 60°C. This is clearly indicated in the figure where the diffusion coefficient decreases by about 20.8% over this range. From eq. (1), this corresponds to an equivalent increase in the hydrodynamic radius. This number is somewhat larger than those measured by Nicoli et al. [13] in the absence of Guanidine-HCl under varying conditions of pH and KCl concentration. Nicoli et al. showed that the apparent protein swelling increases with increasing ionic strength. Our large apparent swelling was obtained at an ionic strength of 1 which is larger than those utilized by Nicoli et al.

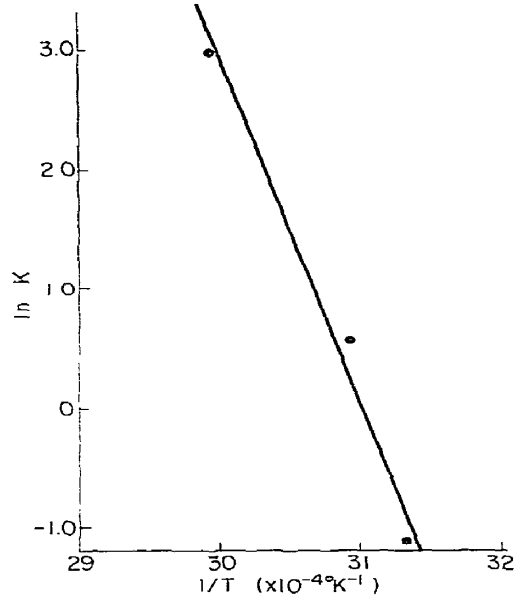

Fig. 3. Logarithm of the "equilibrium constant" for the two state model versus the reciprocal of the absolute temperature. The $T^{-1} = 0$ intercept gives $\Delta S/R$ and the slope gives $-\Delta H/R$.

A notable feature of the curve in fig. 3 is the decrease in diffusion coefficient before the transition begins. The protein thus appears to become slightly larger. This "pretransition" phenomenon has also been observed by heat capacity measurements [9] and proteolytic sensitivity [11].

The observation of a gradual pretransition decrease in the translational diffusion coefficient can be explained by either of two models: 1) a general loosening of the structure of 2) the existence of local structural fluctuations which increase with temperature. The results studying this pretransition using calorimetry and sensitivity to proteolytic enzymes [9,11] have been interpreted as an indication of an increase in structural fluctuations as the temperature is increased. Prior to this work measurements of size changes preceding the thermal transition using sedimentation and viscosity measurements had been negative [7]. However, centrifugal techniques are probably not capable of resolving the small changes measured here.

4. The two state model

The thermal transition curve shown in fig. 3 contains three temperature regions. First, from 4.5°C, there is a slightly linear decrease in diffusion coefficient with respect to temperature. This small temperature effect may, as discussed in section 3, be due to the thermal expansion of the native form of ribonuclease. We call the molecules in native form – state A. The dashed line beyond 40°C is an extrapolation of the linear temperature effect of state A beyond this region.

The second region is from 40°C to about 70°C, in which an abrupt transition takes place. The large standard deviations of data in this region could be caused by fluctuations in diffusion coefficients due to small variations in temperature.

In the third region, between 70°C and 80°C, the diffusion coefficient decreases at the same rate as in the first region. We assume that the molecules in this region are all unfolded and are in a state we shall call state B. The dashed line extrapolated to the lower temperature region also gives the temperature dependence of the diffusion coefficient of state B. Note that the two dashed lines representing the thermal expansion of state A and state B are parallel to each other.

Assuming that the population of intermediate states other than states A and B in the transition region is negligible compared to those of A and B, the equilibrium transition between two states at a given temperature can be expressed by

$$\mathrm{A} \overset{K}{\rightleftharpoons} \mathrm{B} \tag{3}$$

where

$$K = [\mathrm{B}]/[\mathrm{A}] = C_{\mathrm{B}}/C_{\mathrm{A}} , \tag{4}$$

is the equilibrium constant of the transition and [A], C_{A} and [B], C_{B} are the molar and weight concentrations of state A and state B, respectively. It can be shown (2) that the measured diffusion coefficient representing the weighted average for the mixture of two states is related to $C_{\mathrm{A}}, D_{\mathrm{A}}, C_{\mathrm{B}}$ and D_{B} by

$$\frac{C_{\mathrm{A}}^2(D-D_{\mathrm{A}})}{(D+D_{\mathrm{A}})^2}+\frac{4C_{\mathrm{A}}C_{\mathrm{B}}[2D-D_{\mathrm{A}}-D_{\mathrm{B}}]}{(2D+D_{\mathrm{A}}+D_{\mathrm{B}})^2}+\frac{C_{\mathrm{B}}^2(D-D_{\mathrm{B}})}{(D+D_{\mathrm{B}})^2}=0. \tag{5}$$

If D_{A} and D_{B} are not greatly different, this equation may be reduced to a simpler form

$$D = D_A + \frac{C_B}{C_A + C_B}(D_B - D_A) . \quad (6)$$

From eqs. (4) and (6), we can show that

$$K = (D_A - D)/(D - D_B) . \quad (7)$$

The equilibrium constant K is temperature dependent and is related to the standard free energy change by

$$\Delta G = -RT \ln K = \Delta H - T\Delta S , \quad (8)$$

where ΔG, ΔH and ΔS are the changes of standard free energy, enthalpy and entropy in the transition, respectively; T is the absolute temperature and R is the gas constant. Rearranging eq (8), we have

$$\ln K = \frac{\Delta S}{R} - \frac{\Delta H}{R}\frac{1}{T} . \quad (9)$$

Fig. 4 shows the plot of $\ln K$ versus $1/T$ in the transition region from which we have calculated $\Delta S = 179.6$ cal/mole K and $\Delta H = 57.9$ kcal/mole for this two state transition. These numbers are comparable to those found by other authors [7] in the absence of Guanidine-HCl.

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