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DIELECTRIC RELAXATION, ROTATIONAL DIFFUSION AND THE HEAT DENATURATION TRANSITION IN AQUEOUS SOLUTIONS OF RNAse A

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The heat denaturation transition of pancreatic RNAse A was investigated by time-domain spectroscopy. In the temperature interval 5–90°C the protein molecule may be in three states: native, temperature denaturated and unfolded. The heat denaturation process occurs in two stages: In the first stage the system behaves as a macromolecule, maintaining its globular structure and increasing its volume two times and its dipole moment 1.5 times; in the second stage the transition into a statistical coil state occurs.

1. Introduction

Until recently investigation into the dielectric relaxation of globular proteins in aqueous solution was restricted to measurements of the complex dielectric permittivity $\epsilon^*(\omega)$ in the frequency range 10^5–10^7 Hz (β dispersion). As a rule the measurements were made in deionized solvents, at a *pH* close to the isoelectric point, in the narrow temperature interval corresponding to the native state of the protein. Investigations into the β-dispersion region resulted in the establishment of polarisation mechanisms and determination of the parameters of a given relaxation process (τ_μ^β, the dielectric relaxation time, and $\delta = \Delta/C$, the specific dielectric increment, where Δ is the amplitude of the β dispersion and C the concentration in kg m^{-3}) [1,2].

In ref. [3] it was shown that the application of the time-domain spectroscopy (TDS) to the study of a given system enables one to obtain new information on the structural and dynamical parameters of protein globules in the process of temperature-induced conformation transitions. Such investigations have become practical due to the ability of TDS methods to overlap wide characteristic time ranges (10^{-5}–10^{-9} s) essentially in a single measurement and the possibility of obtaining all the information of interest in the time domain in terms of the response dielectric function $\Phi(t)$ ($\epsilon^*(\omega) = \epsilon_\infty + \mathcal{L}[\dot{\Phi}(t)]$).

2. Materials and methods

Pancreatic ribonuclease A (RNAse A) from Sigma was used in this study. It was completely homogeneous according to disc electrophoresis in PAAG with sodium dodecyl sulphate. 2% RNAse solutions in 0.02 M glycine buffer ($pH = 2.6$) were used. The temperature was maintained in a thermostabilized sample holder to an accuracy of 0.1°C in the range 5–90°C.

A TDS computer-controlled system was used to accumulate measurements in the characteristic time interval 10^{-6}–10^{-8} s. The difference method was applied. Usually dioxan was taken as the reference sample but in some cases it was also the solvent. The data analysis was made in the time domain [3]. The response dielectric fucntion $\Phi(t)$, which was obtained from the integral equation [3–7] connecting the registered TDS signals and $\Phi(t)$, has the following form:

$$\Phi(t) = (\epsilon_s - \epsilon_\infty)[1 - \alpha(t)], \quad (1)$$

where $\Delta = \epsilon_s - \epsilon_\infty$ is the amplitude of the observed dielectric relaxation process, ϵ_∞ is the high frequency limit of the dielectric constant, ϵ_s is the static dielectric permittivity and $\alpha(t)$ is the dielectric polarization decay function. In our case this is equal to the macroscopic dipole correlation function (DCF) $G(t)$ [1,3],

$$G(t)=\langle \boldsymbol{M}(0)\cdot\boldsymbol{M}(t)\rangle/\langle \boldsymbol{M}(0)\cdot\boldsymbol{M}(0)\rangle, \quad (2)$$

where $\boldsymbol{M}(t)$ is the fluctuational macroscopic dipole moment of unit sample volume (equal to the vector sum of both solvent and solute molecular dipoles). A detailed description of our apparatus and the principles of the method, algorithms and programs have been given in our previous papers [3–9].

3. Results and discussion

At all temperatures the observed macroscopic DCF for the protein in its native state are simple exponential decay functions in the overlap time window [3]. However, at high temperatures (above 70°C) the DCFs have a more complicated character and in the first approximation may be represented as a sum of two exponentials. As was shown previously [3,4], the slow exponential decays of DCF are due to dielectric relaxation of the protein globule and correspond to β dispersion in the frequency domain. This means that the rotational motion of rigid dipoles in viscous media occurs in the characteristic time range 10^{-7}–10^{-8} s and may be described by rotational diffusion equations. In this case the rotational correlation time of the macromolecule is connected to its volume by the well known Debye equation

$$\tau_0 = 3\eta V/kT \quad (3)$$

and the amplitude of the β process A is proportional to the dipole moment μ of the protein globule. They are also connected by the Onsager–Oncley equation [1,3]:

$$A=\mu^2 NC/2kT\epsilon_0 M. \quad (4)$$

In eqs. (3) and (4) η is the solvent viscosity. V is the effective volume of the rotating protein molecule, N is Avogadro's number, M the molecular weight, ϵ_0 the permittivity of a vacuum (8.85×10 f m^{-1}), k is Boltzmann's constant and T is the absolute temperature.

The temperature dependences of the relaxation times and specific dielectric increments are presented in figs. 1 and 2, the dependence of τ_μ^β is plotted against $3\eta/kT$. Figs. 1 and 2, show the complicated temperature behaviour of τ_μ^β and δ. There are two

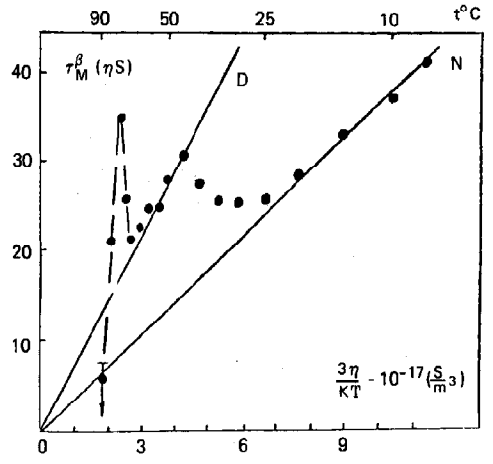

Fig. 1. The dependence of τ_μ^β against $3\eta/kT$ for RNAse. The solid lines N and D represent the calculated magnitudes of τ_0 for corresponding values of the apparent globule volumes of $V_{ap}^N = 38$ nm^3 and $V_{ap}^D = 78$ nm^3.

temperature ranges where the temperature variation of the parameter τ_μ^β seems to follow Arrhenius's law. It is characterised by an activation energy ΔE_τ of ≈ 20 kJ/mole, which is similar to the value for viscous processes. The intermediate temperature interval of the anomalous increase of τ_μ^β corresponds to the heat denaturation transition detected by other methods [10,11].

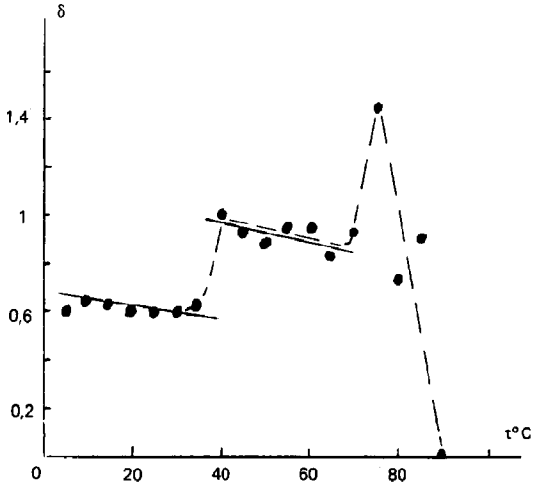

Fig. 2. The temperature dependence of δ for RNAse A. The solid lines represent the calculated magnitudes of δ for corresponding values of the dipole moment of $\mu^N = 300$ D and $\mu^D = 390$ D.

We now consider the results in more detail. In the temperature ranges 5–25°C and 50–75°C the behaviour of τ_μ^β corresponds to the prediction of the Debye theory (eq. (3)). In fig. 1 the calculated magnitude of τ_μ^β for the corresponding values of the apparent globule volumes V_{ap} are shown by the solid lines N and D for the two temperature ranges, respectively.

The behaviour of δ in the same temperature range is also in agreement with theory (eq. (4)). The calculated values of the corresponding macromolecule dipole moments [3] are shown in fig. 2 (solid line).

In the intermediate interval (25–50°C) there is an anomalous increase of τ_μ^β and δ. This testifies to the presence of structural alterations in the globule which results in increases in both the volume and dipole moment.

The next temperature range for anomalous behaviour begins above 75°C. After increasing sharply τ_μ^β decreases by more than an order of magnitude. A similar dependence is observed for δ. In this temperature interval (75–90°C) the registered DCFs have a complicated character. A fast relaxation component with a characteristic time of nanoseconds appears. At the highest temperatures the signals from the protein in the overlapping time window disappear and only part of some fast relaxation processes with small values of τ_μ^β and δ is registered. Probably at these temperatures a transition to the unfolded state occurs for which the group-segment relaxation mechanism is dominant.

Before continuing the discussion we consider the well known effect of experimental values of τ_μ^β exceeding theoretical ones. As was shown in ref. [12], the most probable reason for such discrepancies is the poor description of kinetic protein–water interactions in Brownian rotation theory.

A simple "modified Stokes' friction" model based on the introduction of explicit macromolecule–solvent interaction potentials was proposed in ref. [13]. Such an approach allows one to explain the increase in τ_0 without any increase in the macromolecule volume in solution compared to the molecular dimensions of the globule from X-ray analysis. Assuming the Debye equation to be accurate up to the coefficient, one can determine the apparent volume of the protein globule V_{ap} from the experimental value τ_μ^β

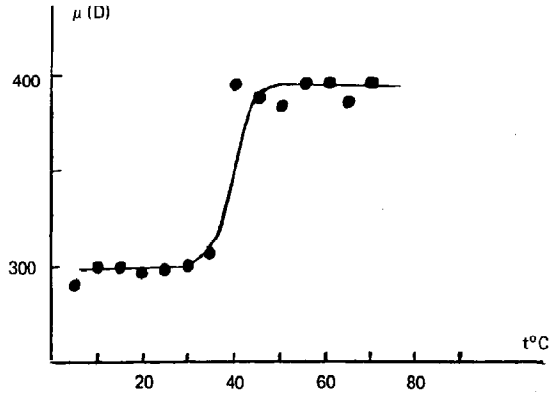

Fig. 3. The temperature dependence of the dipole moment μ for RNAse A.

or as the slope angle tangent from the plot against $3\eta/kT$ (fig. 1). Moreover, it should be borne in mind that V_{ap} exceeds the real value 2–3 times.

We now consider the main features of the temperature dependences of τ_μ^β and δ. It is known that RNAse at acid *pH* does not aggregate or coagulate up to the highest temperature (>90°C) [14].

The whole temperature range 5–90°C can be conventionally divided into three regions corresponding to three protein states: native, temperature denaturated and completely unfolded. In the first two regions the relaxation mechanism connected with the Brownian rotation of the macromolecule as a whole dominates. So one can calculate V_{ap} and μ from τ_μ^β and δ.

The temperature dependence of the dipole moment μ and the relative volume V_{ap}/V_{ap}^0 for RNAse A (V_{ap}^0 is the volume of the protein globule in its native state) are presented in figs. 3 and 4. As mentioned above [3], the dipole moments are in agreement with the corresponding experimental values of other authors.

The behaviour of μ and the V_{ap}/V_{ap}^0 temperature dependence (figs. 3 and 4) shows that during the denaturation transition both the macromolecule volume and its dipole moment increase. This means that in RNAse temperature denaturation we have no complete globule unfolding and a "globule–globule" transition takes place, recalling the "molten globule" state detected by other methods [16,17]. However, in the previous work [16,17] the increase in the pro-

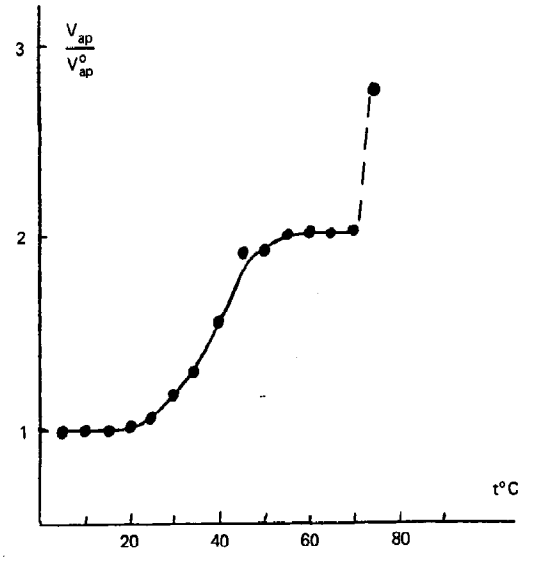

Fig. 4. The temperature dependence of V_{ap}/V^0_{ap} for RNAse A.

tein volume during the transition to the "molten globule" is much less than that detected in our experiments.

In summary our TDS investigation of heat denaturation in pancreatic RNAse A shows that the denaturation process occurs in two stages: The first stage is characterised by the macromolecule retaining its globular structure, doubling its volume and increasing its dipole moment by 50%. In the second stage there is a transition into a statistical coil state.

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