CALORIMETRIC INVESTIGATION OF LYSOZYME THERMAL DENATURATION

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1. Introduction

We have previously shown that prior to the process of thermal denaturation a protein molecule undergoes some changes accompanied by an increase of its partial heat capacity (see for example [1]). However, the relatively low thermostability of the proteins previously investigated – chymotrypsinogen and ribonuclease – made it extremely complicated to distinctly separate pre-denaturational and denaturational effects and obscured the interpretation of the process observed. In this respect lysozyme is a significantly more convenient object of study. Its high thermostability permits one to investigate in more detail the changes it undergoes before the process of denaturation. At the same time, its reversibility to thermal treatment is not less than that of such classical examples used in the investigation of the denaturation process as chymotrypsinogen and ribonuclease. The above feature makes it possible to effectively apply methods of equilibrium thermodynamics to the analysis of changes occurring in lysozyme.

Calorimetric studies of the thermal denaturation of lysozyme have already been reported [2,3], but unfortunately they do not give a picture of the changes of the partial heat capacity of the protein over a wide range of temperature, without which the task posed cannot be solved. The high sensitivity of the precision scanning microcalorimeter installed in our laboratory [4] and the great stability of its baseline enabled us to obtain an adequate picture of the thermal changes taking place in lysozyme during heating.

2. Methods

A commercial preparation of hen egg white lysozyme (Reanal Company, Hungary) with additional chromatographic purification on IRC-50 amberlite was used. The homogeneity of the preparation was checked by electrophoresis in polyacrylamide gel in potassium-formate buffer at pH 3.5. The activity of lysozyme was checked by examining the degree of lysis in a suspension of *Micrococcus lysodeikticus* cells [5].

The lysozyme solutions were investigated in 0.01 M glycine and acetic buffers in which aggregation of denaturated molecules, which reduces the reversibility, was very small. The pH range was 2–5.

The protein concentration in solution was estimated spectrophotometrically taking the optical density of a 1% solution at 280 nm as 26.9 [6].

Calorimetric measurements were made by the automatic differential scanning microcalorimeter at a 1 deg/min heating rate. The operational volume of a gold measuring cell was 1.3 ml. The protein concentration of the solutions under investigation varied from 0.1% to 0.5%. The stability of the baseline allowed the partial heat capacity of the protein to be determined with an error of not more than 3%.

Spectrophotometric measurements were carried out on a Hitachi 124 automatic instrument with differential thermostating of the cells. The temperature of the heated solution was measured directly by a Hewlett-Packard quartz thermometer. Recordings of the melting-point curves and of the differential spectra were made during the heating of one of the cells and the thermostating of the other. The lysozyme

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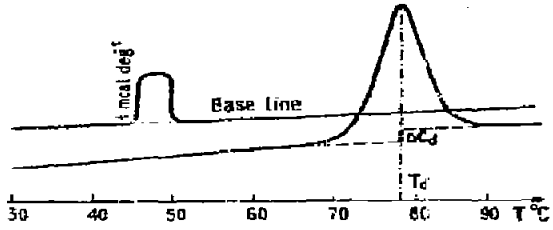

Fig. 1. Microcalorimetric recording of heated lysozyme solution. The concentration of the solution was 1.6 mg/ml.

differential spectra were registered at 5° intervals throughout the whole temperature range.

3. Results and discussion

Fig. 1 presents a microcalorimetric recording of lysozyme thermal denaturation and also the baseline with the calibration mark obtained for the solvent. The relative heat capacity of the solution investigated and its change with the change of temperature can be estimated from the deflection of the recorded curve from the baseline. As is seen from fig. 1, the heat capacity of the lysozyme solution is significantly lower than that of the solvent. The stability of the baseline allows one to calculate the value of the lysozyme partial heat capacity at any temperature. The results of such a calculation for lysozyme solution at different pH values are given in fig. 2.

It is clearly seen that the heat capacity of the solution changes from the very start of heating and at first increases strictly linearly. At higher temperatures

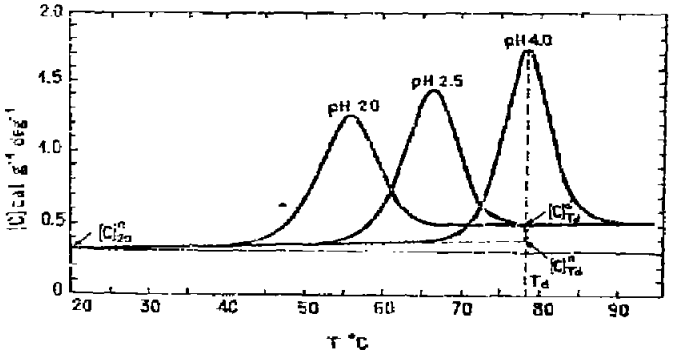

Fig. 2. Dependence of the partial heat capacity of lysozyme upon temperature at different pH values.

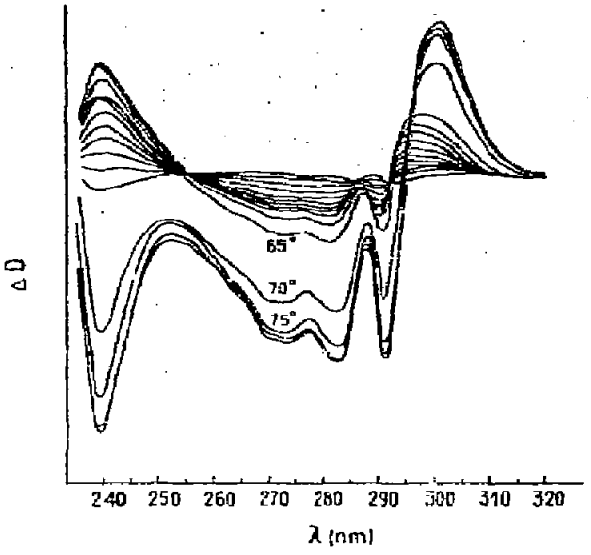

Fig. 3. Temperature effect on the differential spectra of lysozyme at pH 3.0.

the heat capacity increases rapidly to a sharp peak, resulting in intensive heat absorption, which is apparently connected with the main process of thermal denaturation.

The protein heat capacity at 20° is equal to 0.300 cal/g deg and does not depend upon the pH value of the solution.

As a result of denaturation, the partial heat capacity undergoes additional changes, in the denaturated state the heat capacity becomes independent of the temperature.

The jump in heat capacity on denaturation, ΔC_d, may be determined by linearly extrapolating the partial heat capacity change of the native $[C]^n$ and denaturated $[C]^d$ protein to the mid-transition temperature T_d. Then:

$$\Delta C_d = [C]^d_{T_d} - [C]^n_{T_d}.$$

The ΔC_d values are the same, within the measurement error, at all pH values of the solution; either 0.11 ± 0.01 cal g^{-1} deg^{-1} or 1.6 ± 0.1 kcal mol^{-1} deg^{-1}.

The linear extrapolation of heat capacities to the mid-transition temperature also permits one to determine the value of the thermal effect of transition from the peak area above the extrapolation lines.

However, the question arises whether this area of the heat absorption curve is indeed a thermal effect of an independent process or whether it includes the linear increase of heat capacity as well.

The answer to this question can be found by investigating the change of the lysozyme differential spectrum with change of temperature.

Fig. 3, which presents the differential spectra of lysozyme at 5° intervals, shows that with the increase of temperature the protein suffers two qualitatively different changes: the changes in the differential spectra below the denaturation temperature (before intensive heat absorption begins) differ sharply from those observed at higher temperatures. The main difference, which merits particular attention, is the presence of isosbestic points in the pre-denaturational temperature range and their disappearance in the temperature range of the denaturation heat absorption peak. A similar picture to that shown in fig. 3 was observed at all pH values investigated by us.

From the changes in the differential spectrum with temperature, we can choose wavelengths which are not sensitive to pre-denaturational protein changes. By temperature scanning at these wavelengths we obtain a simple sigmoid curve (fig. 4b), the temperature range of which completely corresponds to the temperature range during which intensive heat absorption occurs. Treating this sigmoid curve as the curve of an equilibrium transition from one state into another, the effective enthalpy of this transition can be calculated by using the van 't Hoff plot (fig. 5).

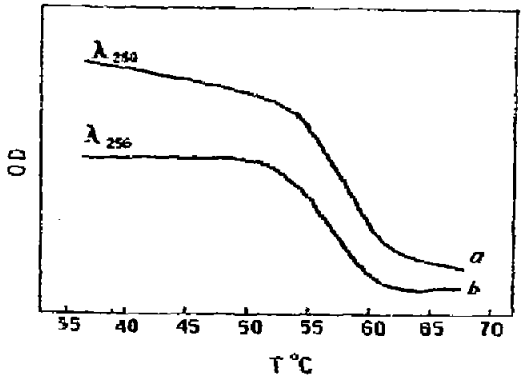

Fig. 4. Temperature dependence of the optical density of lysozyme solution at different wavelengths.

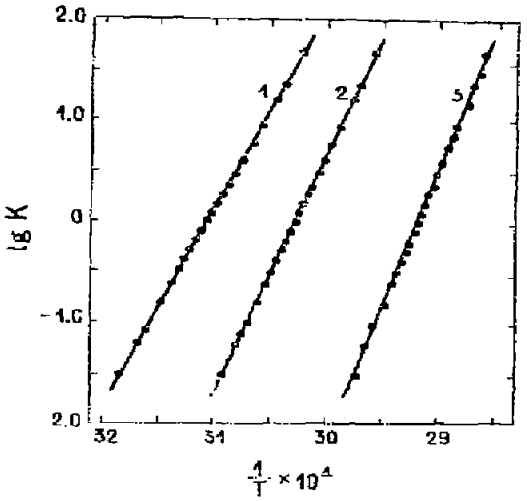

Fig. 5. Van 't Hoff plots of the optical density change in lysozyme solutions at different pH values: 1, pH 2.0; 2, pH 2.25; 3, pH 3.5.

The effective enthalpy of transition may be also estimated from a melting-point curve obtained calorimetrically. In this case, it is suitable to apply the following approximate formula which is easily deduced from the van 't Hoff equation:

$$\Delta H^{eff} = \frac{4RT_d^2}{\Delta T_{\frac{1}{2}}},$$

where $\Delta T_{\frac{1}{2}}$ is the half-width of the heat absorption peak.

It is seen from fig. 6 that the effective protein denaturation enthalpies calculated both from optical and calorimetric data are in good agreement. But, even more interesting is that in good accord with these effective enthalpies are the values of the denaturation enthalpy determined from the area of the heat absorption peak, i.e., the calorimetric or true enthalpy of the process. Together with the conclusion that we correctly singled out the process of denaturation as a process occurring only in the temperature range of a heat absorption peak there is a more essential conclusion implying that this transition is well expressed by the van 't Hoff equation for a monomolecular reaction and, hence, the denaturation of lysozyme may be considered as a transition between two states without any intermediate thermodynamically stable forms.

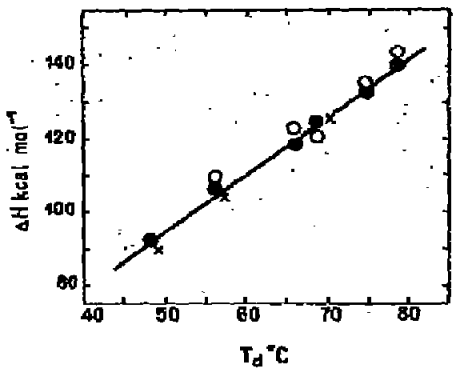

Fig. 6. Temperature dependence of the enthalpy of transition: •, ΔH_d^{cal}; ○, ΔH_{cal}^{eff}; ×, ΔH_{opt}^{eff}.

As regards the pre-denaturational process during which a linear increase of heat capacity takes place, it would be tempting to assume that it is a trivial temperature effect. Unfortunately, it is not at all easy to obtain good agreement between the picture presented of changes of differential spectra (fig. 3) with such a simple explanation as this. Indeed, the presence of isosbestic points below pre-denaturational temperatures indicates that the chromophores which are responsible for absorption in this region of the spectrum have two states, and that the population of the second state increases with the rise in temperature. Naturally, such a situation is difficult to explain by assuming that the protein structure changes gradually, for example, by a slow loosening of its globule. It is more probable that either a two-state transition or a number of transitions between near states occur. It is not excluded that these transitions may be considered as fluctuations of the native structure at weak points [7].

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