Biophysical Chemistry 22 (1985) 323–336
Elsevier

BPC 01019

DSC STUDY OF REVERSIBLE AND IRREVERSIBLE THERMAL DENATURATION OF CONCENTRATED GLOBULAR PROTEIN SOLUTIONS

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Received 20th July 1984
Revised manuscript received 12th July 1985
Accepted 17th July 1985

Key words: Calorimetry; Protein; Concentrated solution; Reversible denaturation; Irreversible denaturation; Aggregation

A calorimetric study of the thermal denaturation of bovine serum albumin, RNAase and catalase in concentrated solutions (crystals) has been carried out. The results obtained for RNAase studied within the pH range 2.5–8.5 show that for concentrated solutions there is an interval of pH where, on cooling of the solution which had undergone denaturation, its renaturation is observed. In the case of concentrated and dilute solutions of RNAase these intervals coincide. The study of RNAase under such conditions at various heating rates shows that there is a range of rates in which the process of denaturation of concentrated solutions can be considered as reversible. The dependences of T_d and H_d on pH and concentration of solutions have been determined. The denaturation enthalpy of concentrated solutions like in dilute ones, has been found to be independent of the pH of solutions, and the experimentally registered change has been proved to be the result of its dependence on temperature. A new method of determination of protein denaturation enthalpy under the conditions of intensive molecule aggregation is suggested. The forms of irreversibility as appearing in the calorimetric experiment were determined by comparing reversible and irreversible denaturation under continuous and step-heating regimes. It is shown that the decrease in T_{max} and the narrowing of the heat absorption peak in the case of decreasing heating rates of protein solutions, observed under certain environmental conditions, results from the irreversibility of the denaturation process.

1. Introduction

In calorimetric studies of the thermal denaturation of protein solutions, the most commonly used method is that of differential scanning calorimetry (DSC). While studying the decay of the native protein structure, one can obtain a set of thermodynamic parameters characteristic of its thermostability. For exact quantitative determination of the parameters of a denaturation transition, it is necessary that the conditions of the protein's environment should be such as to allow the reversibility of the process of thermal denaturation. However, the conditions favourable for reversible denaturation proved to be rather exceptional, and, for most proteins, they can be found within a very narrow range of concentrations, pH, and ionic strength of the solution. More typical is the case of irreversible denaturation. As a rule, biological systems such as concentrated solutions of biomacromolecules, crystals of globular proteins, fibrils of intact collagen, DNA-protein and protein-low-M_r addition complexes, etc., on increase in temperature, undergo irreversible denaturation. At the same time, the above-mentioned systems are much closer to the natural state of proteins and DNA in vivo than commonly studied dilute solutions. It is this closeness to the natural state that accounts for the ever-growing interest in denaturation of biomacromolecules in concentrated solutions, irrespective of the limitations imposed by the irreversibility of the process in its quantitative description and interpretation of the experimental data [1–6].

In this connection, it seems quite necessary to undertake a systematic investigation of concentrated solution of proteins in both reversible and irreversible denaturation. Such investigations are certain to help in choosing optimal experimental conditions under which the effect of irreversibility could be reduced to a minimum. In addition, it is only on the basis of the study of forms of irreversibility of a denaturation transition by calorimetry that one can obtain correct qualitative and some quantitative information on the structure and stability of biomacromolecules under various conditions.

To date, considerable experimental data have been accumulated on the structure and stability of isolated biomacromolecules in dilute solutions [7,8], which seem sufficient to allow further complication of the problem and an attempt at solving it. Identification of the common and characteristic features of the calorimetric properties of denaturation processes in strong and dilute solutions should also help to clarify the influence of interaction between molecules and the excluded volume on the parameters of denaturation of a single protein globule.

Presented here are the results of our recent investigations on calorimetry of concentrated solutions of pancreatic ribonuclease (RNAase) during both reversible and irreversible denaturation, and also on the irreversible denaturation of concentrated solutions of bovine serum albumin and catalase carried out earlier.

2. Materials and methods

The thermal effect during the denaturation of concentrated solutions is large enough for industrial differential scanning calorimeters, such as the DSM-2 (U.S.S.R.) and DSC-III (France), to be used in the work. The former is convenient for high scanning rates because of low thermal inertia; the latter, due to its high stability and its ability to reproduce the baseline, as well as because of its higher sensitivity and larger size of its calorimetric ampoules (1×10^{-4} and 7×10^{-6} cal s^{-1}, 60 and 140 μl, respectively) is preferable for investigations at low rates. In determining the parameters of thermal denaturation of each of the proteins studied at various pH values, a wide range of heating rates (0.2–50 K/min) were used, depending on the purpose in mind. In the case of catalase solutions and crystals, in order to determine the T_{max} (the temperature of maximum heat absorption) dependence on heating time, the range of heating rate was artificially extended to $V_h = 0.003$ K/min on account of isothermic annealing. $V_h =$ 0.2 K/min was the minimum rate at which the thermal effect of protein denaturation (ΔQ) of 15–20% solutions could be obtained with satisfactory accuracy, and was found to be 8–10 cal g^{-1}. The error in the value of the thermal effect obtained appeared to increase with decrease in protein concentration, but never exceeded 5%. The accuracy in determining T_{max} depended on the heating rate: for rates lower than 1 K/min, ± 0.2°C; for 5–10 K/min rates, ± 0.5°C. With high rates, there was an instrumental effect of temperature lag due to the thermal resistivity of a calorimetric cell, and also owing to the fact that the thermal conductivity of aqueous protein is finite. This effect has been thoroughly studied for each of the calorimeters used. Accordingly, the necessary corrections were made in the experimental values of T_{max}.

This paper presents the results of investigations of concentrated solutions of three globular proteins.

2.1. Ribonuclease

The material for investigation was a commercial specimen of pancreatic RNAase A (Serva, F.R.G.), which had been recrystallized five times, and in some cases had been chromatographically purified. Solutions of known pH were prepared by means of the conventional method of dialysis of aqueous solutions of the specimen with respect to a suitable buffer solution. Denaturation of RNAase was studied over a wide range of pH (2.5–9.5). Throughout the range glycine buffer was used; glycine-HCl for the acid region, and glycine-NaOH for the alkaline one. The pH region where the buffer loses its properties (pH 4–8) coincides with the pH region [9] where no change in pH of the solution is observed during the denaturation tran-

sition of RNAase. The buffer is used in this region only for setting the required pH value and for maintaining constant ionic strength of the solution.

For all solutions of all concentrations studied (25–1.5%) 0.5 M buffer solutions were used. The protein concentration being high, and because of the possible change of pH of the buffer solution due to heating, the pH of the solution under test was controlled by additional measurements over the denaturation temperature interval. All pH values referred to here were determined at the T_{max} of the denaturation transition. [10].

Several experiments were carried out using aqueous solutions of RNAase, to which special reference will be made.

2.2. Catalase

Catalase solutions (crystals) extracted from bovine liver in 0.1 M phosphate buffer, at 15–25% protein concentration, pH 7.0 and 7.5 ($T = 20°C$), were studied.

2.3. Bovine serum albumin

Aqueous solutions of defatted bovine serum albumin (Ciech, Poland; and Serva, F.R.G.) were investigated. Protein concentration was 35% and the pH 7.0.

Concentrations of all solutions were calculated from the dry protein weight, which was experimentally determined by the evacuation of ampoules with test specimens, and correcting for the weight of the buffer salts.

3. Results and discussion

3.1. Use of high heating rates for determination of denaturation enthalpy in the irreversible transition

The main difficulty in investigating the denaturation of globular proteins in concentrated solutions lies with the phenomenon of aggregation. The general consequence of post-denaturation aggregation is the irreversibility of denaturation. Irreversibility, in turn, imparts a number of characteristic properties to the general picture of denaturation, irrespective of the method used under conditions of continuous heating of the solution. These properties will be considered below. Furthermore, in calorimetry, there is an additional complication, i.e., heat release due to aggregation. Since the temperature interval of globular protein denaturation is rather long, the processes of denaturation and aggregation may become superimposed. In such cases, the peak of heat absorption is reduced, and the position of the baseline after denaturation becomes uncertain. In order to avoid distortions imposed by the heat released by aggregation on the experimental ΔQ value of irreversible denaturation, we suggest the use of high heating rates [6]. Indeed, taking into consideration that the breaking of bonds during denaturation (heat absorption) and the subsequent aggregation of molecules (heat release) are separated in time, one would expect that, in the case of rapid heating of the solution, the thermal effect of denaturation may be registered before the appearance of the first aggregation bonds. Our initial experiments with bovine serum albumin, carried out with the aid of the DSM-2 apparatus, gave positive results [6]. On the one hand, they proved the dependence of the thermal effect, recorded by the calorimeter, on the heating rate: the longer the time afforded for the molecules to aggregate, the smaller the total thermal effect recorded by the calorimeter in the temperature interval of denaturation (fig. 1). On the other hand, the thermal effect obtained for the maximum experimental heating rate (50 K/min) was close, while that extrapolated to high rates, $1/V_h \ll 1$, was equal to the denaturation enthalpy value of the bovine serum albumin molecule obtained previously in experiments with dilute solutions of this protein (0.1%) under similar environmental conditions [11].

Thus, the experiment demonstrated the prospects of a method to investigate irreversible thermal denaturation using high heating rates. The use of high rates of heating for studying the thermal denaturation of globular proteins in solution is a suitable manner in which to avoid aggregational distortions and to obtain data on the enthalpy of denaturation of a single protein molecule in solutions at any concentration. Subsequent work on

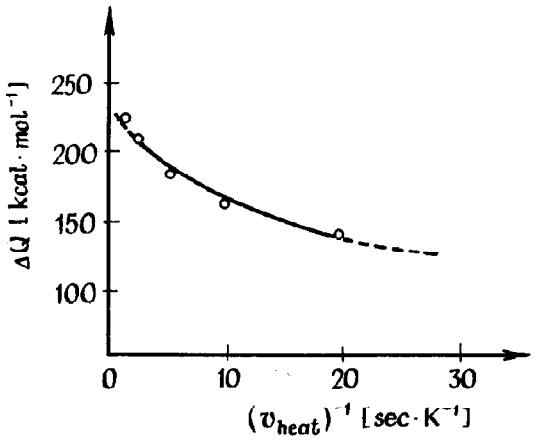

Fig. 1. Thermal effect as a function of heating time for denaturation transition in concentrated bovine serum albumin solution; pH 7, $C = 35\%$.

the irreversible denaturation of catalase and RNAase has supported this conclusion (see below).

As to the equilibrium temperature of denaturation, it cannot be determined at high rates of heating. However, as will be shown further, in the case of an irreversible process, the equilibrium temperature of denaturation cannot be determined even during slow heating of a solution.

3.2. Reversible denaturation of concentrated RNAase solutions

It is known that dilute solutions of pancreatic RNAase undergo reversible denaturation at pH 1.0–4.0. [12]. It was necessary to determine how the increase in protein concentration affected the reversibility of the process. Consider the example of denaturation of a 15% RNAase solution at pH 2.9, which was heated and then cooled within the denaturation temperature interval at 1 K/min (fig. 2) (similar experiments were performed at pH 4.0). This experiment showed that the thermal effect and the maximum temperature in both scanning directions coincided within experimental error. This means that complete restoration of the native structure of RNAase destroyed during heating is accomplished within the same temperature interval of the transition during a time not exceeding 1 K V_h^{-1}. Therefore, in this case, an increase in protein

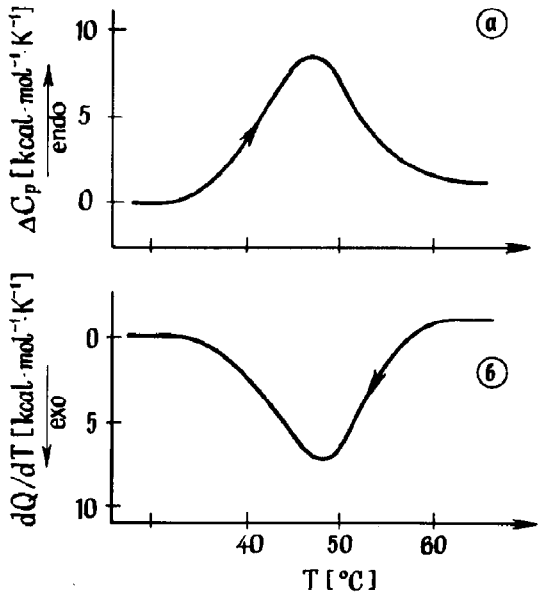

Fig. 2. Experimental thermograms for denaturation (a) and renaturation (b) processes of concentrated RNAase solutions; pH 2.9, $C = 15\%$, $V_h = 1$ K/min, solution mass 120 mg.

concentration up to 15% does not violate the reversibility of the denaturation process [10].

It is worth mentioning that deviation from reversibility may appear for both high and low scanning rates. In particular, at high rates deviation may occur if denaturation proceeds more slowly than $t = 1$ K V_h^{-1} or, if the melt forming under these conditions is not in equilibrium. At low rates this is possible when aggregation commences during the heating time. As will be seen below, at high rates a considerable deviation from equilibrium (reversibility) of RNAase denaturation was observed at $V_h > 5$ K/min. It is common knowledge that RNAase coiling is a very rapid process [13], hence irreversibility in this case is seen as a consequence of the melt not being in equilibrium. During slow heating, the first signs of irreversibility were observed at $V_h = 0.1$ K/min. Here, the thermal effect of denaturation on the second heating was somewhat less than that on the first due to aggregation.

Therefore, within the pH range 0.1–4.0 and heating rates of 3–0.2 K/min, denaturation of RNAase is reversible in spite of the high protein concentration in solution.

The ability of RNAase to undergo reversible denaturation in a concentrated solution allowed comparison of the features of this transition in two opposite situations: reversible and irreversible processes.

3.3. Determination of the baseline in investigation of thermal denaturation

In interpreting the results on complex thermal processes in calorimetry, proper choice of the baseline is very important. Denaturation, complicated by aggregation, proves to be just the case. Depending on the external conditions, the contribution of these two processes may differ. Therefore, before describing our results, we shall dwell at length on the method of determination of the baseline.

With the aid of some of the characteristics of the DSK-III calorimeter, we managed to show that

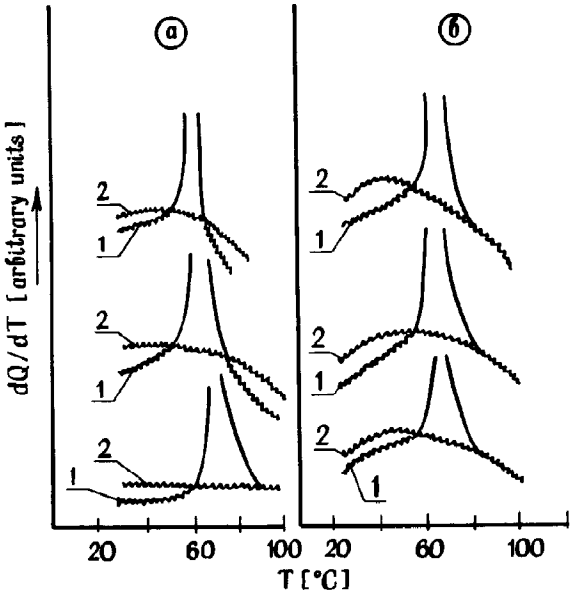

Fig. 3. Experimental thermograms for thermal denaturation of concentrated RNAase solution. (a) Irreversible denaturation of 20% solution, pH 8, recorded at three heating rates: 0.25, 1 and 10 K/min (from top to bottom). (1) First heating of native solution, (2) second heating of the solution after denaturation. (b) Reversible denaturation of aqueous solutions of three concentrations: 26, 13 and 5% (from top to bottom), recorded at a heating rate of 1 K/min. (1) First heating of native solution, (2) second heating of the solution after annealing at 115°C.

this problem can be solved by means of the second heating of the denatured solution [10]. Fig. 3 shows the results of experiments performed at (a) pH 8.0 – irreversible denaturation, and (b) pH 4.0 – reversible denaturation. Fig. 3a shows the thermograms for different heating rates of a 20% RNAase solution and fig. 3b those of aqueous RNAase solutions of different protein concentration at $V_h = 1$ K/min. In both cases, curve 1 corresponds to the first heating and curve 2 to the second. Under conditions of irreversible denaturation, the second heating immediately followed cooling of the solution to room temperature. For the reversible process, the conditions of artificial irreversibility were set; for this purpose the second heating was applied only after 1 h annealing of the solution in the calorimeter at $T = 115$°C. After annealing, the protein could not regain its native structure. The thermograms obtained (2) testify to the absolute irreversibility of denaturation under the conditions in question. From fig. 3 one can see that all the thermograms of the denatured solution (2), before heat absorption, lie above those of the native solution (1). The magnitude of this deviation, which remains constant throughout the temperature range before denaturation begins, as normalized to protein weight, temperature and sensitivity of the instrument, amounted to $\Delta(\Delta C_p) = 1.4 \pm 0.3$ kcal mol^{-1} K^{-1} independent of both the heating rate and the concentration of the solution under test. This increase in heat capacity corresponds to the change in heat capacity during transition of the protein solution from the native to denatured state. It is essential that the value of $\Delta(\Delta C_p)$, obtained for the concentrated RNAase solution, appeared to be close to that of the heat capacity jump found for dilute solutions at $T = T_{max}$ [14].

The post-denaturation situation is more complicated. For the irreversible process at $V_h < 5$ K/min, curves 2 stay above curves 1. However, the difference between the curves within this temperature range depends on the heating rate, decreasing with the latter. At $V_h \geq 5$ K/min, thermograms 1 and 2 after denaturation may be said to coincide within experimental error. For the reversible process, fig. 3b leads to the conclusion that, irrespective of heating rate (experiments carried out in the interval $V_h = 5$–0.25 K/min) and

concentration of the solution, curves 1 and 2 always coincide after denaturation. The question is what do the thermograms of the second heating represent? They can be understood as a record of the baseline for thermograms of the first heating during and after transition. The curve representing the heat capacity of the native specimen can be taken as the baseline before the transition, while after denaturation the baseline is denoted by the heat capacity curve of the denatured specimen during the second heating, where, due to the irreversibility of the process, only the temperature change of the heat capacity is observed. The curves are not extrapolated to the denaturation interval but are determined as follows: in the low-temperature region up to T_{max}, by means of a parallel downward shift in the value of the deviation in the heat capacity line corresponding to the denatured specimen; in the high-temperature region, by the heat capacity of the denatured specimen (fig. 3). The deviation of curve 1 towards the endo effect is determined by protein denaturation, whereas that towards the exo effect is due to the aggregation of denatured molecules. This procedure for determining the baseline turned out to be most fruitful in understanding the complicated picture of the denaturation of concentrated protein solutions under various conditions of reversibility, and was very helpful in increasing the precision in determination of the values of thermal effects under examination.

3.4. Manifestations of irreversibility of protein solution denaturation in calorimetry

In order to determine the effect of irreversibility on the denaturation transition curves, a special comparative calorimetric experiment was carried out [15]. There was another aim in carrying out this experiment, viz., to refute the erroneous conviction that for the determination of true thermodynamic parameters, denaturation of protein solutions should always proceed at the slowest possible rates [16–19]. Two RNAase solutions were studied: $C = 13\%$, pH 4.0 (reversible denaturation) and $C = 20\%$, pH 8.0 (irreversible denaturation), with two different heating techniques. The first technique was a standard regime of continuous heating at 1 K/min, the second being step heating with an alternate regime of continuous heating at 1 K/min for 200 s, followed by isothermal annealing for 400 s. This regime was made possible by one of the automatic programmes for sample heating in the DSK-III calorimeter (Setaram). The thermogram of step heating consists of alternating 'heating-isotherm' parts, which, referred to their respective temperatures and plotted separately for heating and annealing, do not differ from their corresponding records for each of the regimes taken separately.

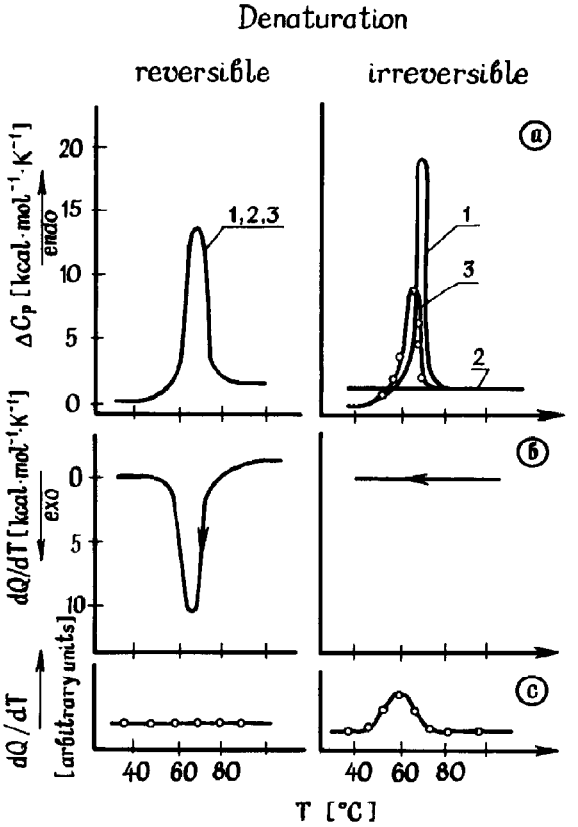

Fig. 4. Heat absorption (heat release) of RNAase solutions during denaturation and renaturation for reversible and irreversible processes. (a) Heating of the solutions, $V_h = 1$ K/min. (1) First heating in continuous regime, (2) second heating in continuous regime, (3) first heating in step regime. (b) Cooling of the solutions, $V_c = 1$ K/min. (c) Temperature dependence of heat absorption, plotted on the data for isothermal annealing at different temperatures.

The temperature dependence of the apparent heat capacity in the range 20–120°C, calculated from the experimental thermograms for both solutions, is shown in fig. 4a. Curves 1 and 2 correspond to the first and second heating under standard conditions (continuous heating), and curves 3 to the first heating applied under the step regime; in the latter case the apparent heat capacity was calculated for heating periods. It can be seen that for reversible transition by either heating technique, the denaturation curves coincided, i.e., the heat capacity value in the denaturation temperature range did not depend on the mode of heating. In the case of irreversible transition, the heat capacity and thermal effect of denaturation in experiments with the use of annealing, step heating appeared to be substantially lower than without annealing ($\Delta Q_h = 61$ vs. 113 kcal mol^{-1} under continuous heating; $V_h = 1$ K/min). A similar result was obtained for the irreversible denaturation of intact collagen under the step heating regime.

We now consider the results obtained for isothermal periods of step heating. Corresponding data are presented in accordance with annealing temperatures as non-normalized thermograms (fig. 4c). Here, the heat flow at different annealing temperatures is different for reversible and irreversible denaturation. The temperature dependence, plotted for irreversible denaturation, appears to be a monotonic curve similar to the baseline of the continuous heating in the absence of denaturation transition. For irreversible transition an endothermic maximum was obtained within the denaturation interval, which may indicate an additional absorption under the isothermal regime ($\Delta Q_{\text{isoth.}} = 39$ kcal mol^{-1}). The total value of the thermal effect under the step heating regime was found to be $\Delta Q = \Delta Q_{\text{isoth.}} + \Delta Q_h = 100$ kcal mol^{-1}, which is in good agreement with ΔQ of the same specimen heated by the standard continuous method.

Our results demonstrate clearly that in a protein solution where denaturation is irreversible, during annealing within the denaturation temperature interval, partial isothermal denaturation occurs. Indeed, according to Bolzmann's factor, the native and denatured parts of the solution must be in dynamic equilibrium. It is not distributed by annealing during reversible denaturation, nor are any changes detected by the calorimeter under the isothermal regime (fig. 4c). At the same time, during the transition from the isothermal regime to that of continuous heating, ΔC_p remains unchanged, as if the heating had not been interrupted (fig. 4a, curves 1 and 3). When molecule aggregation results in irreversible denaturation, dynamic equilibrium is violated, and only one-way transfer (from the native to denatured state) takes place. (Violations of equilibrium may also be caused by considerable kinetic barriers to the reverse process of refolding, for instance, in collagen fibrils.) If the rate of denaturation is high enough, the calorimeter may register thermal absorption under the isothermal regime (fig. 4c). Heat capacity values during continuous heating, following that of the isothermal regime, appear to be lower than in the case of the continuous regime at the same temperatures, since some molecules have already undergone denaturation during the annealing time (fig. 4a, curves 1 and 3).

Thus, in the case of irreversible denaturation, a protein can undergo isothermal denaturation, whereas, in the case of the reversible one it cannot. The ability of a protein to perform isothermal unfolding under the conditions of irreversible denaturation implies that the heat of denaturation may be realized within an infinitely small temperature interval similar to the first type of phase transition, although the process as such will not be cooperative. Continuous heating of such a solution at any rate would involve denaturation imbalance, where the slower the scanning, the narrower and higher would be the heat absorption curve, and the lower the corresponding T_{max}. The above-mentioned peculiarities of denaturation transition curves are typical of protein solution denaturation irreversibility as studied under a dynamic regime irrespective of the method used. The results obtained for reversible RNAase denaturation, which prove experimentally that denaturation parameters are independent of the heating regime, lend support to irreversibility being the only cause of such behaviour of denaturation transition curves in our experiment.

Hence, as expected, the experiment showed that in order to obtain equilibrium curves of denatura-

tion transition, there is no point in investigating both globular and fibrillar protein solution denaturation using very slow heating rates (1 K/min for n h). Irreversible denaturation is a non-equilibrium process, so lowering of heating rates would only increase the degree of non-equilibrium (irreversibility) of the process under consideration.

3.5. Kinetic studies of the thermal effect and temperature maximum of RNAase denaturation transition in concentrated solutions

Here, a 15% RNAase solution was investigated under the conditions of reversible denaturation (pH 2.7), a 20% solution being chosen for irreversible denaturation (pH 8.0). The results are presented in figs. 5–7 according to the thermal effect of denaturation, temperature maximum of heat absorption and denaturation transition curves (temperature dependence of apparent heat capacity during denaturation) plotted vs. time of heating.

3.5.1. Thermal effect of denaturation

Under the conditions of reversible denaturation, the thermal effect throughout the range of heating rates employed (10–0.2 K/min) does not depend on the heating time (fig. 5, curve 1). This confirms the absence of aggregation in highly concentrated protein solutions, allowing one to consider the corresponding value of the thermal effect $\Delta Q = \Delta H_d = 87 \pm 4$ kcal mol^{-1} as being the RNAase molecule denaturation enthalpy in a concentrated solution at pH 2.7. In contrast, for irreversible denaturation the value of the thermal effect decreases with increasing heating time (fig. 5, curve 2), i.e. the slower the solution is heated the stronger is the aggregation of denatured molecules. On extrapolation of the dependence obtained to higher rates, $1/V_h \ll 1$, the heat release due to aggregation is neglected in order to obtain the RNAase molecule denaturation enthalpy at pH 8.0, $\Delta H_d = 122 \pm 4$ kcal mol^{-1}. The difference between the thermal effect at the maximum and minimum rates of the experiment, $\Delta(\Delta Q) = 14$ kcal mol^{-1}, shows that aggregation within the temperature interval is not high. The most active

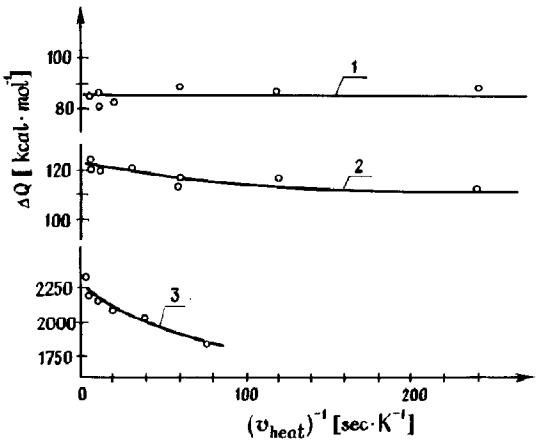

Fig. 5. Thermal effect as a function of heating time for protein denaturation in concentrated solution. (1) RNAase, reversible denaturation, pH 2.7, $C = 15\%$. (2) RNAase, irreversible denaturation, pH 8, $C = 20\%$. (3) Catalase, irreversible denaturation, pH 7.5, $C = 20\%$.

aggregation takes place at higher temperatures. This is supported by the deviation of the thermogram of the first heating of the RNAase solution from the baseline towards the exo effect after denaturation is complete (fig. 3a).

3.5.2. Denaturation temperature

The dependence (fig. 6) of T_{max} of heat absorption on $1/V_h$ for two RNAase solutions (pH 2.7 and 8.0) may, in each case, be divided into two parts. The first is the region of high heating (5 K/min and higher) rates, where a strong dependence of T_{max} on $1/V_h$ is observed. The second is the region of low rates (3 K/min and lower), where, for reversible denaturation, the temperature remains constant within the precision of the experiment while the duration of heating is extended, and for irreversible denaturation under the same conditions, a monotonic decrease in temperature is observed. A similar decrease in T_{max} had been noted earlier during investigation of irreversible denaturation of bovine serum albumin solutions and catalase solutions and crystals (fig. 6, curve 3). The range of low rates used for the latter was substantially extended by use of annealing of specimens down to 0.003 K/min [20]. No saturation of the T_{max} dependence on $1/V_h$ was found

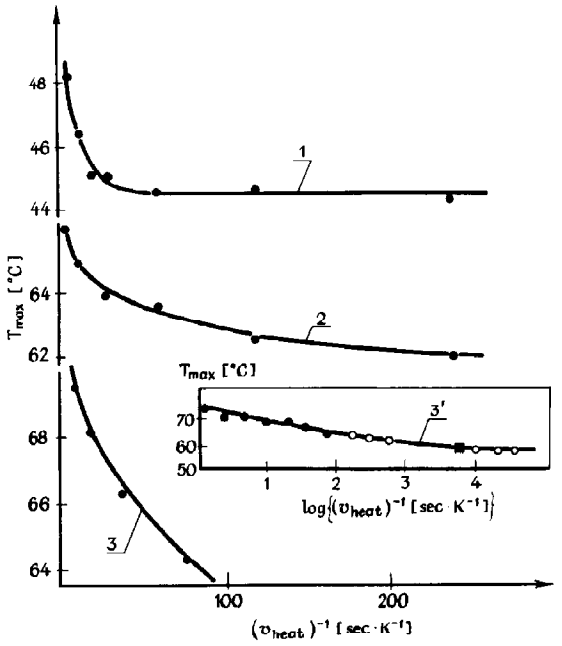

Fig. 6. T_{max} of denaturation transition as a function of heating time for RNAase and catalase in concentrated solutions. (1) RNAase, reversible denaturation, pH 2.7, $C = 15\%$. (2) RNAase, irreversible denaturation, pH 8, $C = 20\%$. (3,-3') Joint results for catalase T_{max} obtained on continuous heating (●) and annealing (○); (■) data from ref. 21.

over a reasonably wide range of heating rates in irreversible denaturation (fig. 6, curve 3). The T_{max} values obtained in these experiments are in agreement with direct measurements of T_{max} for very slow heating rates of catalase crystals in a highly sensitive calorimeter [21].

Thus, in reversible denaturation of RNAase, there is a range of temperatures (3–0.2 K/min), wide enough in terms of heating time (20–300 s K^{-1}), in which T_{max} with an accuracy of 0.5°C does not depend on the heating rate, while during irreversible denaturation, whatever the protein (RNAase, catalase, bovine serum albumin), T_{max} decreases with decreasing heating rate at all scanning rates. It has been shown above that, if the process is irreversible, then the isothermal denaturation as well as the heat release due to aggregation, which is subtracted more intensively from the high-temperature part of the curve, would involve a decrease in T_{max} of the denaturation transition at lower V_h. Kinetic studies of T_{max} for irreversible denaturation of three globular proteins support this conclusion. Therefore, the concept of a denaturation temperature (T_d) ceases to exist in its strict sense in the case of irreversible denaturation. In contrast, under the conditions of reversible denaturation, T_{max} at heating rates below 3 K/min no longer depends on the rate, and can be termed equilibrium denaturation temperature (T_d) of a protein in a concentrated solution.

At high heating rates above 5 K/min, the dependence of T_{max} on $1/V_h$ turned out to be the same for both solutions, and rather strong as well (fig. 6). Considering the relationship $T_d = \Delta H_d/\Delta S_d$ inter-relating all thermodynamic parameters of reversible denaturation, one can state that, since ΔH_d does not depend on V_h, overheating of the globule at high heating rates is of purely entropic nature. The native state having a definite entropy in no way connected with the rate of heating, the change in ΔS_d (decrease) must be accounted for by the entropy decrease of an unfolded molecule at high heating rates. Indeed, the forming melt will have a more ordered structure than the equilibrium melt, if the time of unfolding of the globule is $t_{unf} > 1$ K/V_h. Therefore, heating rates of 5 K/min and higher do not provide sufficient enough time for the equilibrium structure to be attained in a protein molecule ($t_{unf} > 12$ s). At these V_h values, denaturation in all cases under investigation ceased to be in equilibrium and, hence, a reversible process.

3.5.3. Denaturation transition curves

It can be seen (fig. 7) that in the case of irreversible denaturation, the change of V_h causes displacement of the denaturation transition curves along the temperature scale with simultaneous change in their shapes. Two causes are likely to be responsible: isothermal denaturation at low rates, and overheating of a globule at high rates, both resulting in similar changes in the shapes of the curves, i.e., T_{max} decreases with decreasing V_h, the curves becoming narrower and higher. It seems unlikely that these factors can be separated for irreversible denaturation. In contrast, for reversible denaturation, at rates providing for the equilibrium process, the denaturation transition curves

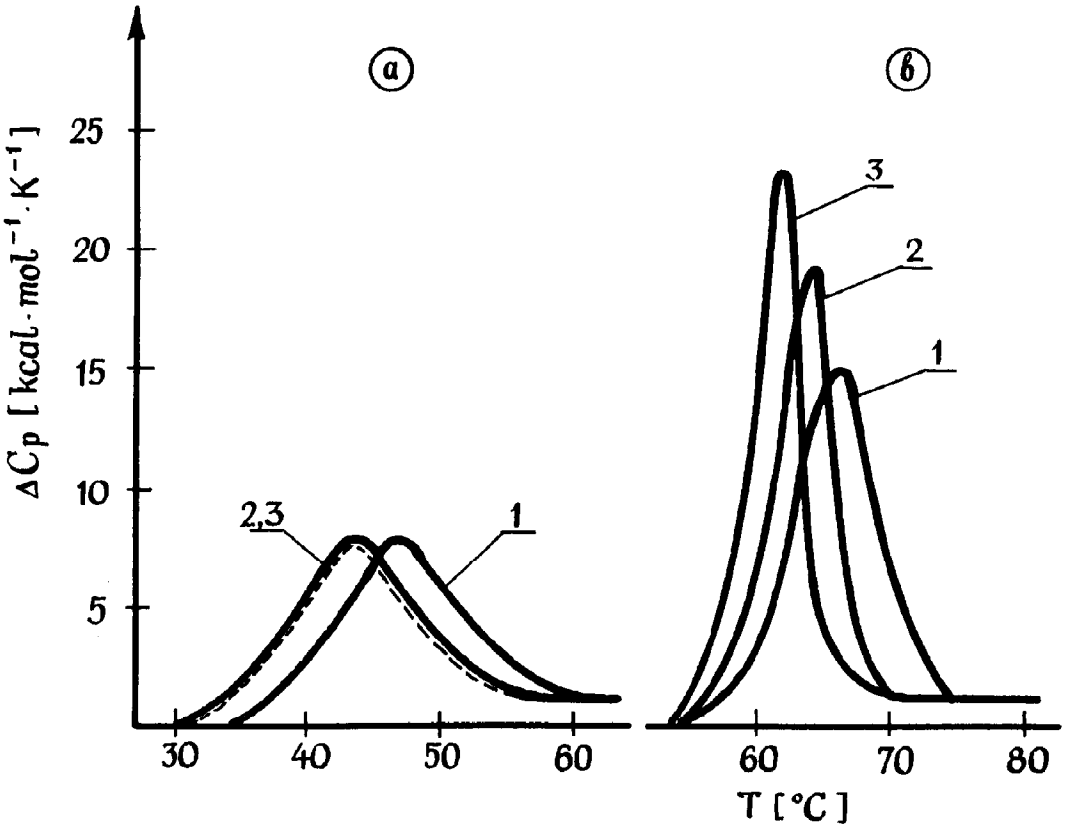

Fig. 7. Denaturation transition curves as a function of heating rate of concentrated RNAase solutions for reversible and irreversible denaturation. (a) Reversible denaturation, pH 2.7, $C = 15\%$; 1, 5 K/min; 2, 1 K/min; 3, 0.25 K/min. (b) Irreversible denaturation, pH 8, $C = 20\%$; 1, 5 K/min; 2, 1 K/min; 3, 0.25 K/min.

coincide (fig. 7a, curves 2 and 3). It is only at higher rates than those at which equilibrium unfolding takes place that overheating of a globule becomes significant, with the respective curves shifting towards higher temperatures (fig. 7a, curve 1).

From the above, it can be seen that the difference between reversible and irreversible denaturation processes becomes most conspicuous during kinetic investigations. Such procedures, where necessary, enable one to choose an optimal regime to study irreversible denaturation.

3.6. Dependence of RNAase denaturation parameters on pH of solutions

The enthalpy values and T_{max} of the RNAase denaturation transition in concentrated solutions were determined within the range pH 2.5–8.5 (fig. 8). RNAase reversible denaturation was accomplished with an accuracy of 5% within the range pH 2.5–4.5. Within the range pH 4.5–7.0, partial irreversibility was observed, registered as a decrease in the value of the thermal effect on second heating. At pH > 7.0, complete irreversibility occurred. The value of H_d for the reversible process was determined at $V_h = 1$ or 5 K/min, depending on the value of the thermal effect. For partially reversible and irreversible processes, it was determined at $V_h = 10$ K/min. As shown by the experiment, even under conditions of maximum irreversibility, aggregation is not very high and can be overtaken even at $V_h = 10$ K/min (fig. 3a). The temperature values of the maximum transition for all pH values studied were determined at

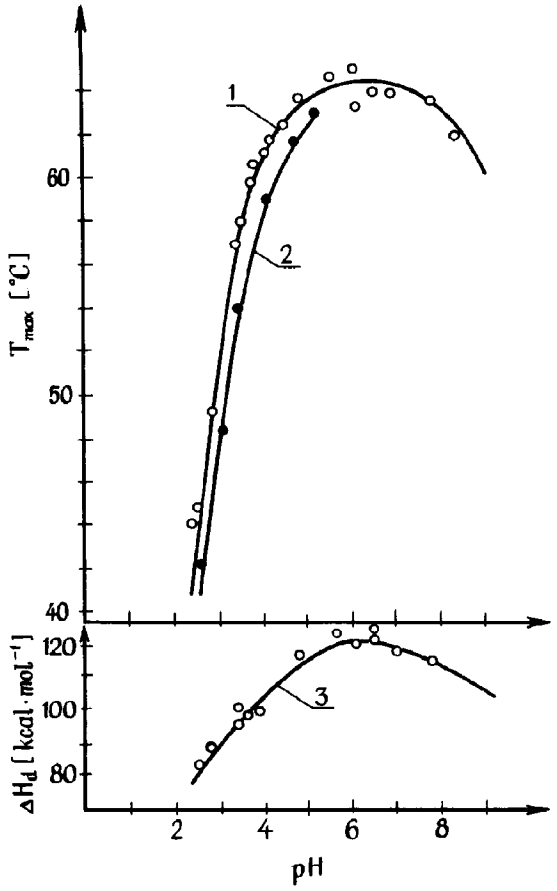

Fig. 8. pH dependence of temperature of maximum and enthalpy of RNAase denaturation transition. (1, 3) $C = 15\%$. (2) $C = 3\%$.

$V_h = 1$ K/min (fig. 8, curve 1). For the reversible process the data obtained correspond to the equilibrium denaturation temperature T_d of the solution under investigation. For the irreversible process such a temperature (T_d) does not exist but for the sake of completeness, the T_{max} values obtained are also given (fig. 8, curve 1). The dome-like enthalpy and temperature dependences of RNAase denaturation on the solution pH (fig. 8, curves 1 and 3) show that maximum protein thermal stability in a concentrated solution is in the region of neutral pH.

Furthermore, these curves could indicate a strong pH influence on both T_d and ΔH_d. However, the denaturation enthalpy, according to Kirhgoff's law ($d(\Delta Q)/dT = \Delta(\Delta C_p)$) depends on T_d, which is, as has been shown, a function of pH. Therefore, the question of the immediate effect of pH on ΔH_d requires further study. If, on the basis of the data on reversible denaturation given above, the dependence of ΔH_d on T_d is plotted at the same value of pH, it turns out that the tangent of the angle of the straight line obtained is equal to the experimental value of the heat capacity jump during the RNAase denaturation transition (fig. 9). Since the situation is the same with dilute solutions, following the reasoning in ref. 7, one can also conclude that no direct influence of pH on the denaturation enthalpy is observed in concentrated solutions. This influence, as in dilute solutions, arises only through the dependence of the denaturation temperature on pH.

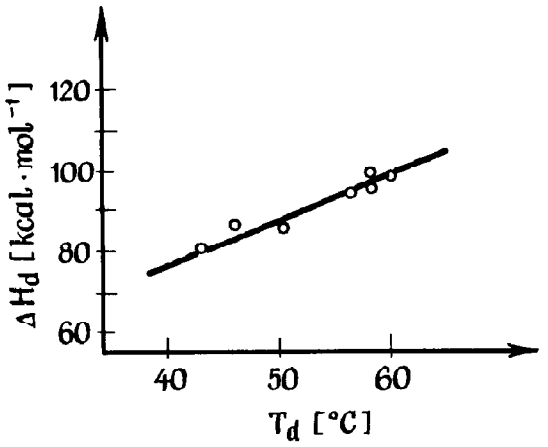

Fig. 9. Temperature dependence of denaturation enthalpy for concentrated RNAase solution (pH 2.5–4.5).

3.7. Dependence of RNAase denaturation parameters on protein concentration in solutions

The equilibrium values of T_d, obtained in experiments with 15–17% RNAase solutions for reversible denaturation are higher than the corresponding T_d values for dilute solutions at the same pH quoted in the literature [12,14]. In order to avoid a possible error due to variations in the materials under study and the instruments used, measurements of T_d were also carried out on 3%

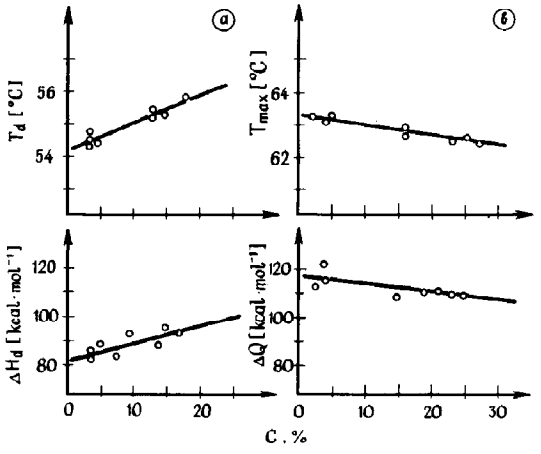

Fig. 10. Thermal effect and temperature of maximum of denaturation transition as a function of RNAase concentration in the solution. (a) Reversible denaturation, (b) irreversible denaturation.

RNAase solution at pH 2.5–5.0. The data on T_d thus obtained (fig. 8, curve 2) coincide with those of ref. 14. Thus, it has been found that throughout the studied range of pH, the T_d of the 15% solution is higher than that of the 3% solution by 2–3 K on average.

The results of a more detailed investigation of the relationship between the parameters of denaturation and protein concentration for two RNAase solutions at pH 3.4 and 8.5 (reversible and irreversible, respectively) are given in fig. 10a and b. One can see that, for reversible denaturation, an increase in protein concentration results in an increase in both T_d and ΔH_d, whereas, for irreversible denaturation, the opposite takes place: T_{max} and ΔQ decrease with increasing C%. It should be borne in mind that for reversible denaturation under the chosen conditions ($V_h = 1$ K/min), the temperature and heat of transition are thermodynamic parameters of denaturation; for irreversible denaturation, they are just the characteristics of thermal absorption of denaturation at a known heating rate. Thus far it remains unclear whether the dependence obtained for reversible denaturation can be accounted for by a direct influence of concentration on the unfolding of a single protein globule, as assumed in ref. 22, or whether there are some other causes, connected, for example, with the change in ionic strength of the solution along with the increase in protein concentration. Two points should be noted. First, the change in denaturation temperature in connection with the change in protein concentration is always accompanied by a change in the thermal effect with the same sign. Second, for reversible and irreversible denaturation, opposite dependences are observed. It seems that whatever the nature of the dependence on concentration seen in reversible denaturation, the change in sign during the transition to irreversible denaturation is a direct consequence of irreversibility. Indeed, when carrying out irreversible denaturation of solutions of different concentrations at the same V_h (1 K/min), we are comparing the thermograms of denaturation of solutions having different degrees of irreversibility at the same time. The influence of aggregation on T_{max} in a concentrated solution must be stronger than in a dilute one. Moreover, since aggregation results in heat release, the latter would proceed more intensely in the concentrated solution, and the corresponding total effect registered by the calorimeter would be less than that in the dilute solution.

It is worth pointing out that the influence of concentration on the denaturation temperature and enthalpy in the case of a reversible transition is certain to be observed for an irreversible transition as well. The experimental data on the decrease in T_{max} and ΔQ with increasing protein concentration in irreversible denaturation is evidence in favour of irreversibility being a stronger factor compared to the concentration effect.

3.8. Comparison of thermal denaturation in a concentrated solution and crystal

The influence of the aggregation state on the process of thermal denaturation is the next step in studying the influence of molecular interaction on the thermodynamic characteristics of a protein during denaturation. These investigations have been carried out with catalase [23]. Since globular protein crystals contain 70% water, they actually do not differ much from the solutions already considered. Only one new factor appears: an order element. Of great interest is a quantitative descrip-

tion and comparison of conformation changes during the denaturation of biomacromolecules in a solution and in a crystal, making it possible to evaluate the validity of identification of conformations of molecules detected by X-ray methods in protein crystals with their respective conformations in solutions, i.e., in vivo.

Here, we present data on kinetic investigations of the denaturation of 20% catalase solutions in phosphate buffer at pH 7.5 and of a trigonal modification of catalase crystals under the same conditions. The investigations were carried out within the heating rate range 50–0.8 K/min. The most significant results are as follows:

Within the temperature range 30–120°C, the thermograms of crystals, as well as those of solutions, have a single heat absorption peak (fig. 11). The difference is in the shape of the peak: with crystals, the peak is narrower and higher. At the same V_h, the T_{max} for crystals is 2 K higher than that for the solution. In both cases the process of denaturation is irreversible.

The decrease in V_h, with either crystals or solutions, involves a decrease in the thermal effect (fig. 5, curve 3) and T_{max} of the heat absorption peak (fig. 6, curves 3 and 3′).

The values of the thermal effect for both crystals and solutions coincide at the same V_h. The denaturation enthalpy value of catalase molecules, obtained on extrapolation towards higher rates, $1/V_h \ll 1$, is $\Delta H_d = 2400 \pm 100$ kcal mol^{-1} and is the same for crystals and solutions.

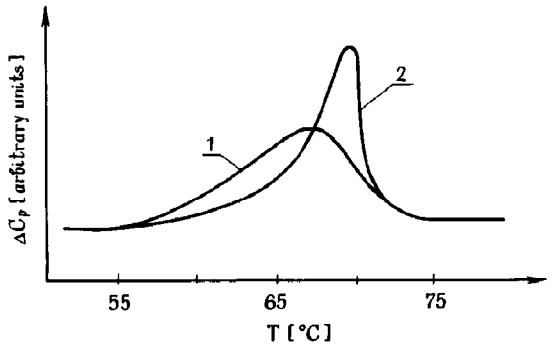

Fig. 11. Experimental thermograms for catalase solution (1) and crystal (2); pH 7.5, $C = 20\%$, $V_h = 3$ K/min.

The absence of an additional heat absorption peak in the thermograms of crystals (fig. 11) proves that crystal melting proceeds simultaneously with the destruction of the native structure of its globules, probably when denaturing globules break molecular bonds in the crystals. Comparison of crystal and solution thermograms suggests that the crystal lattice may retard denaturation ($\Delta T_{max} = 2$°C), so that denaturation in crystals takes place at higher temperatures and, hence, more cooperatively than in solutions. The initiation and termination of denaturation in both cases coincide. The plotted dependences of the thermal effect on heating rates are indistinguishable. This means that the melting heat of the crystal lattice is much lower than the heat of globule denaturation. Such factors as a somewhat higher protein concentration in crystals compared to that of solutions ($C \approx 10\%$) under the conditions of irreversible denaturation, or a possible increase in crystal denaturation entropy ($\Delta S_d^c \geq \Delta S_d^s$), would bring about a decrease in the T_{max} of crystals compared to that of solutions. However, the experiment has proved quite the opposite: T_{max}^c is 0.5% higher than T_{max}^s. This increase in T_{max} in a crystal may be considered as an indication of the existence of energy differences during globule denaturation at various aggregation states. Unfortunately, the supposed corresponding change of ΔH_d by 0.5% is, so far, beyond the limits of calorimetry with concentrated protein solutions.

4. Conclusion

What is essential in dynamic calorimetry for the process of protein thermal denaturation to be reversible is not the absence of aggregation but the separation of denaturation and aggregation processes in time. The range of heating rates available in modern differential scanning calorimeters allows one to choose rates at which the corresponding processes are not superimposed within the time of the experiment.

The fulfilled kinetic investigations of protein denaturation under the conditions of a minimal rate of aggregation are a good illustration of these assumptions. It has been proved that there is a

rather wide range of heating rates in which thermal denaturation of concentrated solutions of globular proteins is a reversible process. In this respect, concentrated solutions may differ from dilute ones only in a narrower range of heating rates. If all these conditions are satisfied, it is possible for concentrated solutions to determine all the thermodynamic parameters of denaturation transition. In the case of intensive aggregation, separation of denaturation and aggregation processes is also possible, but takes place at such high heating rates at which the process of denaturation ceases to be in equilibrium (i.e., reversible). It has been shown, however, that such a high rate of heating enables one to determine the value of the thermal effect for irreversible denaturation connected only with the destruction of the native structure of a protein.

All investigations carried out thus far show that, in reversible denaturation, molecular interaction does not bring about any significant changes in the physical properties of the denaturation transition. This makes available the use of calorimetric results on concentrated solutions for the determination of the structure and stability of protein globules.

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