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ANALYSIS OF DSC DATA RELATING TO PROTEINS UNDERGOING IRREVERSIBLE THERMAL DENATURATION

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Abstract

New approaches to the analysis of differential scanning calorimetry (DSC) data relating to proteins undergoing irreversible thermal denaturation have been demonstrated. The experimental approaches include obtaining a set of DSC curves at various scanning rates and protein concentrations, and also reheating experiments. The mathematical methods of analysis include construction of a linear anamorphosis and simultaneous fitting of a theoretical expression for the dependence of the excess heat capacity on temperature to a set of experimental DSC curves. Different kinetic models are discussed: the one-step irreversible model, the model including two consecutive irreversible steps, the Lumry and Eyring model with a fast equilibrating first step, and the whole kinetic Lumry and Eyring model.

Keywords: DSC, kinetic models, protein denaturation

Introduction

Differential scanning calorimetry (DSC) is widely used for the study of thermal protein denaturation [1–5]. When analyzing DSC data, investigators should understand what information can be obtained from the calorimetric curves.

The methods for the analysis of DSC curves for the cases of reversible and equilibrium processes of protein denaturation have been well elaborated [1, 2, 4, 6]. However, there are many proteins whose denaturation is calorimetrically irreversible. Elaboration of methods for the analysis of DSC data relating to such cases was started by Sanchez-Ruiz and co-authors [7].

In the present work, we demonstrate new approaches to the analysis of DSC data concerning proteins undergoing irreversible thermal denaturation.

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Experimental approaches

The reversibility of denaturation is usually checked by reheating: if heat absorption is not observed for the preheated sample, the process is considered irreversible. However, to trace the chemical baseline, the first heating is carried out up to high temperature (about 90–100°C). Irreversibility can therefore be explained in terms of the effect of high temperatures rather than the irreversible character of the denaturation.

To discriminate between the two possibilities, it is necessary to carry out another reheating experiment: to cool the sample immediately after the peak of heat absorption is completed, and then to scan again. Another experimental variant is possible: to stop heating near or before the maximum point, and then to cool and reheat.

We have used such an approach to study the thermal denaturation of creatine kinase from rabbit skeletal muscle [8]. At a scanning rate of 1 K min^{-1}, the main heat absorption is observed between 47 and 59 with maximum at 54.5°C. When heating was stopped at 59°C, no thermal effect was observed in the area of the main peak in the reheating experiment. Moreover, after heating up to 53.5°C and further cooling, the reheating scan showed a peak corresponding to an enthalpy about 25% that for the original peak. Thus, we concluded that in this case the irreversibility is not caused by the effect of high temperatures, but is associated with the denaturation process.

Another example is the study of the thermal denaturation of lipase B from *Candida rugosa* [9]. When the enzyme was heated to 51.2 then cooled to 15°C and reheated at the same scanning rate, the reheating scan differed from the first scan only by a scale factor determined by the difference in the amounts of protein undergoing denaturation (Fig. 1). An analogous result was obtained for Cry IIIA δ-endotoxin from *Bacillus thuringiensis* [10]. Such results testify to a one-step mechanism of denaturation.

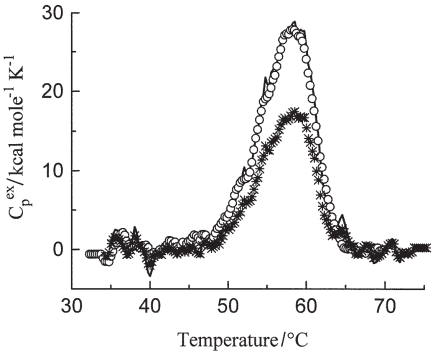


Fig. 1 Original and reheating scans for lipase B from *Candida rugosa* (pH 7.2, scanning rate 59.5 K min^{-1}) [9]. Circles indicate the original scan, asterisks indicate the reheating scan after first heating to 51.1 and cooling to 15°C, and the direct line denotes the reheating scan multiplied by the ratio of the calorimetric enthalpies for the original and reheating scans

If denaturation is irreversible, the process is under kinetic control, and calorimetric profiles are scanning rate-dependent [3, 4, 7, 11]. In this case, one curve obtained at a single scanning rate is not enough for a proper analysis. It is necessary to obtain a set of curves at various scanning rates.

Further, it is necessary to obtain a set of curves at various protein concentrations. In some cases, calorimetric profiles are protein concentration-dependent [12–16]. This means that the kinetic mechanism includes a bimolecular step. One example is the reversible dissociation of an oligomer to monomers, where the step of reassociation of the monomers is bimolecular. Other examples are aggregation and autolysis.

Mathematical methods of analysis

The approach to the analysis of DSC curves for the case of irreversible protein denaturation was first suggested by Sanchez-Ruiz and co-authors [3, 4, 7, 17] and was further developed in our own publications [8, 9, 11, 18]. The basic features of the approach are as follows:

- the fitting of a theoretical equation describing the dependence of excess heat capacity on temperature to experimental DSC curves, by using the original computer programs, or
- the fitting of a system of differential equations to the experimental data, by using the commercial software 'Scientist' (MicroMath Scientific Software, USA);
- simultaneous fitting to a set of experimental curves obtained at various scanning rates;
- the use of many experimental points (the interval between points is about 0.05–0.1 K);
- the comparison of results obtained for different models (using the sum of the squaresed deviations or its derivatives as a criterion).

The main problem is to choose an appropriate model. The simplest model is the two-state (or one-step) model

$$N \xrightarrow{k} D \qquad (1)$$

where k is the rate constant. At present, the denaturation of more than twenty proteins has been described in the framework of this model (Table 1).

One of the most convenient and obvious methods of analysis for this model is the construction of a graphical anamorphosis in the coordinates $\{\ln[\nu C_p^{ex}/(\Delta H - Q)]; 1/T\}$ (or reverse coordinates), where ν is the scanning rate, C_p^{ex} is the excess heat capacity, ΔH is the enthalpy of denaturation, T is the absolute temperature, and Q is the heat absorbed up to temperature T [7, 11]. If the dependence of rate constant k on temperature follows the Arrhenius equation, the anamorphosis is linear.

Additionally, if the one-step model is valid, the $\ln[\nu C_p^{ex}/(\Delta H - Q)]$ value is dependent only on temperature and is independent of the scanning rate. All the points obtained at various scanning rates should therefore lie on a common straight line.

Table 1 Results of description of protein denaturation by the one-step model

Protein	Source	M_w/kDa	Number of subunits	pH	ΔH/ kJ mol^{-1}	E_a/ kJ mol^{-1}	Ref.
Acetylcholinesterase	*Torpedo californica*	130	2	7.3	1603±100	548±33	19
G-actin	rabbit muscle	43	1	8.0	664	245÷285	20
Annexin V E17G	*Escherichia coli*	35.7	1	7.0	690	611±70	21
ATP-synthase	*Bacillus* PS3	550	13	8.0	4120	854±40	22
ATPase	bovine heart mitochondria	370	9	7.0	4600	530±20	23
ATPase F_1 complex	chloroplasts	400	9	7.5	3986	348	24
Bromelain	stem	22.8	1	3.4	334±17	226±11	25
Carbamoyl-phosphate synthetase	*Escherichia coli*	159	2	7.5	2005	434	26
Carboxypeptidase A	pig pancreas	34.8	1	7.5	696÷905	250±14	27
Carboxypeptidase B	pig pancreas	34.5	1	7.5	625÷758	270±4	17
Cellulase	*Streptomyces halstedii* JM8	28	1	6.0	348	553÷590	28
Creatine kinase	rabbit muscle	84	2	8.0	1079	461	8
Cytotoxin RTX-8	*Radianthus macrodactylus*	8	1	7.4		189÷210	29
Cry3A δ–endotoxin	*Bacillus thuringiensis*	67	1	3.0	945÷1300	389÷410	10

Table 1 Continued

Protein	Source	M_w/kDa	Number of subunits	pH	ΔH/ kJ mol^{-1}	E_a/ kJ mol^{-1}	Ref.
5-Enoylpyruvate shikimate-3-phosphate synthetase	*Escherichia coli*	46		7.0	1046±38	553±21	30
Glucose transporter GLUT1	human erythrocytes	53	1	7.4	624±130	582±63	31
Glutamate dehydrogenase	bovine liver	337	6	7.6		276	32
Glutathione reductase	*Spiruline maxima*	96	2	7.0	1000÷1500	411	33
Haemocyanin	lobster	500	6	7.2	13700±1000	383±30	34
Lactate dehydrogenase	pig muscle	134	4	7.0	1763	172	35
Lectin	lentil	48	4	7.4	812÷1056	345÷376	36
Lipase B	*Candida rugosa*	60	1	7.2	996÷1248	239÷275	9
Procarboxypeptidase A	pig pancreas	46.4	1	7.5	835÷974	300±20	27
Procarboxypeptidase B	pig pancreas	45.5	1	7.5	786÷912	342±12	17
Superoxide dismutase	bovine erythrocytes	32	2	7.0	1128÷1459	240	14
Thermolisin	*Bacillus thermoproteolyticus rokko*	37.5	1	7.5	1336±50	282±8	7

Figure 2a shows the graphical anamorphosis for creatine kinase [8]. Practically all the points lie on the common straight line. Therefore, we have concluded that the thermal denaturation of creatine kinase satisfies the one-step model.

However, this criterion demonstrates deviations from the one-step model for some proteins [11, 21, 37]. As an example, Fig. 2b shows the graphical anamorphosis for cellulase from *Streptomyces halstedii* [11]. It may be seen that the points obtained at various scanning rates do not lie on a common line.

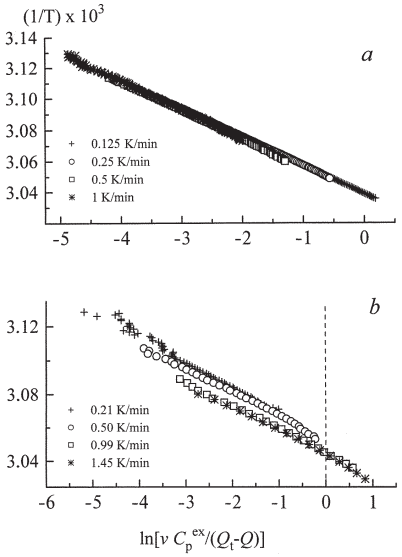


Fig. 2 Dependences of $1/T$ on $\ln[\nu C_p^{ex}/(\Delta H - Q)]$ for a – creatine kinase from rabbit skeletal muscle [8] and b – cellulase from *Streptomyces halstedii* JM8 [11]

Nevertheless, even if the experimental data are satisfactorily described by the one-step model, the real mechanism of denaturation can be more complex. It should be noted that the values of enthalpy of denaturation appear to be higher than the values of energy of activation for most of the proteins listed in Table 1. If it is assumed that the denaturation of these proteins really does have a one-step character, this means that the energy of activation for the reverse process should be negative, i.e. the rate constant of renaturation should decrease with temperature. Such a possibility is discussed in [38]. However, the denaturation of these proteins is most likely to have a multistep character, and the energy of activation values obtained are apparent and characterize the initial rate-limiting step of the process.

When analyzing the one-step model, Sanchez-Ruiz *et al*. [7] noted that the Lumry and Eyring model

$$N \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_2} D \qquad (2)$$

(Where N, U and D are native, partially unfolded and denatured protein forms; and k_1, k_{-1} and k_2 are the rate constants for the corresponding reactions) is more realistic, and the one-step model (1) is a particular case of the Lumry and Eyring model.

Further, Sanchez-Ruiz [39] analyzed two situations when the Lumry and Eyring model is reduced to the one-step irreversible model (1). In the first situation (situation *C*', according to [39]), the value of k_2 is much higher than the values of k_1 and k_{-1}, so that the direct reaction of the first step is rate-limiting and the reverse reaction is practically absent. The second situation (situation *C*, according to [39]) is realized when the rates of the direct and reverse reactions of the first step are much higher than the rate of the second step, but equilibrium for the first step is shifted toward the form *N*.

We have carried out a simulation study to check the possibility of reducing the Lumry and Eyring model (2) to the one-step model (1). In this study, we assumed that the temperature dependences of all the rate constants obey the Arrhenius equation, which we write for the sake of convenience in the following form:

$$k=\exp\left[\frac{E_a}{R}\left(\frac{1}{T^*}-\frac{1}{T}\right)\right] \tag{3}$$

where E_a is the energy of activation, R is the gas constant, and T^* is the absolute temperature at which k=1 min^{-1}.

The simulation study shows that the curves constructed for the Lumry and Eyring model (2) are well described by the one-step model (1) if the value of k_2 is much higher than the value of k_1, irrespective of the value of k_{-1}. In this case, the analysis in the frame of the one-step model (1) results in an apparent value of activation energy, which does not coincide with the activation energy of any real reaction.

Figure 3 depicts the effect of variation of the value of one parameter of the Lumry and Eyring model (2), parameter T^*_{-1} on the apparent value of the energy of activation ($E_{a,app}$), calculated for the one-step model (1). When the values of this param-

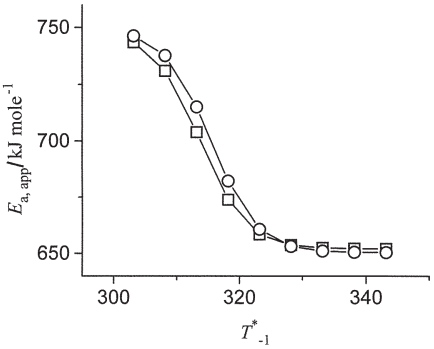


Fig. 3 Effect of variation of the value of parameter T^*_{-1} of the Lumry and Eyring model (2) on the apparent activation energy value calculated for the one-step irreversible model (1). The values of the other parameters of the Lumry and Eyring model: $E_{a,1}$=650, $E_{a,-1}$=250, $E_{a,2}$=350 kJ mol^{-1}, T_1^*=328.2, T_2^*=318.2 K, ΔH_2=200 kJ mol^{-1}. The scanning rates: 0.125 (circles) and 2 K min^{-1} (squares)

eter are high, the value of k_{-1} is low, and the apparent value of energy of activation coincides with the value of the energy of activation of the direct reaction of the first step ($E_{a,1}$). Obviously, this is situation *C*'. When the values of the parameter T^*_{-1} are low, the value of $E_{a,app}$ coincides with the sum of the enthalpy of the first step and the energy of activation of the second step ($E_{a,app}=\Delta H_1+E_{a,2}$). Obviously, this is situation *C*. In the other cases, we obtain intermediate values of $E_{a,app}$, which do not coincide with the activation energy of any reaction.

If the process does not follow the one-step model, the experimental data may in principle give more information, but we should find the appropriate model. One of the most probable models is the Lumry and Eyring model (2). However, use of the whole kinetic Lumry and Eyring model for the quantitative description of DSC curves is difficult because the corresponding system of differential equations does not have an analytical solution at varying temperature. Although there are computer programs which allow the direct fitting of a system of differential equations to experimental data, there are as yet no publications in which DSC data have been described through use of the whole kinetic Lumry and Eyring model (2).

Two other two-step models can be considered as particular cases of the whole Lumry and Eyring model. One of these models is the Lumry and Eyring model with a fast equilibrating first step. This model was studied theoretically by Sanchez-Ruiz [39] and Milardi *et al*. [40]. The model has been used for a quantitative description of the thermal denaturation of azurin [40–44], plastocyanin [45] and some other proteins [21, 46], but the accuracy of fitting does not allow us to consider such a description satisfactory.

The other model is the model involving two consecutive irreversible steps:

$$N \xrightarrow{k_1} U \xrightarrow{k_2} D \qquad (4)$$

This model is realized for the situation when the rate of the reverse reaction of the first step is much less than the rate of the second step. The model was first ana-

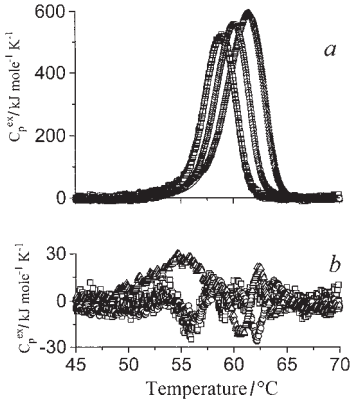


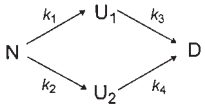
Fig. 4 Result of fitting theoretical Eq. (5) to the experimental calorimetric profiles obtained for uridine phosphorylase from *Escherichia coli* at scanning rates of 0.25 (squares), 0.5 (circles), and 1 (triangles) K min^{-1}; a – solid lines are theoretical curves and points are experimental data; b – residuals [18]

lyzed in our publications [18, 47]. We have solved the system of differential equations for this model and obtained the equations for the dependence of the excess heat capacity on temperature:

$$C_p^{ex} = \frac{\Delta H_1 k_1}{\nu} \exp\left(-\frac{1}{\nu} \int_{T_0}^{T} k_1 dT \right) + \frac{\Delta H_2 k_2}{\nu^2} \exp\left(-\frac{1}{\nu} \int_{T_0}^{T} k_2 dT \right) \int_{T_0}^{T} \left[k_1 \exp\left(\frac{1}{\nu} \int_{T_0}^{T} (k_2 - k_1) dT \right) \right] dT \quad (5)$$

We have applied our approach to analyze the DSC curves obtained for uridine phosphorylase from *Escherichia coli*. We used three models: the one-step model (1), the Lumry and Eyring model with a fast equilibrating first step, and the model involving two consecutive irreversible steps (4). Only the latter model described the experimental data satisfactorily. Figure 4 shows that the theoretical curves coincide well with the experimental ones. Accordingly, we concluded that the thermal denaturation of uridine phosphorylase follows the model involving two consecutive irreversible steps (4) [18].

The description of the thermal denaturation of complex proteins may demand more complex models. The model involving two branches of two consecutive irreversible steps:



(6)

is one such model. We have now obtained the equation for the dependence of the excess heat capacity on temperature:

$$C_p^{ex} = \frac{\Delta H_1 k_1 + \Delta H_2 k_2}{\nu} \exp\left(-\frac{1}{\nu} \int_{T_0}^{T} (k_1 + k_2) dT \right) + \frac{\Delta H_3 k_3}{\nu^2} \exp\left(-\frac{1}{\nu} \int_{T_0}^{T} k_3 dT \right) \int_{T_0}^{T} \left[k_1 \exp\left(\frac{1}{\nu} \int_{T_0}^{T} (k_3 - k_1 - k_2) dT \right) \right] dT + \frac{\Delta H_4 k_4}{\nu^2} \exp\left(-\frac{1}{\nu} \int_{T_0}^{T} k_4 dT \right) \int_{T_0}^{T} \left[k_2 \exp\left(\frac{1}{\nu} \int_{T_0}^{T} (k_4 - k_1 - k_2) dT \right) \right] dT \quad (7)$$

Reverse scanning

Another approach to calorimetric experiments may be proposed. Our recent simulation study shows that reverse scanning could give additional information about the mechanism of the denaturation process. For example, we obtained three practically

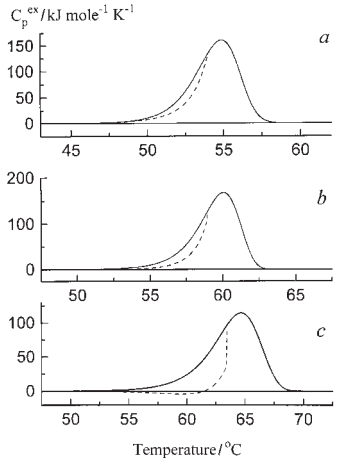


Fig. 5 Theoretical direct (solid lines) and reverse (dashed lines) scans for the Lumry and Eyring model. The values of the parameters of the Lumry and Eyring model: $E_{a,1}$=650, $E_{a,-1}$=250, $E_{a,2}$=350 kJ mol^{-1}, T_1^*=328.2 K, ΔH_2=200 kJ mol^{-1}. The values of parameter T_{-1}^* :343.2 (a) and 313.2 K (b, c); the values of parameter T_2^*: 328.2 (a, b) and 338.2 K (c)

identical calorimetric profiles for the Lumry and Eyring model (2) by using different sets of parameters (Fig. 5a–c, solid lines). However, the curves obtained on reverse scanning are different (Fig. 5a–c, dashed lines). In our opinion, we need calorimeters, which can record not only the direct scan, but also the reverse scan. Such experiments may be useful for a discrimination between the mechanisms of denaturation.

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