What can we get from varying scan rate in protein differential scanning calorimetry?

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Abstract

Differential scanning calorimetry has many advantages over other techniques to study the thermal stability of proteins due to its direct measurement of thermodynamic parameters. Most proteins undergo irreversible thermal denaturation causing their thermogram to be scan rate dependent. We modeled reversible and irreversible protein thermograms at varying scan rates. The complete Lumry-Eyring model was used to model the irreversible thermograms at various values of $T_{1/2}$ (temperature at which equilibrium constant equals unity) and T^* (temperature at which rate constant equals 1 min^{-1}). Our results have shown that the thermal effects of two processes are integrated with decreasing the T^* relative to $T_{1/2}$. It is also shown that the shape of second derivatives of thermograms under different conditions have specific pattern which can be used to judge and estimate the correct model for protein denaturation.

Keywords: *Differential Scanning Calorimetry, Second Derivative, Irreversible Thermal Denaturation, Modeling, Scan Rate.*

1. Introduction

The stability of proteins and their interactions with ligands are topics of special interest in biochemistry, biotechnology and biomedical sciences [1]. Differential scanning calorimetry (DSC) is an experimental technique to measure the heat capacity of a system as a function of temperature under isobaric conditions. DSC has been widely used to acquire the thermodynamic parameters associated with processes such as protein denaturation triggered by changing the temperature.

Proteins show two different kinds of behavior under thermal denaturation named as reversible and irreversible thermal unfolding. Thermal unfolding of many proteins is irreversible in aqueous solution [2]. Reportedly, the stability of many proteins *in vitro* may have a kinetic basis, i.e., stability is controlled by a rate constant that increases with temperature, and the protein denatures in the temperature range in which the denaturation rate becomes significant in the time-scale of the experiment [3]. In DSC experiments, irreversible process is indicated by the lack of the endothermic effect on a second scanning of the sample after rapid cooling [2].

According to Lumry-Eyring model, irreversible protein denaturation is thought to involve, at least, two steps: (a) reversible endothermic unfolding of the native protein (N); (b) irreversible alteration of the unfolded protein (U) to yield a final state (F) that is unable to fold back to the native form which is exothermic process [4].

This model is depicted as:

Scheme I

where $K=k_1/k_{-1}$.

Previously, most researchers supposed the applicability of the equilibrium thermodynamics analysis to irreversible DSC transitions. They believed that the second step proceeds slowly or the enthalpy of the processes responsible for the irreversible step is much lower than that for the cooperative unfolding, so this model is amenable to equilibrium thermodynamics analysis. Later it was shown that most of the DSC transitions were rate limited and highly depended on scan rate [4].

The irreversible DSC profiles are analyzed by two methods: a) graphical method which is boring and time consuming and b) fitting by using different softwares [5,6]. In fitting, selecting the initial values and restrictions to values has an essential role on the righteousness of the results.

The derivatives of curves has being used to identify overlapping components and peak picking in different experiments such as Fourier transform infrared spectroscopy [7]. The most characteristic feature of a second-order derivative is a negative band with minimum at the same position as the maximum on the original data. The balanced wings (satellites) around minimum show the symmetry of the curve [8].

In the present study, we carried out modeling the Lumry-Eyring denaturation mechanism for monomeric proteins under different conditions, and tried to show the effect of varying scan rate on the DSC thermogram and the worth of second derivative to better interpretation of DSC experiments. Such an approach may help researcher to improve fitting results by selecting the reasonable initial variables based on DSC thermograms.

2. Methods

All the model graphs were created by Excel software. Theoretical and experimental data were fitted by least square fitting using solver function of Excel software.

In order to show the consistency of second derivatives of experimental and modeling data, we assessed the second derivatives of DSC profiles of amine oxidase from *Euphorbia characias* (ELAO). Thermal denaturation of ELAO obeys two-state thermal denaturation [9]. The second derivatives of excess heat capacity C_p^{ex} vs. T were calculated and then smoothed using OriginPro 2015 (OriginLab cooperation, Northampton, MA). This software offers different smoothing methods. Considering the high-frequency noise in signals, fast Fourier transform filter with 5 to 7 points of window was used to smooth the second derivatives.

3. Results and discussion

3.1. Effects of scan rate on reversible thermal unfolding

The dependence of C_p^{ex} on temperature for reversible thermal denaturation was described as follows [10]:

$$C_p^{\text{ex}} = \Delta C_p \frac{K}{K+1} + \frac{\Delta H^2}{RT^2} \frac{K}{(1+K)^2} \quad (1)$$

where ΔC_p , K and ΔH are heat capacity difference between denatured and native state, equilibrium constant and enthalpy respectively.

Theoretically the protein thermogram of reversible process should be independent of scan rate. Experimental C_p shows some variation by scan rate due to time response of calorimeter especially at high scan rates. Lopez Mayorga and Freire

proposed an equation to recover actual thermal effects from experimental data as [11]:

$$Y = \frac{1}{A} [Y^* + \tau(dY^*/dt)] = \frac{1}{A} \left[Y^* + \tau v \left(\frac{dY^*}{dT} \right) \right] \quad (2)$$

where Y and Y^* are actual and experimental thermal effects (in this context C_p^{ex}), τ , v and A are calorimetry response time, scan rate and calibration constant, respectively. Adjusting calibration constant to unity also thanks to new DSC instruments with low response time, normally less than 10 sec, these variations are negligible and make no strong changes in C_p . Modeled thermogram of reversible thermal denaturation is a symmetric profile and its second derivative shows equal wings on both side of a minimum corresponding to T_m , temperature with maximum C_p^{ex} (Fig. 1).

[Fig. 1 to be inserted here]

3.2. Scan rate effects on irreversible thermal unfolding

Even after response time corrections, some DSC profiles show scan rate dependence. This may arise from deviation of molecular population from equilibrium due to slow equilibration of $N \xrightarrow{\leftarrow} U$ stage. Different models for irreversible thermal denaturation have been proposed on the basis of various constant rates of Lumry-Eyring model; interested readers are referred to references [12,13]. The complete Lumry-Eyring model was analyzed by Milardy and co-authors [14,15]. They expressed the dependence of C_p^{ex} on temperature as:

$$C_p^{\text{ex}} = \left[\frac{K\Delta H_u}{(K+1)^2} \left(\frac{k}{v} + \frac{\Delta H_u}{RT^2} \right) + \Delta H_i \frac{1}{v} \frac{kK}{K+1} \right] \times \exp \left(-\frac{1}{v} \int_{T_0}^T \frac{kK}{K+1} dT \right) \quad (3)$$

where K , k , ΔH_u , ΔH_i , R , T and ν are equilibrium constant for first step, rate constant for irreversible step, unfolding enthalpy, enthalpy for the second step, universe gas constant, temperature and scan rate, respectively.

Although protein denaturation and aggregation may exhibit a non-Arrhenius behavior, one can typically neglect the temperature dependence of K and k due the factor of $\ln T$ when dealing with the relatively small range of temperatures [16,17].

Thus these values are calculated as

$$K = \frac{k_1}{k_2} = \exp \left[\frac{-\Delta H_u}{R} \left(\frac{1}{T} - \frac{1}{T_{1/2}} \right) \right] \quad (4)$$

$$k = \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T^*} \right) \right] \quad (5)$$

$T_{1/2}$ and T^* are the temperatures at which $K=1$ and $k=1 \text{ min}^{-1}$, respectively.

Based on this equation we studied theoretically the effect of scan rate on DSC profile at various parameters of Eq. (3). Our results showed that enthalpies mostly affect the size of the peaks while E_a determines the sharpness of the second peak. Main determinants of the overall shape of thermogram are the relative values of T^* and $T_{1/2}$, so we focused on these temperatures.

3.2.1. The effect of scan rate on DSC profile when T^* is higher than $T_{1/2}$

Under these conditions the U state accumulates in the denaturation process prior to final state. For most proteins, the conformational change is followed by formation of aggregate forms [18] which is mostly believed to be an exothermic effect.

The DSC thermograms under conditions of $T^* > T_{1/2}$ and $\Delta H_i < 0$ are shown in Fig. 2, as it is clear the apparent T_m (temperature corresponds to highest C_p^{ex})

and $C_p^{\text{ex}}, \text{max}$ (the maximum value of C_p^{ex}) are not changed by increasing the scan rate, while the exothermic and endothermic effects are integrated at low scan rate. Getting the second derivatives will be very helpful to distinct the second step at low scan rate (Fig. 2b).

[Fig. 2 to be inserted here]

Exothermic and endothermic portions of the curve become separated at higher scan rates. The extent of separation depends on activation energy and the differences between $T_{1/2}$ and T^* . Such behavior was seen for amyloid fibrils where T_m is independent of scan rate but some deviations were observed at the high temperature side at different scan rates [19].

Most DSC experiments are conducted at scan rates higher than $1 \text{ K} \cdot \text{min}^{-1}$, so most likely scanning is terminated at separation region which leads to get just endothermic portion of thermogram. However it seems that stopping the experiment under these conditions would not have sharp effects on DSC interpretation and the endothermic part can easily be treated as reversible denaturation. The modeling of Lumry-Eyring equation at high scan rates shows that the sub-peak of endothermic subpeak area is in good agreement with used model. At low scan rates the exothermic and endothermic effects are integrated to some extent which could not simply deconvoluted and getting second derivatives will be very helpful to distinguish these thermal effects (Fig. 2b).

By increasing the E_a , the exothermic portion becomes sharp and well separated from endothermic portion (Fig. 3).

[Fig. 3 to be inserted here]

3.2.2. The effect of scan rate on DSC profile when $T^*=T_{1/2}$

In this situation a small endothermic process appears at higher scan rates, similarly to situation where $T^*>T_{1/2}$. T_m increases while C_p^{ex} (C_p^{ex} at T_m) decreases with increasing the scan rates (Fig. 4a). Calorimetric enthalpy (area under peak) does not change with scan rate and it is the algebraic summation of ΔH_u and ΔH_i . However the DSC profile becomes wider with increasing the scan rate. Meanwhile the DSC profile becomes more symmetric and resembles reversible thermal denaturation at higher scan rate which can simply judged by second derivative curves (Fig. 4b)

[Fig. 4. to be inserted here]

DSC profiles of ferrochelatase [20], protease [21] and recombinant human botulinum protein antigen serotype C [22] become more wider with higher T_m as scan rate increases, similarly to this situation.

3.3. A case when Lumry-Eyring model is reduced to two-state model

The most proteins studied by DSC follows a two-sate irreversible denaturation [23-25]. Due to higher value of K , the rate limiting step would be the second step and it seems that N state directly changes to final state as:

The C_p^{ex} dependence of this model was expressed as:

$$C_p^{ex} = \left[\Delta H_i \frac{1}{v} \times \exp \left[\frac{E_a}{R} \left(\frac{1}{T^*} - \frac{1}{T} \right) \right] \right] \times \exp \left(-\frac{1}{v} \int_{T_0}^T \exp \left[\frac{E_a}{R} \left(\frac{1}{T^*} - \frac{1}{T} \right) \right] dT \right) \quad (7)$$

Kurganov et al. [5] proposed criteria to assess two-state denaturation mechanism for protein irreversible denaturation. They showed linear dependence of $1/T$ on $\ln[\nu C_p^{\text{ex}}/(Q_t - Q)]$ for two-state model. By changing different values of parameters of Eq. (3) in reasonable ranges for protein denaturation, we tried to get a linear dependence of $1/T$ on $[\nu C_p^{\text{ex}}/(Q_t - Q)]$. Comparing the Eq. (3) and Eq. (6) shows that increasing the K value reduces the Lumry-Eyring model to two-state model which can be achieved by a) decreasing $T_{1/2}$ and/or b) decreasing the ΔH (Fig. 5). Under these conditions the curve shape and $C_p^{\text{ex}},_{\text{max}}$ are independent of scan rate meanwhile thermogram shifts toward higher temperatures with scan rate increment, as judged by similar second derivation at different scan rates (Fig. 5c). The difference between T^* and $T_{1/2}$ corresponding to fulfillment of the two-state model inversely depends on E_a .

[Fig. 5 to be inserted here]

The consistency of the results of fitting the DSC data to complete Lumry-Eyring and two-state models was checked for ELAO. Thermal denaturation of ELAO was reported to obey two-state irreversible model. As Table 1 shows, the results of fitting parameters for two models are in very good agreement, but two extra parameters are obtained in Lumry-Eyring model, $T_{1/2}$ and ΔH . The large difference between $T_{1/2}$ and T^* separates thermal effects of two steps similarly to situation **3.2.1** but the second step is an endothermic effect. The low values of ΔH and $T_{1/2}$ cause that the thermal effects of first portion (equilibrium) of Lumry-Eyring model to be omitted. This means that at low temperature the partially unfolded and folded states are in fast equilibrium. It should be noted that only the dimeric form of ELAO is catalytically

active and its monomeric components are inactive due to the contribution of intersubunit contacts in forming the active site. We suggest that the active site is not affected in partially unfolded state and the protein can fold back to catalytically active state. The activity loss of this enzyme is causing by cooperative unlocking of conformational locks between monomers at temperatures higher than 60 °C [26]. It was also shown that retention time for denatured ELAO is a little less than that for folded state in size exclusion chromatography experiments, indicative of a dimeric state of the denatured protein [9]. The probably formed S–S bond in C-terminals of ELAO monomers links two unfolded monomers like a hinge and conserves unfolded state of ELAO in the dimeric form [9]. It would be reasonable to relate the higher enthalpy of the second step mainly to unlocking of conformational locks accompanying with major unfolding of protein. The second derivative of C_p^{ex} vs. T for ELAO resembles that for two-state curve indicating the validity of this model for ELAO (Fig. 6).

[Table 1 to be inserted here]

[Fig. 6 to be inserted here]

3.4. Scan rate effects on irreversible thermal unfolding with $\Delta H_i > 0$

It is thought that the aggregation process is exothermic, but the final state is not just aggregating, other endothermic processes may contribute in final state of protein denaturation. Modeling of Lumry-Eyring by positive values of ΔH_i at $T^* > T_{1/2}$ shows two peaks which are more separated as scan rate increases similarly to situation 3.2.1. The second derivative profile shows two peaks which just second peak is affected by scan rate. The main difference between second derivatives of negative

and positive values ΔH_i is that second peak shows asymmetric shape weighted toward first half at $\Delta H_i > 0$ (Fig 7.b) and toward second half at $\Delta H_i < 0$ (Fig.2.b).

[Fig. 7 to be inserted here]

When T^* equals $T_{1/2}$ and $\Delta H_i > 0$, the DSC modeled profiles become wider with shorter C_p^{ex} by increasing scan rate while second derivatives are being more symmetric. The main difference with situation 3.2.2 is that the negative peak at the end of profile does not appear by increasing scanning rate (Fig. 8).

[Fig. 8 to be inserted here]

4. Conclusion

The complete modeling Lumry-Eyring equations at different scan rates and relative values of $T_{1/2}$ and T^* showed that the endothermic first step and exothermic second step can be integrated or separated. Second derivative of thermogram shows specific pattern to each situations which easily explicate integrated thermal phenomena. An unwanted effect of the derivatization process is that the signal-to-noise ratio decreases as higher orders of derivatives are used. The decrease in signal-to-noise ratio can be reduced by using the smoothing techniques [8]. Although peak picking using the second derivative method is recommended for high-resolution data, this method can be used for DSC analysis because of recent developments in DSC instruments with high signal-to-noise ratio, smoothing software and protein purifications.

Acknowledgments

The supports from Ardabil University of Medical Sciences, University of Tehran are acknowledged. This investigation was also supported by Iran National Science Foundation (grant number 87040071) and Russian Science Foundation (grant number 16-14-10055 to B.I.K.)

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Captions to figures

Fig. 1. The C_p^{ex} of reversible denaturation were modeled using Eq. (1) by values as $\Delta H=200 \text{ kcal}\cdot\text{mol}^{-1}$, $T_{1/2}=335 \text{ K}$, $R=0.0019859 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, and $\Delta C_p=10 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$. C_p^{ex} were corrected using Eq. (2) at scan rate of $2 \text{ K}\cdot\text{min}^{-1}$ and response time of 6 sec. Solid, dashed, dot and thin lines are C_p , C_p^{ex} , base line and second derivative respectively.

Fig. 2. DSC profile (a) and second derivative (b) when $T^*>T_{1/2}$. The theoretical graphs were obtained using Eq. (2) and with values of $\Delta H=200 \text{ kcal}\cdot\text{mol}^{-1}$, $T_{1/2}=335 \text{ K}$, $E_a=100 \text{ kcal}\cdot\text{mol}^{-1}$, $T^*=345 \text{ K}$ and $\Delta H_i=-50 \text{ kcal}\cdot\text{mol}^{-1}$ at scan rates of 0.25 (dot line), 0.5 (dashed line) 1 (dash dot line) 2 (long dashed line) and 4 (continuous line) $\text{K}\cdot\text{min}^{-1}$.

Fig. 3. The effect of activation energy on DSC profile at $E_a=100 \text{ kcal}\cdot\text{mol}^{-1}$ (solid line) and $E_a=200 \text{ kcal}\cdot\text{mol}^{-1}$ (dashed line) at scan rate of $2 \text{ K}\cdot\text{min}^{-1}$. Other parameters are as in Fig. 2.

Fig. 4. The effect of scan rate on DSC profile when $T^*=T_{1/2}=335 \text{ K}$ and $\Delta H=-50 \text{ kcal}\cdot\text{mol}^{-1}$ (a) and on their second derivatives (b). Scan rates are 0.25, 0.5, 1, 2 and $4 \text{ K}\cdot\text{min}^{-1}$ as indicated.

Fig. 5. The thermogram of two-state model (a), dependence of $1/T$ to $\ln[\nu C_p^{\text{ex}}/(Q_t - Q)]$ (b) and second derivative of the DSC curve (c) at parameters of $\Delta H=2053 \text{ kcal}\cdot\text{mol}^{-1}$, $T_{1/2}=330 \text{ K}$, $E_a=407 \text{ kcal}\cdot\text{mol}^{-1}$, $T^*=342.14 \text{ K}$. Scan rates are 0.25 (dot line), 0.5 (dashed line) 1 (dash dot line) 2 (long dashed line) and 4 (continuous line) $\text{K}\cdot\text{min}^{-1}$.

Fig. 6. The second derivative of C_p^{ex} vs. T for *Euphorbia characias* amine oxidase at various scan rates, in $\text{K}\cdot\text{min}^{-1}$, as indicated. Raw data were adopted from Ref. [9].

Fig. 7. The DSC profile (a) and second derivative (b) when $T^*>T_{1/2}$. The theoretical graphs were obtained using Eq. (2) and with values of $\Delta H=200 \text{ kcal}\cdot\text{mol}^{-1}$, $T_{1/2}=335$

K, $E_a=100 \text{ kcal}\cdot\text{mol}^{-1}$, $T^*=345 \text{ K}$ and $\Delta H_i=50 \text{ kcal}\cdot\text{mol}^{-1}$ at scan rates of 0.25, 0.5, 1, 2 and $4 \text{ K}\cdot\text{min}^{-1}$ as indicated.

Fig. 8. The effect of scan rate on DSC profile when $T^*=T_{1/2}=335 \text{ K}$ and $\Delta H=50 \text{ kcal}\cdot\text{mol}^{-1}$ (a) and on their second derivatives (b). Scan rates are 0.25, 0.5, 1, 2 and $4 \text{ K}\cdot\text{min}^{-1}$ as indicated.

Fig. 1.

Fig. 2. a

Fig. 2. b

Fig. 3.

Fig. 4. a

Fig. 4. b

Fig. 5. a

Fig. 5. b

Fig. 5. c

Fig. 6.

Fig. 7. a

Fig. 7. b

Fig. 8. a

Fig. 8. b

Table1. The results of experimental DSC thermogram of amine oxidase fitted to Lumry-Eyring and two-state models

Scan rate (K·min ⁻¹)	Lumry-Eyring model					Two-state Model [‡]		
	$T_{1/2}$ (K)	T^* (K)	ΔH_u (kcal·mol ⁻¹)	ΔH_i (kcal·mol ⁻¹)	E_a (kcal·mol ⁻¹)	T^* (K)	E_a (kcal·mol ⁻¹)	ΔH (kcal·mol ⁻¹)
0.125	302.1	342.1	79.1	471.3	96.3	342.1	96.3	473.5
0.25	302.4	342.1	78.9	490.4	97.3	342.1	97.3	491.6
0.475	302.0	341.0	78.9	520.6	98.2	342.0	98.2	521.3
1	300.9	342.6	78.9	553.8	97.5	342.5	97.5	554.0

[‡] Data were adopted from Ref. [9]