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Differential scanning microcalorimetry study of the thermal denaturation of haemoglobin

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Abstract

A study of thermal denaturation of human haemoglobin A_0 (HbA_0) and methaemoglobin (mHb) was carried out by differential scanning calorimetry. DSC haemoglobin profiles were scan rate dependent and only partly reversible. Thermal unfolding of protein was analysed with the use of both equilibrium thermodynamic and kinetic approaches. The fittings based on the simple equilibrium/dissociation model were good and much more satisfactory than those based on "fully-kinetic" models. However the presence of some kinetic distortion during the unfolding process should be noted due to the scan-rate effect on DSC transitions. The calculated first-order kinetic constant for mHb was higher by two orders than the one for HbA_0 (stabilised form). The average activation energy for HbA0 was found to be 289 ± 28 kJ M^{-1} while for mHb it was about 100 kj M^{-1} lower.

Keywords: Haemoglobin; Methaemoglobin; Protein denaturation; Differential scanning calorimetry

1. Introduction

Haemoglobin (Hb) is haeme-containing protein that share a well-known "globin fold" and reversibly bind molecular oxygen. It belongs to multisubunit proteins since Hb molecule is a tetramer in its functional form. The study of the stability of such proteins has been the subject of great interest over the last decade. Structural stability of haemoglobin and the mechanism of its degradation are not yet fully clear. It has been reported that the melting point and decomposition temperature of powder Hb occurs at about 120 and 210 °C, respectively [1] whereas haemoglobin in water or buffer solutions shows a markedly lower unfolding temperatures ranging from 63 to 67 °C [2–4]. Different aspects of haemoglobin stability are discussed in literature [1–12]. However there are relatively few papers dealing with studies of haemoglobin with the use of differential scanning calorimetry (DSC) measurements. DSC is a helpful technique for characterizing the energetics and the mechanism of temperature-induced conformational changes of biological macromolecules.

The theoretical background for equilibrium thermodynamic analysis of reversible protein unfolding is well established unlike the description of irreversible transitions. However recently various theoretical studies on irreversible denaturation of simple and multimeric proteins have appeared in literature [13–20]. The irreversible process of protein denaturation can be analysed in the Lumry and Eyring models [19] including different situations depending on the rate-limited steps. The simplest model of irreversible protein denaturation is the one-step transformation of native protein to the denatured state. When the transition is more complex and one or more intermediate states can be postulated the Lumry and Eyring model with the fast equilibrating first step and the irreversible second or model involving two consecutive irreversible steps are more appropriate for use. In application to DSC data these models were investigated by Lyubarev et al. [20], Milardi et al. [17]. In the case of oligomeric proteins a change in

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molecularity (e.g., dissociation) during the protein denaturation process should be taken into the consideration.

The purpose of this paper is to investigate the thermal denaturation of stabilized human haemoglobin A_0 (HbA_0) and methaemoglobin (mHb) by differential scanning calorimetry and to analyse the DSC data basing on the chosen equilibrium and kinetic models.

2. Experimental

Human haemoglobin A_0 ferrous (LOT 48H7604) and Human methaemoglobin (LOT 103H9341) were purchased from Sigma and used without further purification. HbA_0 product contained the sucrose and ficoll 400 as non-ionic stabilizers. Degassed aqua pro injection was used as solvent in all experiments.

DSC scans for $0.77 \cdot 10^{-5}$–$7.7 \cdot 10^{-5}$ ML^{-1} haemoglobin solutions (pH = 6.5 ± 0.3) were performed using the VP DSC ultrasensitive microcalorimeter (Microcal Inc., Northampton, MA) with cell volumes 0.5 mL. The protein concentrations were monitored spectrophotometrically at 275 nm. Heat capacity vs. temperature profiles were obtained for scanning rates of 30, 45, 60, 90 °C h^{-1} in the temperature range 20–90 °C at constant pressure of about 1.8 atm over the liquids in the cells.

The calorimetric data were corrected for the instrumental baseline water–water. Next a linear baseline was used to obtain the excess apparent molar heat capacity (C_p^{ex}) related to the denaturation process. DSC curves were analysed with MicroCal Origin Software. The fit of the curves of the theoretical model to the experimental data was achieved by the non-linear Levenberg–Marquardt method.

Statistical analysis of the results was done using ANOVA or Kruskal–Wallis test.

3. Results and discussion

3.1. Overall characteristic of HbA_0 and mHb thermal transitions

In Fig. 1, curves present a typical apparent heat capacity (C_p) profiles (at scanning rate 90 °C h^{-1}) for the thermal denaturation of native HbA_0 and mHb in water solution after subtracted water–water scan and concentration normalization. The observed endothermic transitions are connected with the unfolding of haemoglobin molecules. The methaemoglobin unfolding starts and proceeds in lower temperature than the unfolding of stabilised HbA_0 form. Values of T_m (defined as the temperature at which a local maximum occurs in the excess heat capacity, C_p^{ex}) and HHW (half-high width) at the studied concentrations and scan rates obtained for averaged curves (from 3–5 scans at constant conditions) are shown in Figs. 2 and 3, respectively. The relatively broad peaks visible in Fig. 1 show

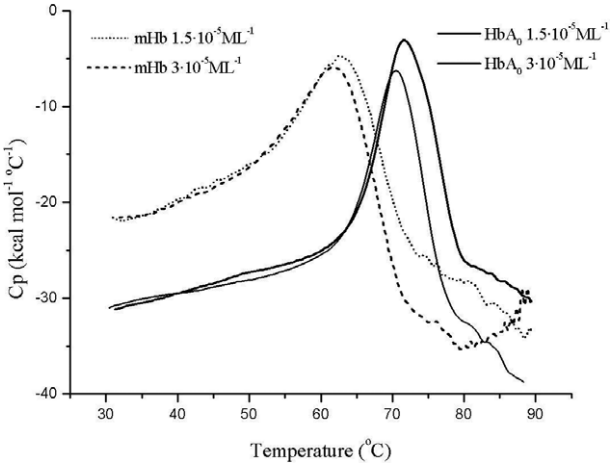

Fig. 1. Representative DSC curves of HbA_0 and mHb (scan rate 90 °C h^{-1}).

maximum at about 71 °C (HbA_0) and 62 °C (mHb) which are close to the T_m value 65.7 °C ± 0.2 °C reported for Hb in phosphate buffer (pH = 6.0) obtained at the same scan rate [2]. The transition seems to be more cooperative in the case of HbA_0 because HHW values are smaller than for mHb. With decreasing protein concentration the endotherm slightly broadens, i.e., HHW increases (Fig. 3). The enthalpy changes ΔH estimated as the area under the peak with SD ≤ 23 kcal M^{-1} are similar for both kinds of Hb (see Table 1).

The shape of DSC profiles and their remarkable asymmetry suggest complex denaturation path. A skew of curves toward the low temperature side of the transition indicates that the unfolding of Hb molecule can be accompanied by the dissociation of this tetrameric protein to form monomers [2].

3.2. Concentration dependence

It is important to check if the calorimetric profiles for haemoglobin are protein concentration-dependent, because the effect of the protein concentration on the position of the transition temperature T_m gives some information about the changes in the molecularity occurring during the thermal denaturation process [14,16,21,22].

One can see from Fig. 2 that T_m increases slightly with increasing HbA_0 concentration. In Fig. 4 a plot of the logarithm of protein concentration versus $1/T_m$ is linear with a negative slope. The negative slope indicates [21,22] that the HbA_0 undergoes dissociation on denaturation.

Fig. 5 shows a set of DSC curves obtained for mHb at various protein concentrations at constant scan rate. T_m is practically independent of protein concentration (the average T_m value for mHb (observed at a scan rate of 60 °C h^{-1}) is 61.8 °C ± 0.4 °C) while the area under the peak (the enthalpy of denaturation ΔH) decreases with increasing protein concentration. Moreover, the peaks are clearly different on their right side. This behaviour suggests that at temperatures above T_m exothermic effects

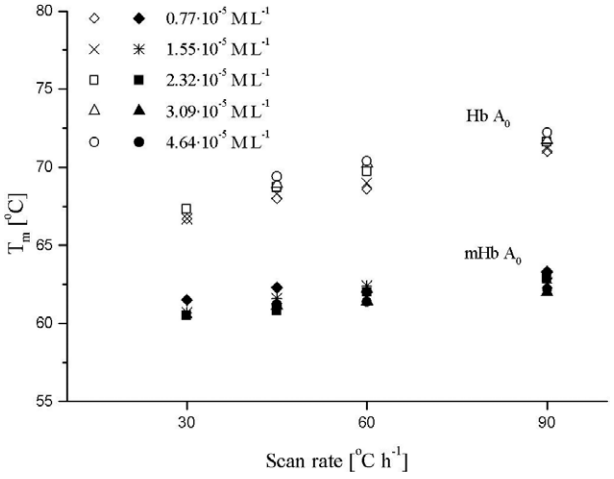

Fig. 2. Effect of scan rate and protein concentration on the T_m of haemoglobin.

depending on protein concentration occur. These effects are probably ascribable to the irreversible aggregation of the unfolded polypeptide chains. The presence of aggregation and precipitation phenomena is well visible in Fig. 5 for the highest mHb concentrations. The constancy of T_m may be a result of two effects (dissociation and aggregation) which cause the shift of T_m in opposite direction.

3.3. Scan rate dependence

It follows from Figs. 2 and 6 that the transition temperature T_m shifts towards higher temperatures with scan rate increasing thus Hb denaturation is scan rate dependent. The shift of T_m due to the scan rate changes from 30 to 90 °C h^{-1} is more marked for HbA_0 (~4.5 °C) than for mHb (~2.0 °C). The downward shift at the end of the transition visible in Fig. 6, probably connected with the exothermic aggregation phenomena, diminishes on the DSC curves when the scan rate increases. However, the denaturation enthalpy practically does not depend on the scanning rate (Table 1).

Table 1
The enthalpy changes ΔH (kcal M^{-1}) obtained for averaged scans (3–5 performed at the given concentration c and scan rate V)

V[K h^{-1}]	$c \cdot 10^5$[M L^{-1}]	0.77	1.55	2.32	3.09	4.64
30	HbA_0	247	223	217		
	mHb	240	268	200		
45	HbA_0	185	187	204	227	230
	mHb	254	235	245	208	178
60	HbA_0	236	250	232	215	245
	mHb	296	266	255	230	211
90	HbA_0	234	230	220	217	235
	mHb	283	280	239	292	273

3.4. Reversibility

The reversibility of Hb denaturation was checked by reheating experiment. If the first heating was carried out to 90 °C, the process was observed as irreversible. However we carried out another experimental variant in which heating was stopped at lower temperatures chosen from the transition region. DSC curves for haemoglobin A_0 and methaemoglobin obtained by successively scanning the protein up to different final temperatures with cooling up to 20 °C (HbA_0) or 25 °C (mHb) between scans are illustrated in Fig. 7. This experiment indicates that the process of Hb denaturation is partly reversible. The rough estimation of the reversibility degree gives value of about 65 ± 5% after heating to 68 C for both forms of haemoglobins. Comparison of the DSC curves in Fig. 7A and B taking into account that T_m is higher for HbA_0 than for mHb

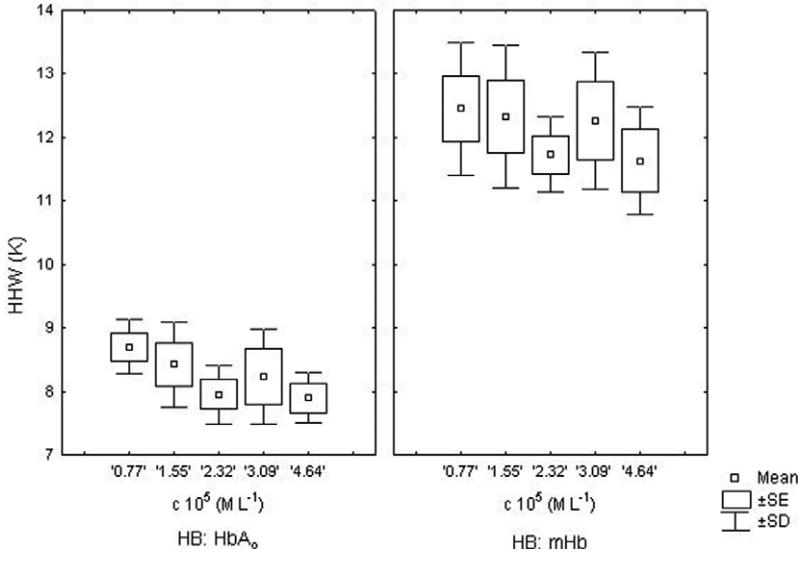

Fig. 3. Half-high width (HHW) versus protein concentration for HbA_0 and mHb.

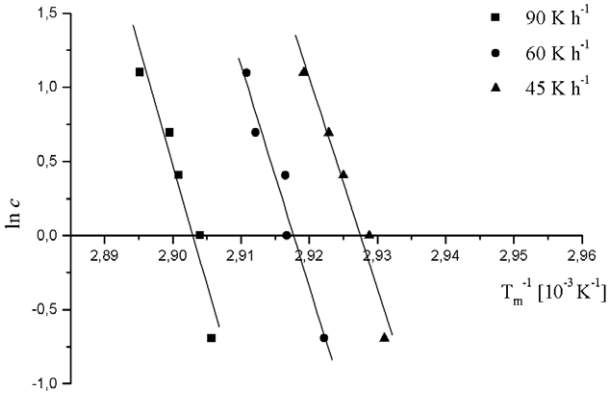

Fig. 4. Dependence of ln c (c-protein concentration in mg ml^{-1}) on $1/T$ for HbA_0.

suggests that reversibility is somewhat higher in the case of mHb than HbA_0.

3.5. Models

The choice of an appropriate model for description of haemoglobin denaturation is not easy because the conclusions of previous sections do not point at such model unambiguously. The partial reversibility, a skew of DSC endothermic peaks, concentration dependence of T_m justify the using of reversible two-state unfolding model with simultaneous dissociation of Hb tetramer into monomers. On the other hand the scan rate dependence and irreversibility (when the absence of an endothermic effect on a second scanning of the sample after heating in the whole temperature range and rapid cooling is taken as a reversibility criterion) of Hb thermal transition suggest that kinetic approach is more adequate.

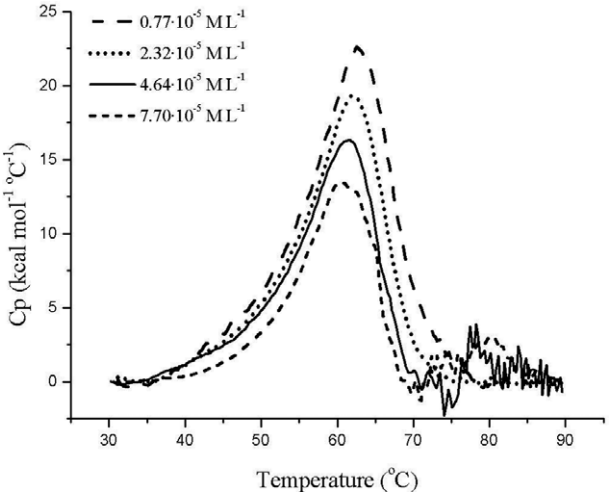

Fig. 5. Concentration dependence of DSC mHb curves (scan rate 60 °C h^{-1}).

3.5.1. Equilibrium model with dissociation

At the beginning the simple one-step dissociation with concomitant denaturation of n-mer (model III, according to [14]) expressed as:

$$N_n \overset{K}{\longleftrightarrow} nU \tag{1}$$

(where N and U are the native and unfolded states of the protein, respectively, and K is an equilibrium constant) was taken into consideration. The fittings of our experimental data were performed in two-state transition model with subunit dissociation and $\Delta C_p \neq 0$ using the software package Origin supplied by MicroCal. Good results were obtained with the value of $n = 2.5 \pm 0.5$ for HbA_0 and $n = 4.2 \pm 0.5$ for mHb (see Fig. 8A) for all studied samples. Only for the lowest protein concentration a slightly higher values: $n = 3.5$ and $n = 5.2$ were found for HbA_0 and mHb, respectively. The better fits with smaller values of χ^2 were received in the case of mHb than HbA_0. Generally, evaluated n values did not depend on the scan rate.

Most theoretical methods developed for the description of protein thermal transitions assume that heat capacity change on denaturation (ΔC_p) is zero. When model (1) with

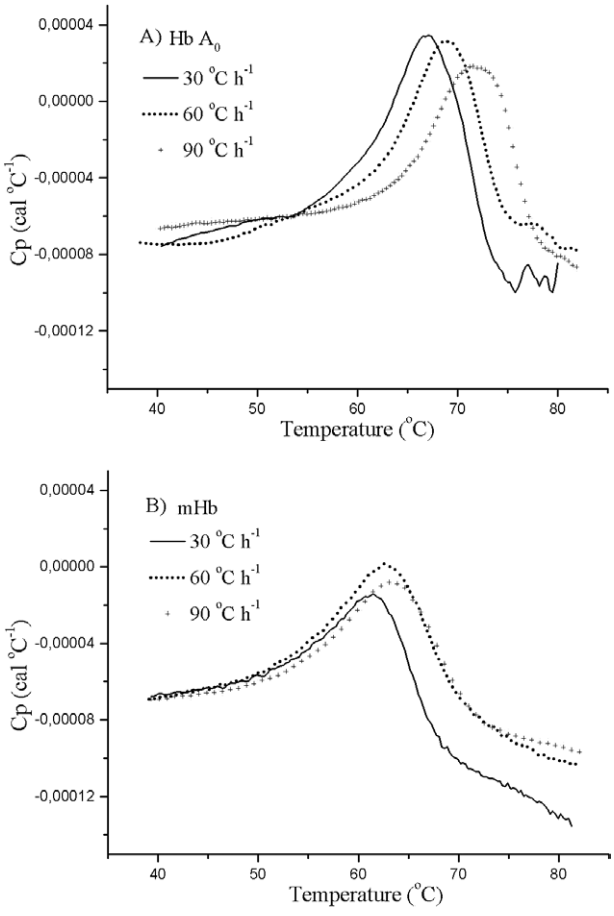

Fig. 6. Scan rate dependence of DSC HbA_0 (A), mHb (B) curves (concentration $7.7 \cdot 10^{-6}$ ML^{-1}).

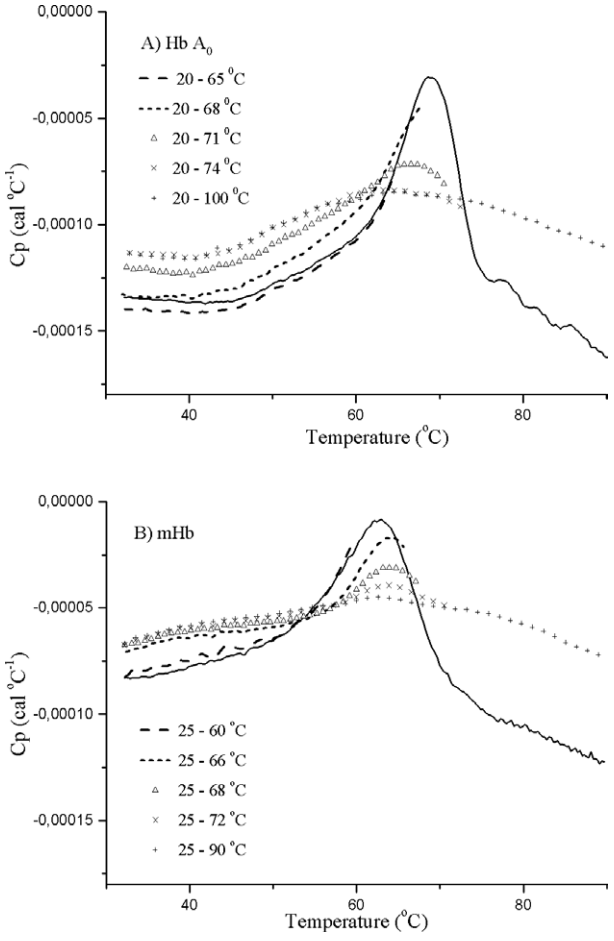

Fig. 7. DSC curves obtained by successive scanning the proteins up to different final temperatures in comparison with individual scanning from 20 to 100 °C and from 25 to 90 °C (solid lines) for: HbA_0 (A) and mHb (B) solutions.

$\Delta C_p = 0$ is considered, the excess heat capacity $C_p^{ex}(T)$ is given by [23,24]:

$$C_p^{ex}(T) = \frac{\Delta H \Delta H^{vH} \theta(1-\theta)}{RT^2[n-\theta(n-1)]} \quad (2)$$

where the degree of advancement of the total process, θ, and the overall enthalpy of denaturation, ΔH, can be evaluated directly from the experimental data: the latter by integration of the total area under the peak, the former by the evaluation of the incremental area divided by the total area. The van't Hoff enthalpy, ΔH^{vH} can be obtained by using the equation:

$$\Delta H^{vH} = \left(\sqrt{n}+1\right)^2 RT_m^2 C_p^{ex}(T_m)/\Delta H \quad (3)$$

where R is the gas constant.

Minimization of the sum of the squared deviations between experimental and calculated C_p^{ex} with using the Microsoft Excel Solver tool let us to find n for which the best fit to the experimental data is achieved. The fits of the heat capacity profiles obtained from Eq. (2) are shown in Fig. 9. The calculated values of n are similar as in the case of $\Delta C_p \neq 0$.

It should be noted that studied form of HbA_0 does not totally dissociate during the denaturation process while mHb tetrameric molecule dissociates into 4 monomers The obtained non-integer value of n ($n \approx 2.5$) for HbA_0 indicates the dissociation of tetramer into dimmers mainly with only small fraction of molecules dissociated into monomers. Although the concentration effect on T_m is more clear in the case of HbA_0, the mHb follows the predictions of equilibrium/dissociation model better (the fits with smaller χ^2).

Generally, the accuracy of fitting is quite good (see Figs. 8,9) thus it seems that the denaturation behaviour of Hb conforms to a equilibrium/dissociation model (1).

3.5.2. Two-state kinetic model

We concluded earlier that the denaturation of HbA_0 and mHb could be treated as kinetically controlled process also. In this case the analysis of DSC data should begin with checking whether experimental data satisfy the one-step model. The criteria of validity of the simplest model of irreversible protein denaturation were discussed by Kurganov et al. [15].

If the one-step model:

$$N \overset{k}{\longleftrightarrow} D \quad (4)$$

(where D a final denatured state and k is a first-order kinetic constant) is valid, the plot $1/T$ versus $\ln[VC_p^{ex}(Q_t - Q)^{-1}]$ (V—scan rate, Q_t—total heat of process, Q—heat evolved at given temperature T) should be linear. Moreover an agreement between the values of kinetic parameters calculated from DSC transitions at different scan rates should be obtained.

Fig. 10 shows that the experimental points describing main endothermic peak can be approximated by a straight line for HbA_0 (A) and for mHb (B). There is a reasonable overlap of the plots for HbA_0 and a little worse in the case of mHb. It follows that in first approximation the denaturation

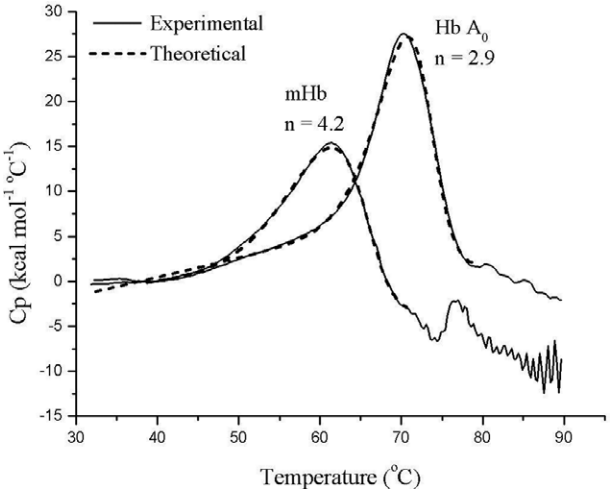

Fig. 8. The results of non-linear least square fittings of the excess molar heat capacity curves to the $N_n \overset{K}{\longleftrightarrow} nU$ model as implemented in the Origin software package (scan rate 60 °C h^{-1}, concentration $3.09 \cdot 10^{-5}$ ML^{-1}).

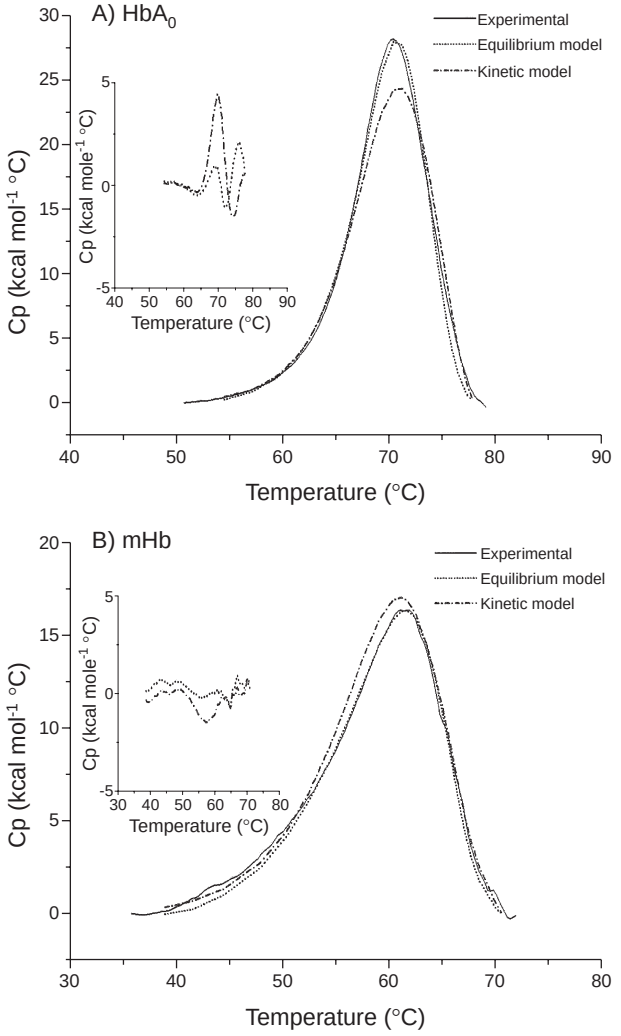

Fig. 9. Comparison of the experimental and theoretical DSC curves obtained in equilibrium model (1) (n=2.2 (A); n=3.9 (B)) and kinetic one-step model (4) (E_a=286 kJ M^{-1}, T*=348.8 K (A); E_a=183 kJ M^{-1}, T*=342.4 K (B)) for HbA_0 (A) and mHb (B) (scan rate 60 °C h^{-1}, concentration $4.64\cdot10^{-5}$ ML^{-1}). Insert: differences between the experimental and theoretical curves.

process of the both samples can be interpreted in terms of a simple first-order one-step kinetic process expressed by Eq. (4). In this model, if the native state N is taken as a reference state, the excess heat capacity $C_p^{ex}(T)$ may be calculated using the equation [15]:

$$C_p^{ex} = \frac{Q_t - Q}{V}\exp\left\{\frac{E_a}{R}\left(\frac{1}{T^*} - \frac{1}{T}\right)\right\} \quad (5)$$

where E_a is the experimental energy of activation, T* is the temperature, at which k=1 min^{-1}. The activation energy of this process can be calculated in several ways proposed by Sanchez-Ruiz et al. [13] on the assumption that heat capacity change on denaturation (ΔC_p) is zero. These methods are described in detail in several places [13,14,25]. We applied four methods based on:

1) the construction of the linear dependence ln k versus $1/T$ where k can be obtained from a DSC profile as

$$k = VC_p^{ex}(Q_t - Q)^{-1} \quad (6)$$

2) the dependence:

$$\ln\left[\ln Q_t(Q_t - Q)^{-1}\right] = \frac{E_a}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) \quad (7)$$

and a plot of $\ln[\ln Q_t(Q_t - Q)^{-1}]$ versus $1/T$.

3) the coordinates of the maximum of the calorimetric peak (T_m, C_p^m) according to:

$$E_a = 2.718RC_p^m T_m^2 Q_t^{-1} \quad (8)$$

4) a set of DSC scans obtained at different scan rates and the dependence

$$\ln\left(\frac{V}{T_m^2}\right) = \ln\left(\frac{AR}{E_a}\right) - \left(\frac{E_a}{R}\right)\left(\frac{1}{T_m}\right) \quad (9)$$

where A is the preexponential factor in the Arrhenius equation.

Excluding the last method, the obtained results do not depend on the calculation way and concentration within the experimental error. The average values for the activation energy (with 95% confidence intervals) based on the three first methods are presented in Fig. 11. Generally the values of E_a are about 100 kJ mol^{-1} higher for HbA_0 than for mHb. There are no statistically essential differences between E_a at various scan rates in the case of HbA_0. However the activation energy of mHb shows a slight tendency to decrease with scan rate increasing and is essentially smaller for 90 °C h^{-1} than for other rates. Our estimation gives the overall average activation energy for HbA_0 equal 289±kJ M^{-1} (69 kcal M^{-1}). This value falls into the range 56–87 kcal M^{-1} determined by Grasso et al. [18] on the base of the procedure of Olsen and optical measurements modified by haemoglobin stabilisers.

The values of T* found in this model are a little below the temperatures in which the endothermic transitions are

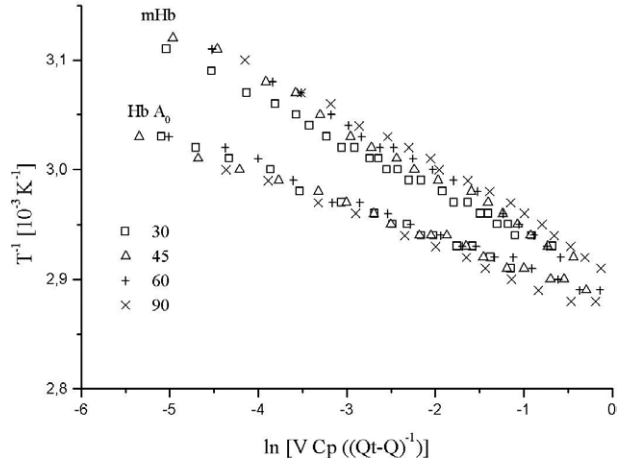

Fig. 10. Dependence of $1/T$ on $\ln[VC_p^{ex}/(Q_t - Q)]$ for HbA_0 and mHb.

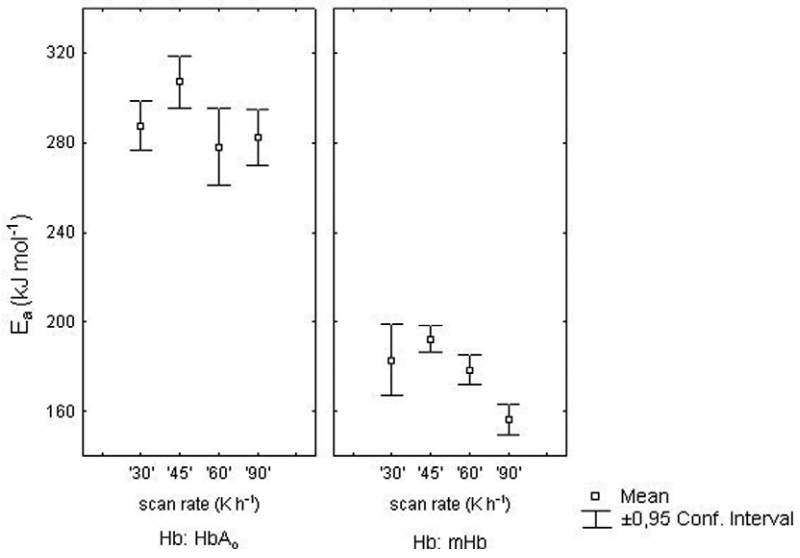

Fig. 11. Effect of scan rate on activation energy of HbA_0 and mHb.

terminated. It means that the kinetic step proceeds at a significant rate only at a temperature above the reversible unfolding. One can see from Fig. 9 that the theoretical C_p^{ex} versus temperature profile calculated with using the Eq. (5) coincides poorly with the experimental curve for both kinds of Hb. The inserts of Fig. 9 show the differential curves between experimental and theoretical results. The fit for HbA_0 is quantitatively good at temperatures below 66 and above 73 °C but the divergences are markedly visible near the point of maximum. In the case of mHb the low-temperature (<63 °C) region of the calorimetric profile is not exactly fitted by the two-state irreversible model. The opposite direction of differences for HbA_0 and mHb should be noted.

The simple kinetic model (4) gives the possibility of estimation of the first-order rate constant k. At 25 °C temperature value of k for mHb ($\sim 10^{-5}$ min^{-1}) is two orders higher than for stabilised form of HbA_0. This value is not influenced by scan rate in the case of HbA_0 while it is higher at the scan rate 90 °C h^{-1} than at slower scan rates for methaemoglobin.

Comparison of the fits to the models (1) and (4) presented in Fig. 9 reveals a more reliable description of the thermal denaturation of both kinds of haemoglobin in the equilibrium model with dissociation than in the analysed kinetic model. Moreover the lack of agreement between all four methods of E_a estimation point out that the one-step mechanism of irreversible Hb denaturation is not fully valid.

Taking into account the criteria of validity, the kinetic model $N \rightarrow D$ with first-order kinetic should be more appropriate for HbA_0 than for mHb. However the quality of fitting is similar for both forms of Hb. It should be noted that even if the experimental data are satisfactorily described by this one-step model, the real mechanism of denaturation can be more complex as was remarked by Lyubarev et al. [26].

3.5.3. The Lumry–Eyring model with pre-equilibrium step involving dissociation

In the next approach we considered the model in which an equilibrium and kinetic irreversible steps are connected. The Lumry–Eyring model with a fast equilibrating first step and the irreversible second (model IV according to [14]), can be expressed as:

$$N_n \overset{K}{\longleftrightarrow} nU \overset{k}{\longleftrightarrow} D \qquad (10)$$

In this model the n-meric native protein N_n undergoes a two-state reversible unfolding with simultaneous dissociation into n monomers and next the unfolded species U undergoes an irreversible alteration to yield a final state D.

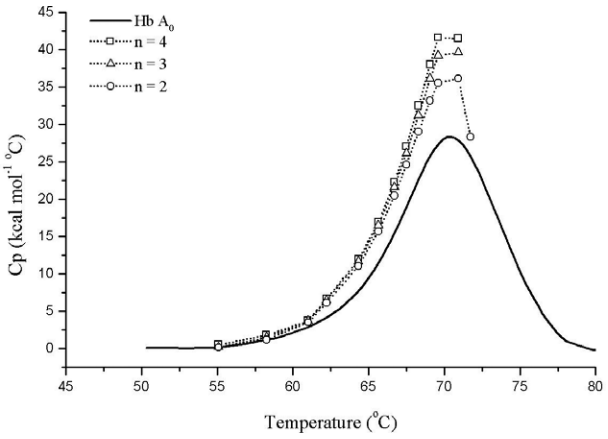

Fig. 12. Comparison of the experimental and theoretical DSC curves calculated by using the Eq. (11) for HbA_0 (scan rate 60 °C h^{-1}, concentration $4.64 \cdot 10^{-5}$ ML^{-1}).

At the assumptions that: 1) the equilibrium between the native state and the unfolded monomeric states, U, is always rapidly established and that the amount of U is low but continuously renewed, 2) all the dissociation and unfolding processes are concomitant and 3) the irreversible step is determined by a first-order rate constant k, the shape of the transitions is given by:

$$C_p^{ex} = \frac{\Delta H E_{app}}{RT_m^2} \exp\left(\frac{E_{app}\Delta T}{RT_m^2}\right)\left[1+\frac{1-n}{n}\exp\left(\frac{E_{app}\Delta T}{RT_m^2}\right)\right]^{\frac{1}{n-1}} \quad (11)$$

where $E_{app}=E_a+\Delta H/n$, E_a is the activation energy of the irreversible step ($U \rightarrow D$), ΔH is the unfolding enthalpy and $\Delta T=T-T_m$. This equation is valid only for the temperatures $T \leq T'$, where $T' = T_m + \frac{RT_m^2}{E_{app}} \ln\frac{n}{n-1}$ what was discussed in [14].

This model with n equal 2, 3 and 4 was used to our experimental data. The results of calculations basing on Eq. (11) (taking only $T \leq T'$) are presented in Fig. 12 for HbA_0. One can see that the application of this model does not give satisfactory results for any n. The value $n=2$ gives the smallest discrepancies between theoretical and experimental curves for both Hb forms. It is in agreement with dissociation/equilibrium model (1) for HbA_0 unlike to mHb.

Generally the last model gives the poorest description of Hb thermal denaturation. It seems that the assumption of low U fraction is wrong. A significant amount of unfolded state in equilibrium with the native one during the temperature-induced Hb denaturation may be expected. In this case the use of the Lumry and Eyring model (10) is difficult because an analytical solution of the corresponding system of differential equations does not exist. In an extreme case when the kinetically controlled process ($U \rightarrow D$) proceeds at a significant rate only at a temperature above the reversible unfolding ($T^* \gg T_{1/2}$, where $T_{1/2}$ is the temperature at which the equilibrium constant $K=1$) an overall irreversible process $N \leftrightarrow U \rightarrow D$ yields results similar to those for the reversible process alone ($N \leftrightarrow U$). Indeed, this case can be probable for Hb because the fits of our experimental data are the best in the equilibrium/dissociation model. It is known that no equilibrium model can explain a scan-rate effect on DSC transitions. Presumably some kinetic distortion occurs within the unfolding pathway, although the unfolded or partially-unfolded states in equilibrium with the native state does not become negligible during Hb thermal denaturation transition.

4. Conclusions

The DSC studies of thermal denaturation process of two forms of haemoglobin: mHb and stabilised HbA_0 show some differences between their unfolding characteristics. In the case of HbA_0 the denaturation proceeds in higher temperatures with a bigger activation energy and the transition is more cooperative than for mHb. However the enthalpies of denaturation are similar. In both cases the heat capacity versus temperature profiles show some features (as scan rate dependence) characteristic for kinetically controlled processes, while others (as partial reversibility, concentration effects, the shape of DSC curves) admit of the equilibrium approach including protein dissociation. For both forms of haemoglobin the best fittings of DSC experimental results are found with simple equilibrium/dissociation model when n is close to 2 and 4 for HbA_0 and mHb, respectively.

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