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Thermochimica Acta 409 (2004) 137–144
thermochimica acta
www.elsevier.com/locate/tca

Differential scanning calorimetric study of the molten globule state of cytochrome *c* induced by sodium *n*-dodecyl sulfate

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Received 19 September 2002; received in revised form 15 May 2003; accepted 17 June 2003

Abstract

The molten globule (MG) state, a compact denatured state with a significantly native-like secondary structure but a largely flexible and disordered tertiary structure, has been proposed to be a major intermediate of protein folding. To explore another approach for characterizing the MG state, sodium dodecyl sulfate (SDS) induced formation of the MG state of horse cytochrome *c* at pH 2 was studied by circular dichroism, visible spectroscopy, isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). These techniques confirmed that the addition of SDS to acid-unfolded state of cytochrome *c* induced the MG state. Although, the DSC thermal denaturation of cytochrome *c* was always calorimetrically irreversible, the MG state induced by SDS at low concentrations showed a reversible profile. The spectroscopic properties demonstrated that the hydrophobic tail of SDS utilized the hydrophobic contribution to stabilizing the heme conformation at MG state in cytochrome *c*. This would be the main reason of thermal profile reversibility of MG state in cytochrome *c*. The reversibility of DSC thermogram would allow its deconvolution and analysis of the energetic domains for this protein.

Keywords: Cytochrome *c*; Molten globule; SDS; Reversibility; Differential scanning calorimetry; Deconvolution

1. Introduction

For a long time, it appeared that the folding of small single domain proteins could be approximated to a two-state cooperative transition between an ensemble of unfolded state, and the folded state. However, it soon became apparent that intermediate states exist in many instances, and since their properties were common to many proteins, the general term molten globule (MG) was coined. These properties include a high degree of compactness, stable secondary structure to varying levels and the lack of stable tertiary interactions [1–4]. It has been argued that folding intermediates investigated so far are either partly folded proteins [5], i.e. they contain several domains and one domain folds to the fully folded state before another (as a sequence of equivalent thermodynamic steps), or they form misfolded structures which can be considered to be "off-pathway" species [6,7]. A large part of the folding literature has recently been dedicated to defining the nature of folding intermediates in an attempt to resolve these arguments.

Cytochrome *c* is probably one of the most heavily investigated proteins in terms of its folding pathway, in that both experimental and theoretical data are widely available. It is unfolded substantially under conditions of low salts at pH 2; the addition of anion from either a salt or an acid stabilizes the molten globule state [8–11].

The acidic intermediate state of cytochrome *c* shows cooperative thermal unfolding, which is not observed for the molten globule of other proteins [12,13]. Calorimetric and two-dimensional nuclear magnetic resonance data suggest that, although the intermediate state of cytochrome *c* is a denatured state separated thermodynamically by a phase transition from the native state, it is closer to the native state than the cases for other molten globules. In addition, confusion has arisen from use of the term "MG" for various

Abbreviations: DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; CD, circular dichroism; ΔH_{cal}, enthalpy change of calorimetry; [θ], molar ellipticity; MG, molten globule state; SDS, sodium *n*-dodecyl sulfate

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doi:10.1016/S0040-6031(03)00358-7

intermediate states with different extents of structure [14]. However, because the anion dependent stability of the intermediate state of various proteins, including cytochrome *c*, is very similar [9,10] suggesting a common mechanism of conformational stability, we use the term "MG" for the acidic intermediate of cytochrome *c* as originally proposed. Although, in a strict sense it dose not satisfy the original definition [8,13].

A series of studies carried out by Goto and Fink, suggested the importance of charge repulsion in the stability of acidic and salt molten globules [15,16]. They examined the salt and acid induced formation of molten globules and proposed that the conformation of molten globule is determined by a balance of charge repulsions between positive groups. The molten globule state induced by salts and acids have similar conformation [15]. In recent years, differential scanning calorimetry (DSC) has established itself as the prime technique for the study of the thermal stability of proteins, especially since the availability of ultrasensitive microcalorimeter and convenient deconvolution algorithms. Most reversibly unfolding protein systems obey the two-state van't Hoff model and hence can be described completely in terms of the thermodynamic properties of the folded and unfolded forms [17,18]. A knowledge of the heat capacity value of the protein as a function of temperature permits the calculation of its molecular partition function and through the appropriate algorithm, the deconvolution of the unfolding thermal profile into two-state process [19–21]. In the case of irreversibly denaturing proteins, the process is often kinetically controlled, restricting any formal thermodynamic analysis. The overall irreversibility of a denaturation process can be established by the reduction in size of the endothermic peak in a rescan of a rapidly frozen sample.

It is known that under most conditions heat denaturation of most proteins is partially reversible or fully irreversible. The usual conclusion drawn from such experiments is that heat causes irreversible changes in the protein structure. It is believed that processes leading to irreversibility involve aggregation, defective disulfide bond, or covalent alterations [22,23].

The folding–unfolding pathway in reversible [24] as well as irreversible denaturation [25] have been shown to include folding intermediates frequently referred to as molten globule. Some studies indicate that these intermediates have a compact structured form. Therefore, these molten globule species play an important role in the thermal denaturation process.

Here differential scanning calorimetric profile of horse cytochrome *c* was carried out, which showed to be irreversible and highly scan-rate dependent. In this paper, we introduce a simple method to convert the irreversibility of DSC thermogram of cytochrome *c* to a reversible profile by inducing the MG state by sodium *n*-dodecyl sulfate (SDS). This allows deconvolution of thermogram and obtaining additional structural information via analyzing the energetic subdomains of cytochrome *c*.

2. Materials and methods

2.1. Materials

Horse cytochrome *c* (type IV), in the oxidized form, was purchased from Sigma. The protein solution was dialyzed against buffers (20 mM HCl in pH 2 and 25 mM phosphate buffer in pH 7). The extinction coefficients were used to calculate the concentration of the native at pH 7 and the denatured at pH 2 for cytochrome *c*. The heme group linked to the two-cystein residues by thioether bonds was removed by reaction with silver sulfate [26]. Apocytochrome *c* was passed through a column of Sephadex G-25 equilibrated with 5 mM sodium acetate buffer at pH 4 containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) and stored at −20 °C. Sodium dodecyl sulfate and heme were purchased from Sigma. Other chemicals were of reagent grade. The concentrations of sodium dodecyl sulfate utilized in all experiments were under the critical micelle concentrations (cmc). The concentration of cytochrome *c* was 5 μM in all experiments. The molecular weight for cytochrome *c* is 12,384 Da.

2.2. Methods

2.2.1. Differential scanning calorimetry (DSC) measurements

DSC experiments were performed on a Scal differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) with cell volumes of 0.48 ml at a scanning rate of 1 K/min (was kept constant in all experiments), interfaced with a personal computer (IBM compatible). Prior to DSC experiments, the protein solutions were dialyzed for 24 h at 4 °C against three changes of a large volume of 20 mM HCl at pH 2, 0.08 mM SDS and also 25 mM phosphate buffer at pH 7. Before the measurements, samples were degassed by stirring in an evacuated chamber at room temperature and then immediately loaded into the calorimeter cell; the final dialysis buffer (also degassed) was loaded into the reference cell. A pressure of 152 kPa (1.5 atm) of dry nitrogen was always kept over the liquids in the cells through out the scans to prevent any degassing during heating. The reversibility of the thermal transitions was checked by a second heating of the cool sample immediately following the first scan. The calorimetric traces were corrected for the instrumental background by subtracting a scan with buffer in both cells. The relative errors of the values of molar enthalpy changes are in the range of 3% and the absolute errors of given transition temperatures T_m are 0.3 °C. The deconvolution analysis and fitting were done based on Privalov and Potekhin theory [21] which was installed as DOS program in software package (named Scal-2) and supplied by Scal (Russia). Scal-2 program, which is installed in DSC instrument, enables to determine the native and denatured lines based on fitting error. The best fitting error is selected as a best

deconvolution. The baseline preparation was done by HCl (20 mM) including 0.08 mM SDS in both sample and reference cells. All experiments were performed at a protein concentration of 1.0 mg/ml. In the calculations of molar quantities the molecular weight used for the protein was 12,384 Da.

2.2.2. Circular dichroism measurements

All measurements in this work were carried out at 20 °C with thermostatically controlled cell holders. CD spectra were measured with a JASCO J-715 spectropolarimeter (Japan) equipped with an interface and a personal computer. The instruments were calibrated with ammonium D-10-camphorsulfonic acid [27]. The results were expressed as the mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100 \times \theta_{obsd}/(lc)$, where θ_{obsd} is the observed ellipticity in degrees, c is the concentration in residue mol/l, and l is the length of the light path in cm. The helical content of the MG state of cytochrome *c* induced by 0.08 mM SDS was calculated by the method of Chen et al. [28]. The CD spectra were measured at protein concentrations of 5 μM with a 1 mm path length cell from 250 to 190 nm.

2.2.3. Absorption measurements

Visible spectra of cytochrome *c*, apocytochrome *c* and heme were measured with a spectrophotometer, Model Shimatzu-3100, at protein concentrations of 5 μM with 1 cm path length cells. The protein concentration was determined spectrophotometrically. Extinction coefficients were used to calculate the concentration of the native form. The ferricytochrome *c* extinction coefficient was $1.06 \times 10^5\ M^{-1}\ cm^{-1}$ at 410 nm at pH 7 [29]. The pH was measured using a Beckman Φ 50 pH-meter at 20 °C.

2.2.4. Isothermal titration calorimetry (ITC) measurements

Stepwise titration calorimetry was carried out with a four-channel commercial microcalorimetric system (Thermal Activity Monitor 2277, Thermometric, Sweden). Each channel is a twin heat-conduction calorimeter where the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between the vessel holder and the surrounding heat sink. The insertion vessel was made from stainless steel. Sodium dodecyl sulfate solution was injected by using of a Hamilton syringe into the calorimetric stirred titration vessel, which contained 1.8 cm^3 cytochrome *c* solution including HCl (20 mM), pH 2. Thin (0.15 mm inner diameter) stainless steel hypodermic needles, permanently fixed to the syringe, reached directly into the calorimeter vessel. The injection of sodium dodecyl sulfate into the perfusion vessel was repeated 20 times, and each injection included 30 μM of reagent. The calorimetric signal was measured with a digital voltmeter, part of a computerized recording system. The enthalpy change for each injection was calculated by a "Digitam" computer program (designed by Thermometric). The enthalpy of dilution and demicellization for sodium dodecyl sulfate micelle was subtracted from the enthalpy of cytochrome *c* and surfactant interaction. The enthalpy of dilution of cytochrome *c* is also negligible. The microcalorimeter was frequently calibrated electrically during the course of the study.

3. Results and discussion

3.1. Circular dichroism (CD) spectra

Fig. 1 shows the far-UV CD spectra of the acid-unfolded state of cytochrome *c* in the presence and the absence of SDS. The dashed curve and curve 1 depict the native form at pH 7 and the unfolded state of cytochrome *c* at pH 2 in the absence of SDS, respectively. The other curves show the addition of SDS at low concentration to unfolded state at pH 2, which induced substantial alpha-helical structure as indicated by lower minima (lower $[\theta]_{208}$, $[\theta]_{222}$) at 208 and 222 nm relative to native spectrum. The value of ellipticity at $[\theta]_{222}$ for both the native and the complexes with 0.08 mM SDS is very similar; but the value of $[\theta]_{208}$ is different because of the addition of SDS. The negative increment of $[\theta]_{208}$ indicates the presence of alpha-helix protein conformation, which is kept in a fixed position for molten globule at a low concentration of SDS. Based on the ellipticity values at 222 nm and the method of Chen et al. [28], the helical content of the MG-like state of cytochrome *c* induced by 0.08 mM SDS is 33.57%, the helical contents of the unfolded state and the native state of cytochrome *c* are 4 and 30%, respectively.

3.2. Visible absorption spectra

The heme absorption of cytochrome *c* in the vicinity of 400 nm reflects the spin state of the iron, which is dependent on the conformational state of the protein. Whereas the native protein (low spin state) shows a maximum at 410 nm with an extinction coefficient of $1.06 \times 10^5\ M^{-1}\ cm^{-1}$; while the unfolded state (high spin state) indicates a maximum at 394 nm with an extinction coefficient of $1.9 \times 10^5\ M^{-1}\ cm^{-1}$ (see Fig. 1B). The absorption coefficient of SDS is negligible at 400 nm. Fig. 1B shows the changes from the unfolded state to the MG state as the result of the addition of corresponding concentration of SDS in Fig. 1. It is apparent that the right shift of wavelength is accompanied with intensity decreases; which indicates the presence of MG state for the protein.

Fig. 2 shows the visible spectra of heme in the presence and absence of apocytochrome *c* and SDS. The presence of apocytochrome *c* induced the right shift for heme interaction and the presence of SDS showed the hypochromicity for the visible peaks. Fig. 2 (inset) shows the effect of SDS on the heme solution, which induced the right shift for heme peak related to the absence of SDS.

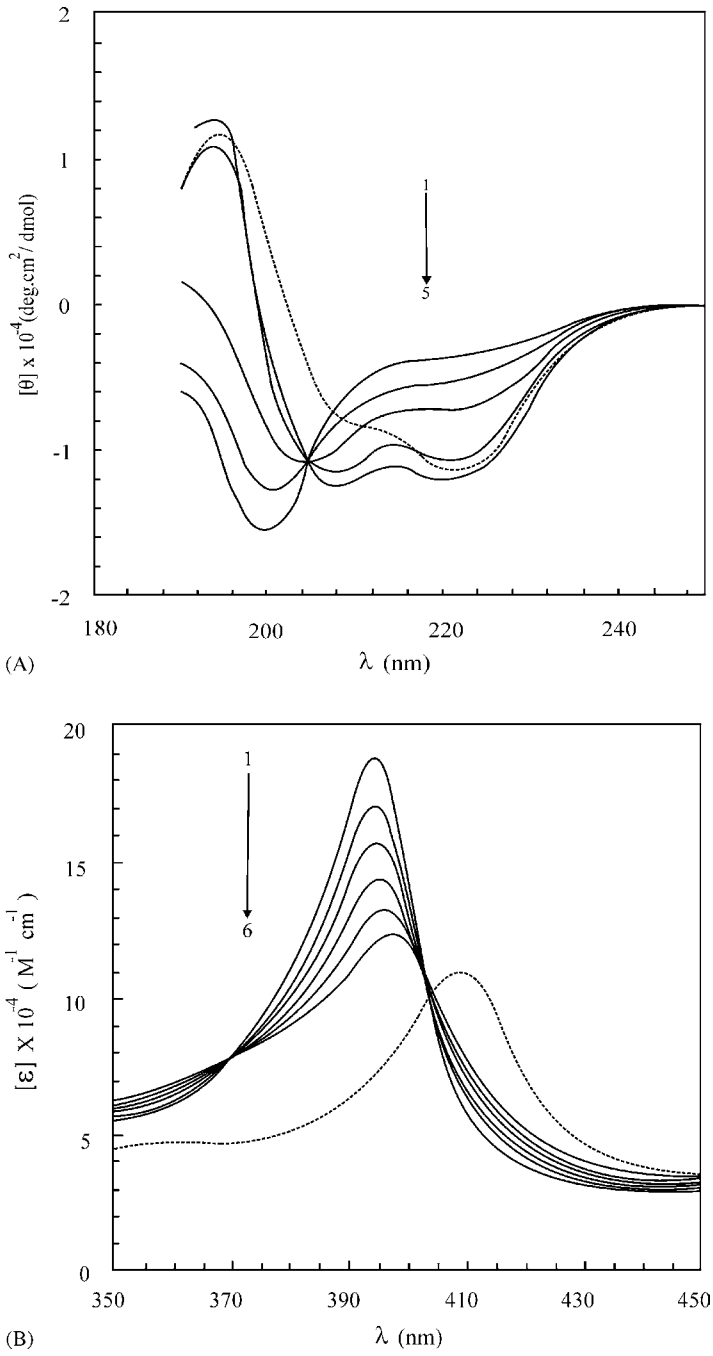

Fig. 1. (A) Far-UV CD spectra of cytochrome *c* as a function of SDS concentration at pH 2 and 20 °C. (**1**) 0 mM SDS (denatured state); (**2**) 0.01 mM SDS; (**3**) 0.037 mM SDS; (**4**) 0.06 mM SDS; (**5**) 0.08 mM SDS. (B) Soret absorption spectra of cytochrome *c* as a function of SDS concentrations at pH 2 and 20 °C. (**1**) 0 mM SDS (denatured state); (**2**) 0.02 mM SDS; (**3**) 0.04 mM SDS; (**4**) 0.06 mM SDS; (**5**) 0.07 mM SDS; (**6**) 0.08 mM SDS. Dashed line shows the native state in 25 mM phosphate buffer at pH 7. Protein concentration was 5 μM.

3.3. Isothermal titration calorimetry

Table 1 shows the enthalpy change of calorimetry (ΔH_{cal}) versus concentrations of SDS that is affected the acid-unfolded state of cytochrome *c*. These results indicate that the lowest negative values of ΔH_{cal} belong to SDS (0.08 mM) that is consistent with lowest value of molar ellipticity at 222 nm ($[\theta]_{222}$) and 208 nm ($[\theta]_{208}$) (see Fig. 1 and Table 1).

3.4. Differential scanning calorimetry

Fig. 3A shows the DSC profiles for MG-like state of cytochrome *c* induced by SDS, native and acid-unfolded forms.

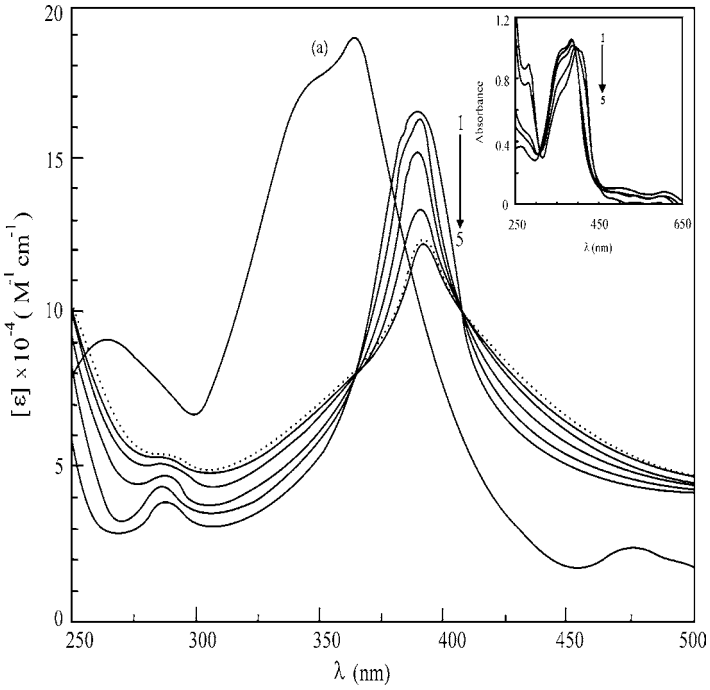

Fig. 2. Absorption spectra of heme (a); (**1**) apocytochrome *c* interacted with heme (heme–apocytochrome *c* complex); (**2**) SDS (0.02 mM) interacted with heme–apocytochrome *c* complex; (**3**) SDS (0.04 mM); (**4**) SDS (0.06 mM); (**5**) SDS (0.08 mM). Dotted curve shows the MG state that was induced with addition of 0.08 mM SDS to holocytochrome *c* at pH 2. Inset: absorption spectra of heme as a function of SDS concentrations at pH 2 and 20 °C. (**1**) SDS (0 mM); (**2**) SDS (0.02 mM); (**3**) SDS (0.04 mM); (**4**) SDS (0.06 mM); (**5**) SDS (0.08 mM).

The native state of cytochrome *c* showed a sharp heat absorption peak representation of cooperative melting of the native structure. DSC profile of the MG-like state of cytochrome *c* exhibited a small and broad curve with a 221 ± 3 kJ/mol calorimetric enthalpy change value. The transition points (T_m) of native and molten globule states were 60 and 44 °C, respectively (see Fig. 3A). Here we have used low concentration of protein solution (5 μM) because cytochrome *c* has a tendency to form soluble aggregates upon addition of SDS at pH 2. The DSC scan was repeated twice, keeping the same sample solution in the cell. As can be seen from the superimposition of the DSC curves for MG state, the unfolding transition was reversible for molten globule state inducing by SDS, but it was irreversible for native state of cytochrome *c*. Due to reversibility of MG state of cytochrome *c* induced by SDS, it is possible to deconvolute the profile and obtain the energetic domains for cytochrome *c* which is shown in Fig. 3B and Table 2.

We have previously reported that the anionic surfactants are the most commonly used denaturants for proteins at high concentrations. They are also responsible for the folded state for some proteins at low concentrations [30]. The structure and stabilizing mechanism of molten globule states of proteins have been widely investigated. It has been demonstrated that the main driving force of the salt induced MG state is due to the reduction of the electrostatic repulsion between the charged groups of protein molecule [10,31,32]. Horse cytochrome *c* is substantially unfolded under conditions of low salt at pH 2 by charge repulsion between the positive groups. Added anions bind to the positive groups, shielding the charge repulsion, and this results in the manifestation of the intrinsic forces stabilizing the MG state [9]. In this manuscript we provide evidence for the MG state of

Table 1
The enthalpy change of calorimetry (ΔH_{cal}) and molar ellipticity at 222 nm ($[\theta]_{222}$) of acid-unfolded and different structure states at various concentrations of SDS for cytochrome *c*

Protein states	ΔH_{cal} (kJ/mol)	$[\theta]_{222}$ (° deg cm^2/dmol)
Acid-unfolded	–	−3200
SDS (0.02 mM)	−2.5	−5470
SDS (0.05 mM)	−6.285	−10500
SDS (0.08 mM)[a]	−11.7	−11700

[a] MG state induced by SDS that is obtained from acid-unfolded state of cytochrome *c*.

Table 2
The transition point (T_m) and the enthalpy change of calorimetric (ΔH) values of energetic subdomains for MG state of cytochrome *c* induced by SDS

Energetic subdomains[a]	T_m (°C)	ΔH (kJ/mol)
I	27.2	36.4
II	37.1	49.5
III	46.4	65.1
IV	55	73

[a] See Fig. 3B.

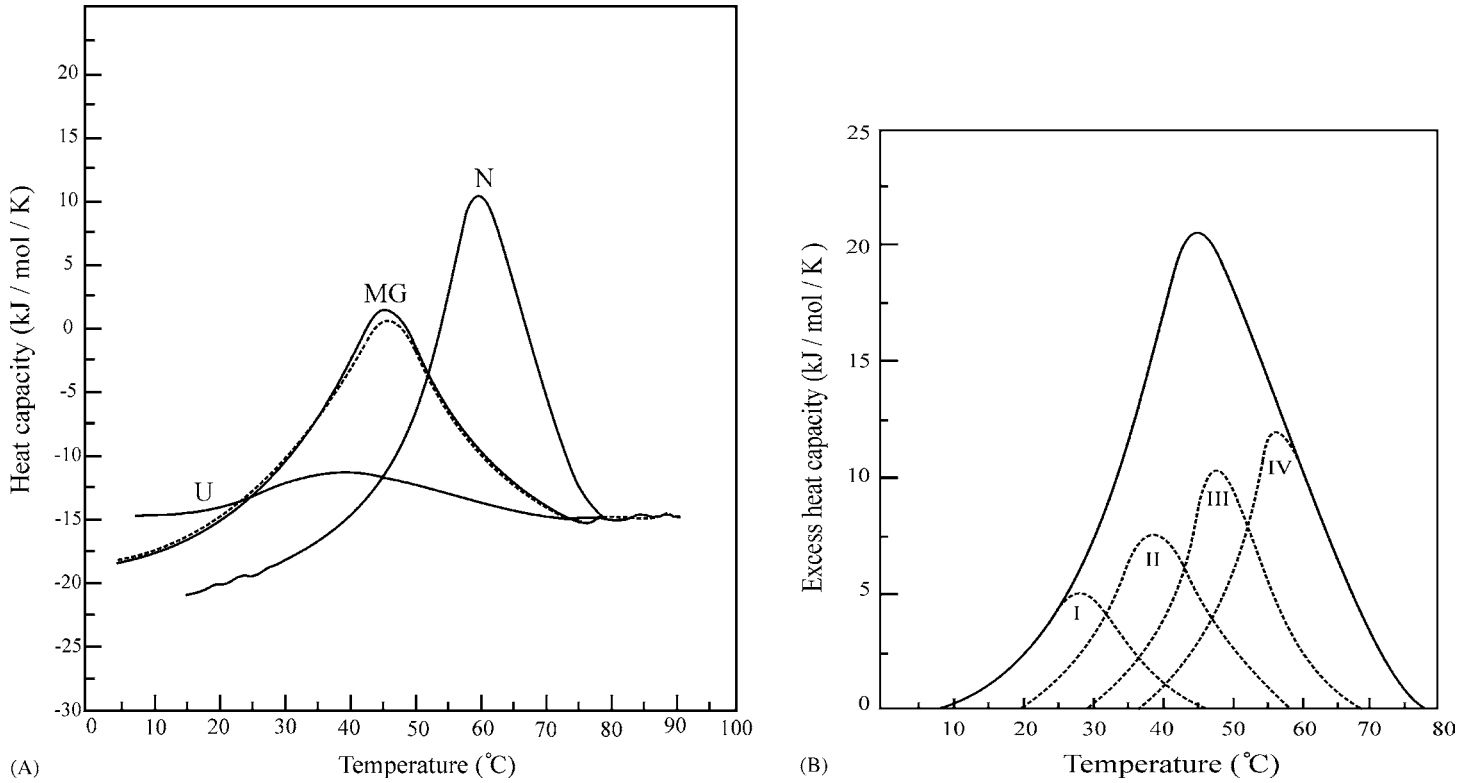

Fig. 3. (A) DSC thermograms for the various conformational states of cytochrome *c*. U, acid-unfolded state of cytochrome c at pH 2; MG, molten globule state of cytochrome *c* induced by SDS at pH 2; N, native state of cytochrome *c* at pH 7. Dashed curve shows the repeated scan of MG state that is keeping the same sample solution of MG state in the cell. (B) Deconvolution of thermograms of MG state of cytochrome *c* induced by SDS. Solid line represents the experimented curve after subtraction of chemical baseline, the dashed curves obtained from fitting the data for the multi-state model and represent the elementary transitions as energetic subdomains.

cytochrome *c* induced by SDS, as a salt with hydrophobic chain (hydrophobic salt). We also demonstrate a reversible DSC thermogram from an irreversible case by inducing a native-like structure of cytochrome *c*.

The spectroscopic properties of the MG state strongly support the view that SDS stabilizes the MG state of cytochrome *c*. The circular dichroism (CD) spectra of the MG relative to acid-unfolded state strongly support the view that SDS confirms the MG state of cytochrome *c*. The MG state shows a native-like amount of alpha-helix conformation. The spectroscopic properties of the MG state closely resemble those reported by Goto et al. [10], thus implying a close structural similarity.

Isothermal titration calorimetry (ITC) provides a reliable calorimetric enthalpy change for the formation of MG states of cytochrome *c* induced by titration of the acid-unfolded state with SDS. SDS was selected because of its dual interactions as electrostatic and hydrophobic. It is an amphipatic material and has a polar head and a nonpolar tail group. The calorimetric enthalpy change values (ΔH_{cal}) indicated that the deepest minima (highest negative values of ΔH_{cal}) belong to SDS at 0.08 mM concentration. These values were correlated with the spectroscopic properties of the MG state of cytochrome *c* induced by SDS. Therefore, the ΔH_{cal} values show the deepest minima belong to MG state of cytochrome *c*. This means that ITC has the ability to detect the MG state of cytochrome *c* via the highest exothermic value for ΔH_{cal}.

The thermal denaturation of heme proteins including cytochrome *c*, myoglobin and horseradish peroxidase at pH 2 give rise to a well defined DSC transition, whose apparent T_m (temperature at the maximum of the heat capacity profile) depended on the scan rate [33–35]. The thermal denaturation of cytochrome *c* was always calorimetrically irreversible. That is in a second heating of the protein solution no thermal effect was observed. The effect of the scan rate on the calorimetric profiles clearly indicated that they corresponded to irreversible, kinetically controlled transitions. It is thus evident that equilibrium thermodynamics cannot be applied in their analysis [36]. The DSC curve for MG state induced by SDS showed the reversible thermogram. This is advantageous for MG state allowing deconvolution of thermogram for cytochrome *c* and determination of the T_m and enthalpy of each energetic subdomains (Table 2). The four energetic subdomains emphasize the multi-state denaturation for unfolding of cytochrome *c*. The ratio of ΔH_{vH} (261 ± 1 kJ/mol) to ΔH (221 ± 3 kJ/mol) for corresponding state also indicates the multi-state denaturation. The deconvolution of MG state of cytochrome *c* shows the four domains that are markedly consistent substantially with the results of Fisher and Taniuchi [37]. Their results had allowed assigning four core domains of the cytochrome *c*. A core domain is a structural region containing a hydrophobic core and the surrounding shell, which folds and unfolds as a unit. Core domain 1 folds by itself and consists essentially of the right channel structure and a part of the heme. Core domains 2, 3 and 4, respectively, are assigned based on the cores located on the left (the Fe–S bond), on the right, and at the bottom of heme [37].

The native state of cytochrome *c* is more stable than the MG state induced by SDS (see T_m's in Fig. 3A), whereas DSC thermogram of the native state is irreversible and DSC profile of the MG state is reversible. Spectroscopic properties can help us to comment on this matter. Apocytochrome *c*, lacking the heme has been considered to be unfolded completely under aqueous conditions [38]. Thermal unfolding of MG state induced by perchloric acid showed an irreversible DSC profile [39]. DSC thermograms a native-like state of apocytochrome *c* in the presence of various concentrations of SDS was also irreversible (data not shown). Therefore, heme plays an important role in the reversibility of the thermal unfolding of heme proteins.

The heme is a prosthetic group essential for stabilizing the native conformation [40]. In the native state of cytochrome *c*, the N-terminal and C-terminal alpha helices, along with one edge of the heme group, form a central hydrophobic core. Nonpolar side chains provided from these in addition to the heme group, constitute a major part of the hydrophobic core of cytochrome *c*. Jeng et al. indicated that the helices and the hydrophobic core are also preserved in the acidic of MG state. This suggests that the native-like helix-to-heme contacts are critical for stabilization of the MG state [11]. Thus the helices and hydrophobic core can be formed even in the absence of heme even though the structure is much less stable than that of the holoprotein because of the lack of the contributions from the heme group. It has been reported in the literature that apocytochrome *c* shows a tendency to aggregate after the transition is over. High tendency to aggregate is one of the properties of the MG states [10]. It is considered that two kinds of heme interactions contribute to stabilizing the native structure and the MG state. First, the axial ligations of iron to His18 and Met80 may be important in stabilizing the compact and rigid structure of cytochrome *c*. Second, the heme is a predominantly hydrophobic compound. Therefore, it may provide an environment favorable for formation of the hydrophobic core [41].

In the acidic state of cytochrome *c*, heme is removed from hydrophobic core prior to unfolding of the protein. The stability of MG state is determined by a delicate balance of interactions such as electrostatic repulsion between charged residues and opposing forces such as hydrogen bond and hydrophobic interaction [3,15,42]. SDS is an amphipatic material and has a negative polar head. Addition of SDS reduces the electrostatic repulsion and reinforces the hydrophobic interactions twin at low concentration, producing the net force favorable for MG formation. The spectroscopic data demonstrate that the addition of SDS to acid-unfolded state of cytochrome *c* cause heme to move to an essential place. However, the SDS surrounds it when SDS concentration is increased. Therefore, although the MG state of cytochrome *c* is less stable than the native state but its heme junction to the hydrophobic core is much tight. It is noted that the

heme plays an important role in the reversibility of the DSC thermogram. Thus, allowing deconvolution of the thermal profile of the MG state of cytochrome *c* induced by SDS and obtaining additional information about energetic subdomains for cytochrome *c*.

4. Conclusions

SDS induces the MG state of cytochrome *c* at acidic conditions similar to salts but with higher affinity and stability. This allows the reversibility of DSC profile and deconvolution of the curve. The reason for reversibility can be attributed to the heme junction at the hydrophobic core of cytochrome *c* in the presence of SDS (an amphipatic molecule). The ability to deconvolute the DSC profile of the MG state will allow the analysis of the energetic subdomains of cytochrome *c*, which is not possible to do for the native state due to irreversibility of thermogram.

Acknowledgements

The authors thank Dr. C.M. Sorenson and Dr. N. Sheibani from University of Wisconsin Medical School for their valuable comments. The financial support provided by the Research Council of the University of Tehran and Azad University of Mashad are gratefully appreciated.

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