Stabilization of Partially Folded States of Cytochrome C in Aqueous Surfactant: Effects of Ionic and Hydrophobic Interactions†

Krishnananda Chattopadhyay‡ and Shyamalava Mazumdar*

Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400005, India

Received July 7, 2003; Revised Manuscript Received October 14, 2003

ABSTRACT: The interaction of submicellar concentrations of sodium dodecyl sulfate (SDS) with horse heart cytochrome *c* has been found to stabilize two spectroscopically distinct partially folded intermediates at pH 7. The first intermediate is formed by the interaction of SDS with native cytochrome *c*, and this intermediate retains the majority of the secondary structure while the tertiary structure of the protein is lost. The unfolding of this intermediate with urea leads to the formation of a second intermediate, which is also formed on refolding of the unfolded protein (unfolded by urea) by SDS. The second intermediate retains about 50% of the native secondary structure with no tertiary structure of the protein. The second intermediate was found to be absent at low pH. While induction of helical structure of a protein by SDS in the native condition has been reported earlier, this is possibly the first report of the refolding of a protein in a strongly denaturing condition (in the presence of 10 M urea). The relative contributions of the hydrophobic and the electrostatic interactions of the surfactants with cytochrome *c* have been determined from the formation of the molten globule species from the acid-induced unfolded protein in the presence of SDS or lauryl maltoside.

Formation of the three-dimensional structure of a protein by folding of its amino acid chains remains a puzzle still today (*1*−*4*). Extensive studies on the mechanism of folding of a large number of proteins suggest the existence of intermediate states in the folding pathway, although the factors that lead to the formation and stabilization of the folding intermediates are only partially understood (*5*). Folding of mitochondrial respiratory protein cytochrome *c*[1] has been well-studied in the literature (*5*−*10*), because of the relatively small size (12 kDa) and presence of covalently bound heme in the protein. At mild unfolding conditions (such as low denaturant concentrations), the methionine-iron bond present in native cytochrome *c* breaks to form a bis-histidine misfolded species, which gives rise to a complex refolding kinetics of cytochrome *c* (*6*, *11*−*13*). At low pH, the bis-histidine species is not formed, and the population of the misfolded species in equilibrium is not significant (*14*).

Cytochrome *c* resides in the intermembrane space of mitochondria and transfers electrons to cytochrome oxidase in the inner mitochondrial membrane (*15*). Biosynthesis of this protein involves transport of apocytochrome *c* that is coded by nuclear DNA, to the intermembrane space of mitochondria, where heme ligation to the protein and formation of the folded holoprotein takes place. Although apocytochrome *c* is in an unfolded random coil form (*16*), interaction of mitochondrial membrane induces significant α helical structure (*17*, *18*) to form a partially folded conformation of apocytochrome *c* (*19*). On the other hand, interaction of folded cytochrome *c* with lipids was shown to partially unfold the protein. The partially folded intermediate formed by the interaction of cytochrome *c* and apocytochrome *c* with lipids has been proposed to represent a common folding intermediate (*19*−*21*) of the protein. Cytochrome *c* was shown to exist in equilibrium between a soluble state and a membrane-bound state at physiological pH. The anionic lipid membrane forms a loosely packed structure of cytochrome *c*, which has been proposed to be responsible for the apoptotic activity of cytochrome *c* in eukaryotic cells (*21*). Such lipid-induced intermediate formation has also been reported in the case of some bacterial toxins, such as colicin A (*22*).

The present paper describes detailed equilibrium and kinetic studies of the interaction of ionic and neutral surfactants with cytochrome *c*. Interaction of SDS with native cytochrome *c* has been found to form a partially unfolded species similar to that observed in the presence of negatively charged lipids (*20*). We also show that the interaction of SDS with the unfolded cytochrome *c* results in partial refolding of the protein. Although at pH 7, these two intermediate states are distinctly different, they are analogous at low pH. SDS has earlier been noted to induce a considerable amount of helix formation in the native conditions (*18*, *20*); however, refolding in a protein by SDS in strongly denaturing conditions (10 M urea), as reported in the present paper, is unique to the best of our knowledge. The effects of

† This work was supported by the Tata Institute of Fundamental Research.

* Address for correspondence: Prof. S. Mazumdar, Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400005, India. FAX: +91 22 2280 4610. E-mail: shyamal@tifr.res.in. Home page: http://www.tifr.res.in/~shyamal/.

‡ Present address: Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA.

[1] Abbreviations: Cytc: cytochrome *c*, SDS: sodium dodecyl sulphate, LM: lauryl maltoside, CD: circular dichroism, CMC: critical micellar concentration.

10.1021/bi0351662 CCC: $25.00
Published on Web 11/20/2003

hydrophobic and ionic interactions on the formation and the stability of these folding intermediates have also been investigated.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI A), sodium dodecyl sulfate (SDS), and lauryl maltoside (LM) were purchased from Sigma Chemical Company, St. Louis, MO. All the other reagents used were of the highest purity. Cytochrome *c* was further purified by passing it through a Sephadex G25 column equilibrated with 50 mM sodium phosphate buffer, pH 7, and its concentration was determined by the absorption spectrum of the reduced protein using an extinction coefficient of 29 mM^{-1} cm^{-1} at 550 nm.

Optical spectra between 250 and 900 nm were recorded using a Shimatzu UV2100 spectrophotometer attached to an IBM PC-486 and equipped with a thermostated cell holder. Steady-state fluorescence studies were carried out using a SPEX (Fluorolog 16810.22m) and also using a Shimadzu (RF540) spectrofluorimeters. The fluorescence emission spectra were recorded from 310 to 450 nm using an excitation wavelength of 295 nm, which eliminated emission from amino acids other than tryptophan. Concentration of the cytochrome *c* used for the steady-state fluorescence and absorbance measurements was 5 μM.

Circular dichrosim (CD) studies were carried out using a Jasco J-600 spectropolarimeter equipped with a thermostated cell holder for constant temperature experiments. Visible and near UV CD experiments were carried out using protein concentrations of 10−20 μM, and the path length of the cuvette was 10 mm. Far-UV CD studies were carried out using a 1-mm path-length cuvette with 2−5 μM protein concentration. Far-UV CD spectra were measured over the 200−260 nm range, and each spectrum was taken by averaging over 20 scans. Near-UV and visible CD spectra were collected in the spectral ranges of 250−300 nm and 300−700 nm, respectively.

Stopped-flow experiments were carried out using a SF61MX (Hi-TECH Scientific, UK) stopped-flow spectrometer. The sample-handling unit was mounted inside a thermostated-bath compartment and temperature of the compartment was maintained (within ± 1 °C) using a circulating water bath. The absorption-detected and fluorescence-detected measurements were performed with a 75 W quartz tungsten halogen visible lamp and a 100 W xenon lamp (HBO 100W/2), respectively. The stopped-flow total-fluorescence emission was measured by excitation at 295 nm (5 nm bandwidth) with a WG-320 cutoff filter. Kinetic traces were analyzed by averaging 10 traces and then by fitting them to a multiexponential nonlinear least-squares model.

RESULTS

Interaction of SDS with Cytochrome c: Formation of I'_s. The optical spectrum of cytochrome *c* at pH 7 shows a distinct blue shift of the Soret absorption band from 409 to 407 nm, with a concomitant disappearance of the 695 nm band on addition of a small amount of SDS, which has been reported earlier (*23*). Disappearance of the 695 nm band of cytochrome *c* on treatment with the surfactant indicates breaking of the heme−methionine ligation (*24*) and the

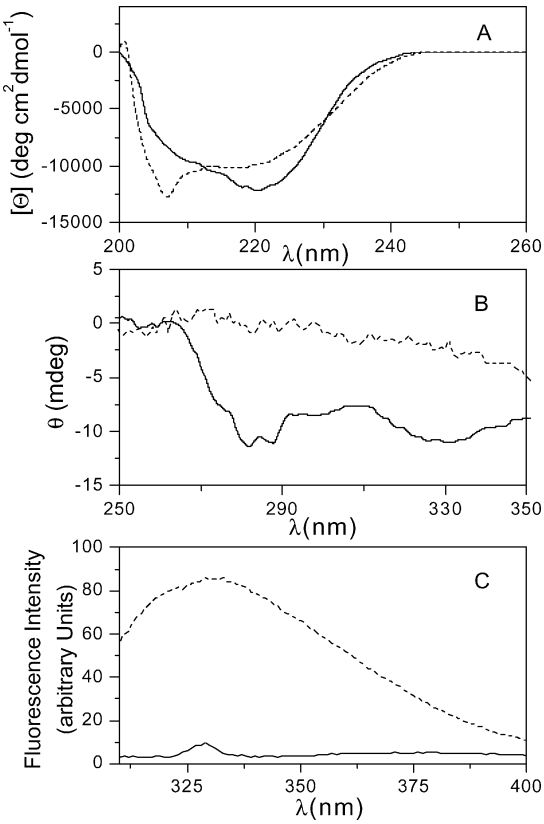


FIGURE 1: (A) Far-UV circular dichroism spectra of cytochrome *c* in the absence (solid line) and in the presence of 2 mM SDS (dotted line) in 50 mM phosphate buffer at pH 7. Molar ellipticity (mean residue) of the protein was plotted against wavelength. (B) Near-UV CD spectra of cytochrome *c* (20 μM) in the absence (solid line) and in the presence of 2 mM SDS (dotted line) in 50 mM phosphate buffer at pH 7, CD values were baseline corrected and ellipticity at 260 nm was adjusted to zero. (C) Fluorescence emission spectra of cytochrome *c* (5 μM) in the absence (solid line) and in the presence of 2 mM SDS (dotted line) in 50mM phosphate buffer at pH 7.

spectrum shows formation of characteristic misligated bis-histidine adduct of cytochrome *c* as shown earlier by Resonance Raman studies (*23*). The UV−visible spectrum of cytochrome *c* at pH 7 in SDS solution matches that in the presence of phospholipids reported recently (*20*).

The far-UV CD spectra of cytochrome *c* in the presence and in the absence of SDS are shown in Figure 1A, and analysis of the far-UV CD spectra using the neural network program CDNN (*25*) shows that there is no significant change in the secondary structure of the protein in the presence of SDS. Furthermore, the far-UV CD of the protein in the presence of SDS is analogous to that reported for that in the presence of lipid micelles solution (*20*), and it also closely resembles the far-UV CD of apo-cytochrome *c* in the lipid micelle (*26*). The near-UV CD spectrum of the protein, however, shows a significant difference in the presence and in absence of the surfactant (Figure 1B). Cytochrome *c* has four phenylalanine, one tryptophan, four tyrosine, and two thioether linkages, which contribute to the near-UV CD. Some of the heme transitions in the range 240−300 nm (*27*) may also contribute to the CD in this region. Tyrosine residues give CD bands between 275 and 282 nm, while phenylalanine residues show weak CD bands between 255 and 270 nm (*28*). The near-UV CD spectrum of cytochrome *c* (Figure 1B) in aqueous buffer shows minima

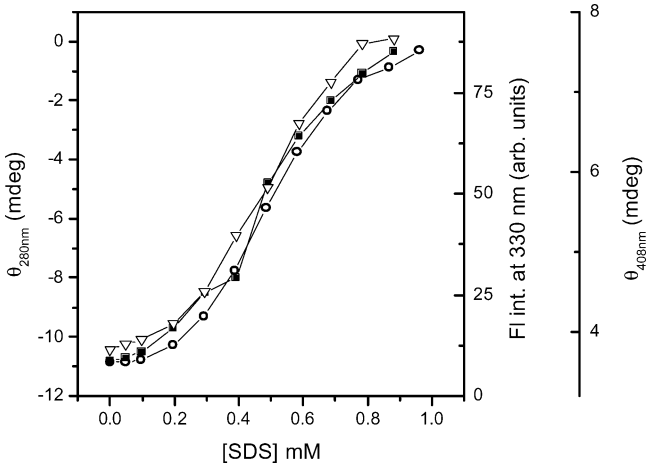


FIGURE 2: Change in ellipticity of cytochrome *c* (20 μM) at 408 nm (triangle) and in ellipticity at 280 nm (square) and change in the fluorescence intensity (circle) of cytochrome *c* (5 μM) (λ_{ex} = 295 nm) with SDS concentration. The experiments were carried out in 50 mM phosphate buffer at pH 7.

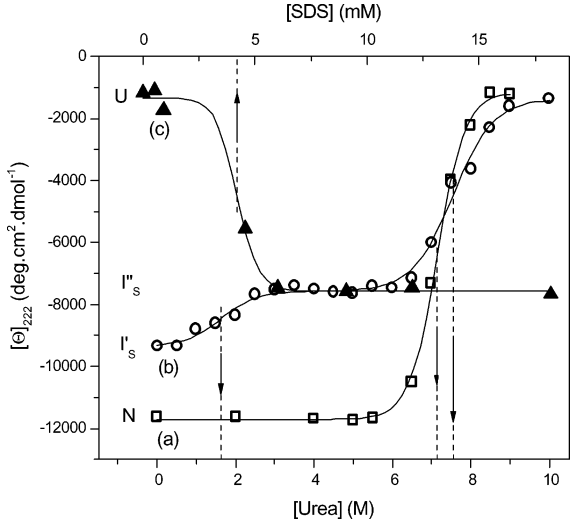


FIGURE 3: (a) Urea-induced unfolding of native cytochrome *c* (N): mean residue molar ellipticity of N at 222 nm, plotted against urea concentration (hollow square), showed a two-state transition. (b) Urea-induced unfolding of the molten-globule like intermediate formed with the cytochrome *c* at 2 mM SDS (I'_s): Change in mean residue ellipticity of I'_s at 222 nm, plotted against urea concentration (hollow circles) followed biphasic transition with the platue region corresponding to formation of intermediate, I''_s. (c) Formation of I''_s from unfolded protein (U) on titration with SDS. Change in mean residue ellipticity of unfolded cytochrome *c* (U, in the presence of 10 M urea) at 222 nm was plotted against SDS concentration (filled triangles); upper axis represents SDS concentration. All the experiments were carried out at pH 7 in 50 mM phosphate buffer.

at 282 and 288 nm, which have earlier been assigned by site-directed mutagenesis to be due to the Trp59 residue (*29*, *30*). The interaction of cytochrome *c* with SDS leads to the complete disappearance of these sharp bands (Figure 1B). Similar CD spectra were obtained in the collapsed intermediate state of cytochrome *c* formed in lipid micelles (*20*) and also in the completely unfolded cytochrome *c* in aqueous buffer in the presence of 10 M urea or 6 M Gdn. HCl (*30*). Present CD results thus indicate the formation of "a molten globule like" partially unfolded (I'_s) form of cytochrome *c* at pH 7 in the presence of SDS. The CD spectrum of cytochrome *c* in the visible region also changes on addition of SDS solution as reported earlier (*23*). The CD activity of the heme absorption arises due to the interaction of the heme electronic transitions with those of the aromatic amino acid residues in the protein. Change in the axial ligation of cytochrome *c* or some conformational change in the heme cavity may cause the observed change in the visible CD of the protein in the presence of the surfactant (see later).

The fluorescence emission from the lone tryptophan residue (Trp59) of cytochrome *c* is weak because of efficient energy transfer from the Trp59 to the heme center (Figure 1C). The interaction of the surfactant with cytochrome *c* resulted in a large increase in the fluorescence intensity of the protein (Figure 1C) with a small red shift of the emission maximum of tryptophan fluorescence from 328 nm to about 334 nm. The addition of the surfactant causes partial unfolding of the protein leading to increase in the energy-transfer distance between the heme and the tryptophan residue. Unfolding of the protein also often leads to a red shift in the fluorescence maximum as a result of increase in polarity of the microenvironment around the tryptophan as it is exposed to the solvent on unfolding.

Figure 2 shows plot of the CD intensity at 280 nm (θ_{280}), the CD intensity at 408 nm (θ_{408}), and the fluorescence intensity at 330 nm of the protein at different concentrations of SDS at pH 7. The observation of almost coinciding transition curves for the near-UV CD, the visible CD, and the fluorescence intensities of the protein with SDS indicates that they all correspond to the same structural change in the protein. All these data could be fitted to a simple two-state model, $N \rightleftharpoons I'_s$ between the native (N) and the molten globule like species (I'_s) and a midpoint SDS concentration of 0.5 mM was observed. The free energy change at zero SDS concentration was calculated using a linear free energy relationship, and the value of $\Delta G = 15$ kJ mol^{-1} agrees with the earlier report (*23*).

Equilibrium Unfolding of the Native and the Molten Globule Like Species (I'_s) by Urea: Formation of the Second Partially Unfolded Form of Cytochrome c in SDS (I''_s). Figure 3 (trace a) shows the variation of mean residue ellipticity of native cytochrome *c* (N) with urea concentrations in the absence of the surfactant at 222 nm at pH 7 at room temperature. The unfolding of the native protein shows a typical two-state transition and the concentration of urea at the midpoint of the transition (C_m) and the free energy change at zero urea concentration (ΔG_{aq}) have been calculated. The values of ΔG_{aq} (34 kJ mol^{-1}) and C_m (7.2 M urea) for the transition agreed with the earlier report (*31*).

The unfolding of I'_s by urea, monitored by the far UV CD at 222 nm in the presence of SDS, indicates a three-state unfolding transition (Figure 3, trace b) and can be described as follows:

$$I'_s \rightleftharpoons I''_s \rightleftharpoons U \qquad (1)$$

The intermediate state (I''_s) with a midpoint at 1.6 M urea was found to contain ~50% of the native secondary structure. The near-UV CD spectrum of I''_s is featureless and similar to that of I'_s which indicates the absence of any tertiary structure. I''_s is stable at ~4−6 M urea in the presence of 2 mM SDS but unfolds completely at ~8 M urea. The midpoint of the second transition (I''_s to U) is 7.5 M urea.

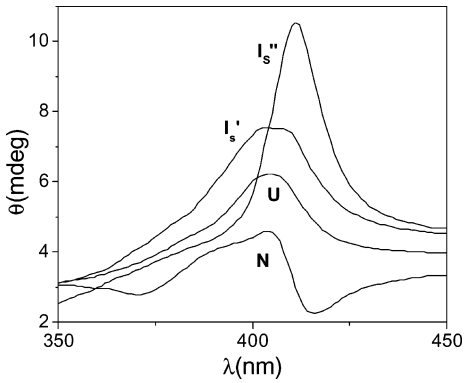


FIGURE 4: Visible CD spectra of (N) native cytochrome *c*, (I'_s) cytochrome in the presence of 2 mM SDS, (I'_s) cytochrome *c* in 10 M urea and 6 mM SDS at pH 7. Protein concentration was 20 μM.

Analogous to the far-UV CD, the visible CD spectrum of I'_s was also found to show biphasic transition with urea. The visible CD of I''_s (in the presence of 2 mM SDS and 4 M urea) was distinctly different with higher intensity than that of I'_s and U at 408 nm (Figure 4) indicating that I''_s is not merely an average of I'_s and U but a distinctly different species. The unfolding of I'_s by urea in the presence of different concentrations of SDS showed that I''_s was preferentially stabilized in SDS and at the surfactant concentrations higher than 3.5 mM, I''_s was the only species observed, and the completely unfolded species (U) was not formed even in the presence of 10 M urea.

The addition of SDS to the unfolded cytochrome *c* (U, in the presence of ~10 M urea) led to partial refolding of the protein. The variation of the far-UV CD at 222 nm of the unfolded protein in 10 M urea, pH 7, plotted against added SDS concentration (Figure 3, trace c) indicates that the secondary structure of the protein was ~50% recovered at ~6 mM SDS. This species is spectroscopically identical to the intermediate I''_s with the same CD and fluorescence spectra. Any conversion of I''_s to I'_s was however not observed even on addition of 18 mM SDS to the 10 M urea solution of the protein.

Effect of pH on the Interaction of SDS with Cytochrome c. The interaction of SDS with cytochrome *c* was also monitored at pH 4 where the misfolded bis-histidine species of the protein do not form. The addition of SDS to the protein solution caused a two-state transition at pH 4, which was analogous to that observed at pH 7. The far-UV CD spectrum of the protein in the presence of 2 mM SDS at pH 4 was similar to that of I'_s at pH 7, and there was no characteristic CD band in the near-UV region indicating that a molten globule like species ($I'_{s(lowpH)}$) analogous to I'_s is formed at pH 4. This species however would be devoid of any misfolded bis-histidine ligation unlike in case of the I'_s at pH 7. When the solution of $I'_{s(lowpH)}$ in the presence of 2 mM SDS was titrated with urea at pH 4, no change in the far-UV CD or the fluorescence was observed. This indicates that the $I'_{s(lowpH)}$ is more stable against unfolding by urea compared to that at pH 7 (I'_s) and any species analogous to I''_s was not formed at low pH.

The far-UV CD and the fluorescence studies on the interaction of the unfolded cytochrome *c* (unfolded by 10 M urea) at pH 4 with 6 mM SDS was found to form $I'_{s(lowpH)}$. Moreover, unlike at pH 7, formation of any species analogous to I''_s was not observed with the urea-unfolded protein at pH 4. Similar result was observed when acid-unfolded cytochrome *c* at pH 2 was refolded by 6 mM SDS, and only $I'_{s(lowpH)}$ was formed (see later).

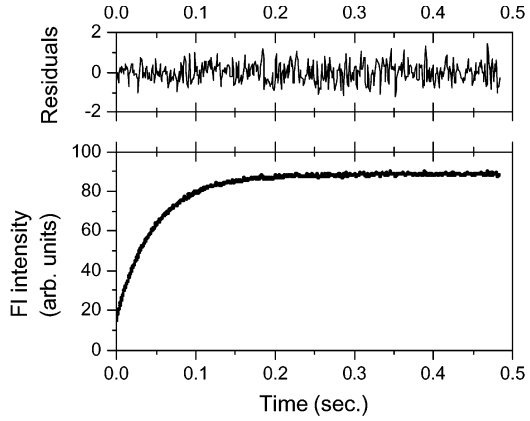


FIGURE 5: Typical stopped-flow trace of change in fluorescence intensity of 5 μM cytochrome *c* with time on the interaction with 3.5 mM SDS in 50 mM phosphate buffer at pH 7. The solid line is the fit of the experimental data to a two-exponential model. Rate constants obtained are shown in Table 1. Residuals distribution is plotted in the upper curve to show the goodness of the fit.

Kinetics of Interaction of SDS with Cytochrome c. The formation of I'_s was associated with a large increase in the steady-state fluorescence intensity as well as a change in the absorbances at 406 nm and at 695 nm. The stopped-flow kinetics of the formation of I'_s on addition of 3.5 mM SDS to the protein at pH 7 showed biexponential behavior of the fluorescence emission as well as the absorbance at 406 nm, and a typical stopped flow trace of the change in the fluorescence intensity associated with the formation of I'_s is shown in Figure 5. The absorbance of the protein at 695 nm, however, showed single-exponential decay kinetics on treatment with SDS. The rate constants (k_1 and k_2) and the normalized amplitudes (a_1 and a_2) obtained from the analysis of the stopped-flow data are tabulated in Table 1. The slower rate component (k_2) obtained from the fluorescence as well as from the absorbance at 406 nm (Table 1) is close to the rate constant obtained from the variation of the absorbance at 695 nm (~13−17 s^{-1}), which can be assigned to the breaking of the axial ligation to the heme. The faster rate constant (k_1) hence should correspond to a conformational event, which takes place before the breaking of iron−methionine bond (k_2). This can be confirmed by the kinetic experiments in the presence of excess imidazole (when sixth coordination site of the heme is occupied by imidazole) or at low pH (when sixth coordination site is either vacant or is occupied by water) (Table 1), because in these experimental conditions, the kinetic event involving the iron−methionine bond should be absent. In both conditions, the stopped flow data could be fitted to a single-exponential model with much faster (~200−250 s^{-1}) rate constants (Table 1). It is important to note that the rate constants were found to increase nonlinearly with the concentration of SDS, and the first component (k_1) could not be detected at SDS concentrations above 8 mM, indicating possible cooperative binding of SDS molecules in the rate-determining step.

The refolding of the molten globule like species, I'_s of cytochrome *c* to the native protein was monitored using stopped flow by 7-fold dilution of a solution of cytochrome *c* in 2 mM SDS with buffer. A large decrease in the

Table 1: Kinetic Parameters of the Reactions of Folded and Unfolded Cytochrome *c* with SDS

start species	end species	reagent	method	k_1, s^{-1} (a_1, %)	k_2, s^{-1} (a_2, %)
Cytc, pH 7	I'_s	3.5 mM SDS	absorption (at 406 nm)	33 (13)	16 (87)
Cytc, pH 7	I'_s	3.5 mM SDS	absorption (at 695 nm)		17 (100)
Cytc, pH 7	I'_s	3.5 mM SDS	total fluorescence[a]	30 (30)	13.4 (70)
Im-Cytc, pH 7	Im-I'_s	3.5 mM SDS + imidazole	absorption (at 406 nm)	190 (100)	
Im-Cytc, pH 7	Im-I'_s	3.5 mM SDS + imidazole	total fluorescence[a]	170 (100)	
Cytc, pH 4.4	$I'_{s(low\ pH)}$	3.5 mM SDS pH 4.4	absorbance (at 396 nm)	250 (100)	
Cytc, pH 4.4	$I'_{s(low\ pH)}$	3.5 mM SDS pH 4.4	total fluorescence[a]	260 (100)	
I'_s	Cytc, pH 7	7-fold dilution with buffer pH 7	total fluorescence[a]	0.016 (20)	0.003 (80)
Im-I'_s	Im-Cytc, pH 7	7-fold dilution with buffer pH 7 + imidazole	total fluorescence[a]	0.23 (100)	
unfolded Cytc in 10 M urea, pH 7	I''_s, pH 7	6 mM SDS	total fluorescence[a]	completed in < 2 ms	
unfolded Cytc at 10 M urea pH 4.4	$I'_{s(low\ pH)}$, pH 4.4	6 mM SDS	total fluorescence[a]	completed in < 2 ms	
unfolded Cytc at pH 2	$I'_{s(low\ pH)}$, pH 2	6 mM SDS	total fluorescence[a]	completed in < 2 ms	
I'_s, pH 7	I''_s, pH 7	8 M urea	total fluorescence[a]	0.013 (100)	
I''_s, pH 7	I'_{ss}, pH 7	7-fold dilution with 6 mM SDS	total fluorescence[a]	10 (100)	

[a] $\lambda_{ex} = 295$ nm, $\lambda_{em} > 310$ nm, k_1 and k_2 are rate constants and a_1 and a_2 are the normalized amplitudes.

tryptophan fluorescence accompanied by this process could be fitted to a sum of two exponentials, and the rate constants are shown in Table 1. A minor step (20%) was observed with a rate constant of 0.016 s^{-1} (k_{1r}), which was followed by a very slow rate constant of 0.003 s^{-1} (k_{2r}). The refolding of I'_s to the native cytochrome *c* (N) involves formation of tertiary structure and the change in the axial ligation from non-native His to Met80 coordination at the heme at pH 7.0. The refolding of I'_s in the presence of excess imidazole (200 mM) was relatively fast and involved only one kinetic process with rate constant of 0.23 s^{-1}.

Kinetics of Interaction of SDS with the Unfolded Protein. The addition of SDS to a solution of unfolded cytochrome *c* (U) at pH 7 results in the formation of I''_s, which was characterized by a decrease in the fluorescence intensity and increase in the far-UV CD of the protein. Kinetics of the formation of I''_s was monitored using stopped flow fluorescence by mixing 6 mM SDS to the unfolded (10 M urea) cytochrome *c* (U) solution at pH 7. The formation of I''_s from U was found be very fast and the reaction was complete within the dead time of the instrument (2 ms) (Table 1).

Analogously, the formation of $I'_{s(lowpH)}$ on the treatment of the acid-unfolded (at pH 2) or the urea-unfolded (at pH 4) cytochrome *c* with SDS (6 mM) was also found to be extremely rapid and was completed within the dead time (2 ms) of the stopped flow instrument (Table 1).

Kinetics of Conversion between I'_s and I''_s. The kinetics of formation of I''_s was monitored using stopped flow fluorescence by mixing 8 M urea to a solution of I'_s in the presence of 3.5 mM SDS at pH 7, and the rate was found to be very slow (0.013 s^{-1}). The rate of formation of I'_s from I''_s was monitored by 7-fold dilution of a cytochrome *c* solution containing 10 M urea and 6 mM SDS (I''_s) with a 6 mM SDS solution in aqueous buffer at pH 7. The rate constant of this step was found to be 10 s^{-1} (Table 1).

Kinetics of the Refolding of Urea-Unfolded Cytochrome c at pH 7. We studied the refolding of cytochrome *c* by diluting a 10 M solution of the protein by 7-fold with aqueous buffer, and the time-dependent decrease in the tryptophan fluorescence intensity was monitored using stopped flow method. Four kinetic steps were identified, which agreed with earlier results (*6*, *13*). The first folding step with the time constant smaller than the dead time (2 ms) of the instrument corresponds to the burst phase (intermediate I″), which was followed by a fast phase (2−100 ms) (intermediate I′). This phase was associated with a substantial decrease in the tryptophan fluorescence (*32*). The fast phase is then followed by an intermediate phase (200−500 ms) (intermediate I) and a slow phase (>1 s) to form the folded protein (*6*). A partially folded state with fluctuating secondary structure is formed in the burst phase. The fast phase was accompanied by a major conformation change, while the intermediate phase was characterized by major heme absorbance change. The intermediate phase was not observed for unfolding of the protein in the presence of 100 mM imidazole or at pH 4, indicating that this phase corresponds to binding of Met80 at the heme (*32*). The slow phase was associated with a small change in fluorescence, and has been assigned to proline isomerization (*33*, *34*) giving the final folded conformation of the protein.

Stabilization of the Molten Globule Intermediates: Effects of Electrostatic and Hydrophobic Interactions. The addition of salt (∼100 mM NaCl) to an acid-unfolded cytochrome *c* solution (at pH 2) leads to the formation of a molten globule intermediate (*31*, *35*, *36*). Our results indicate that an analogous molten globule species ($I'_{s(lowpH)}$) is formed in the presence of only ∼6 mM SDS even in the absence of salt at pH 2. Thus, the ionic surfactant seems to facilitate formation of the helical structure of the protein, indicating that both ionic as well hydrophobic interactions between the surfactant and the protein might be important for the formation of the secondary structure of the protein. To understand the role of hydrophobic interactions on stabilization of the molten globule intermediate, we carried out CD studies of the acid-unfolded cytochrome *c* in the presence of 6mM lauryl maltoside, which has the same hydrophobic tail as that of SDS (C_{12}) with a neutral headgroup (maltoside) instead of an ionic (sulfate) headgroup. Figure 6 shows variation of the far-UV CD at 222 nm of a solution of acid-unfolded cytochrome *c* at pH 2 with NaCl concentration. The titration of a solution of the unfolded cytochrome *c* at pH 2 by NaCl showed gradual increase in the far-UV CD intensity (negative), and at salt concentrations above ∼100 mM NaCl the far-UV CD spectrum of the protein matched with that

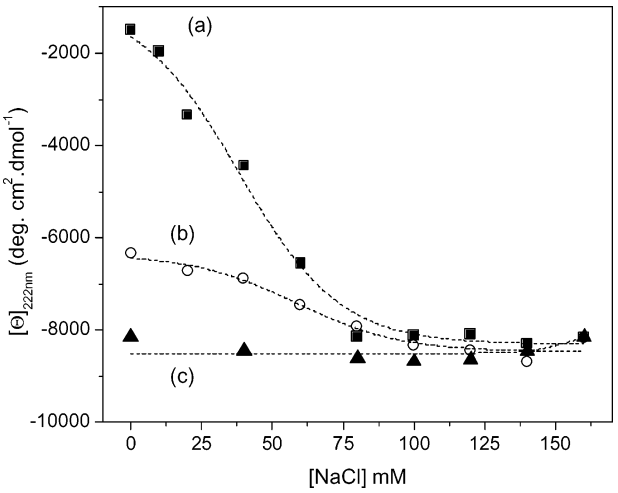


FIGURE 6: Change in mean residue molar ellipticity with NaCl concentration for (a) cytochrome *c* at pH 2 (filled squares), (b) cytochrome *c* at pH 2 in the presence of 6 mM lauryl maltoside (hollow circles), and (c) cytochrome *c* at pH 2 in the presence of 6mM SDS (filled triangles). Dashed lines represent sigmoidal fits to the data.

reported for the characteristic molten globule species (*35*). The addition of 6 mM lauryl maltoside to the unfolded protein at pH 2 led to a significant increase in the far-UV CD at 222 nm, which corresponds to >50% helical content of the protein. The addition of NaCl to this solution further increased the far-UV CD and resulted in the formation of secondary structure analogous to $I'_{s(lowpH)}$. On the other hand, the addition of 6 mM SDS to the cytochrome *c* (unfolded) solution at pH 2 however resulted in the complete formation of the secondary structure ($I'_{s(lowpH)}$), and further addition of salt did not have any effect on the secondary structure of this species. These results indicate that the formation of the molten globule like species $I'_{s(lowpH)}$ involves both hydrophobic as well as ionic interactions.

DISCUSSION

It has been shown that cytochrome *c* exists in equilibrium between soluble state and membrane-bound conformation at physiological pH (*37*). The membrane-bound form is partially unfolded, and it has a reduced electron transfer activity. The interaction of cytochrome *c* with negatively charged lipid vesicles was earlier shown to stabilize an intermediate state with nativelike secondary structure but without any tertiary interactions. The lipid membrane, with its negatively charged surface formed by the phosphate groups, can electrostatically bind cytochrome *c*, which has a large positive patch on the surface. On the other hand, free lipid molecules, which are not involved in the formation of the membrane, could interact with the protein through the ionic headgroups as well as through the hydrophobic tail of the lipid causing a large change in the conformation of the protein. Thus, lipid molecules dissociated from the membrane would have a larger effect on the structure of cytochrome *c*. The SDS surfactant molecules mimic the lipids, and thus studies on interaction of SDS with the protein would provide significant insight on the possible effects of the lipid monomers on the protein in vivo.

A recent study suggested formation of a common folding intermediate in lipid micelles with holo and apo-cytochrome *c* (*20*). Our studies indicate that the anionic surfactant SDS also interacts with cytochrome *c* leading to formation of an analogous intermediate state. These amphiphilic molecules can form micelles at concentrations above the critical micellar concentration (CMC). The formation of the micelles is a dynamic self-aggregation process, and thus a micellar solution contains an equilibrium mixture of micelles and monomers. A submicellar concentration (i.e., surfactant concentration less than the CMC of the micelles) of the surfactant, however, would predominantly consist of the monomeric surfactant molecules in solution. The present study on interaction of SDS in the submicellar concentration range thus shows that the monomeric surfactant molecules interact with the protein and this leads to formation of partially unfolded intermediates of cytochrome *c*.

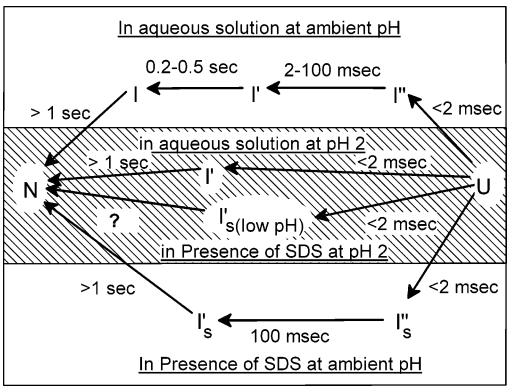
Equilibrium unfolding of cytochrome *c* in the presence of SDS shows the existence of at least two stable intermediate states at physiological pH (I'_s and I''_s). I'_s is a misligated bis-His complex, which retains more than 80% of the secondary structure of the protein while the tertiary structure is completely lost. The formation of I'_s is associated with a large increase in the tryptophan fluorescence emission intensity indicating that the Trp59 residue moves away from the heme as a result of the partial unfolding of the protein. The transition from native cytochrome *c* to I'_s is a two-state equilibrium with a midpoint at 0.5 mM SDS indicating that formation of I'_s takes place at SDS concentration lower than the critical micellar concentration (CMC) of the surfactant (~8 mM) (*38*).

I''_s is formed as an equilibrium intermediate between the fully unfolded protein (U) and I'_s at physiological pH. The CD results of I''_s indicated that it retained ~50% of the helical content of the native protein with no tertiary structure. The environments around the heme center in these species (N, I'_s, I''_s, and U) are distinctly different from each other as they show different CD spectra (Figure 4) of the heme transition. The results also suggest that I'_s is possibly more compact compared to I''_s, while both of these intermediates could be classified as molten-globule intermediates of the enzyme.

The formation of I'_s from the native protein on addition of SDS is a complex process with at least two kinetic steps. The rate-determining step of formation of I'_s was assigned to the breaking of the methionine coordination at the heme (~17 s^{-1}). In the presence of excess imidazole or at low pH, the heme−methionine bond does not exist, and hence the rate of the reaction of the protein with SDS is much faster and monophasic in such case (Table 1). The formation of the native like structure from I'_s also has multiple steps (rate constants 0.016 and 0.003 s^{-1}).

The refolding kinetics of cytochrome *c* in aqueous buffer solution and in SDS show marked similarity to each other indicating possible similarity in the folding mechanism of the protein. Scheme 1 summarizes the results of kinetic studies of refolding of cytochrome *c* in the presence and absence of the surfactant. The burst phase intermediate I″ and the species I''_s are formed with time constant <2 ms, and they both are associated with partial formation of the collapsed structure of the protein. Although I″ was detected only in the kinetic study, I''_s was observed as an equilibrium intermediate stabilized by the surfactant.

Scheme 1: Schematic Model of Refolding of Cytochrome *c* in the Presence and in the Absence of Surfactants



The fast phase of folding of cytochrome *c* in aqueous solution is known to be associated with formation of most of the secondary structure of the protein (*6*). The intermediate (I′) formed during this phase was suggested to contain a subdomain with N helix (residues 3−14) and C helix (residues 87−103) of the protein interacting through extensive hydrophobic interactions similar to that found in the native structure (*39*). Similarly, the formation of I'_s involves generation of most of the secondary structure of the protein. The acid-unfolded cytochrome *c* (at pH 2) as well as the folded protein at low pH (pH 4) forms $I'_{s(lowpH)}$ which was analogous to I'_s.

The stabilization of these intermediate states by SDS possibly arises due to cooperative interaction between the protein and the surfactant molecule. The formation of molten globule states was earlier proposed to be facilitated by hydrophobic and electrostatic interactions (*31*, *35*). SDS, being a large amphiphilic molecule, may provide the hydrophobic interactions for the formation of the collapsed intermediate states. On the other hand, the negatively charged headgroup of SDS can exert some electrostatic effect. To understand the possible role of these two effects, we used LM, which is a nonionic (electrostatic effect is absent) surfactant with similar hydrophobic chain length as that of SDS. The total charge of cytochrome *c* at pH 2 is +24 (19 Lys + 2 Arg + 2His + 1 of heme) while at pH 7 the total charge of the protein is +8 (19 Lys + 2 Arg − 3 Glu − 9 Asp − 1 of heme). The strong electrostatic repulsion between the charged amino acids at pH 2 leads to the formation of an extended unfolded structure of the protein at this pH. The increase in solution dielectric constant by addition of NaCl to the acid-unfolded cytochrome *c* solution at pH 2 helps to mask the repulsive interaction ($F \propto 1/\epsilon$, ϵ is the dielectric constant of the medium) between the charges and thus stabilizes the molten-globule intermediate (*35*) at high salt concentrations at this pH. The molten-globule intermediate ($I'_{s(lowpH)}$) analogous to that formed at a high NaCl concentration (100 mM, Figure 6) was stabilized by 6 mM SDS, which provides both electrostatic as well as hydrophobic effects to the protein structure. In the presence of 6 mM LM (Figure 6), a significant amount of secondary structure of the protein was formed in the solution, which was still less than that obtained in the presence of salt or SDS. LM, as a neutral surfactant, can offer hydrophobic interaction as that of SDS, but it does not have any electrostatic effect on the protein. The electrostatic contribution can be provided by addition of NaCl to the LM treated cytochrome *c* at pH 2, which forms the rest of the secondary structure of the protein. Moreover, the molten globule intermediate $I'_{s(lowpH)}$ was more stable toward urea-induced denaturation compared to I'_s, suggesting that binding of SDS to the protein in $I'_{s(lowpH)}$ form is probably much stronger compared to that in I'_s. These results support that stabilization of the cytochrome *c* intermediates by SDS is achieved by virtue of its hydrophobic as well as electrostatic contributions.

Earlier studies by flow-cytometry detected a conformational change in cytochrome *c* in an early event of apoptosis in mouse (*21*). These cytochrome *c* molecules were shown to be associated with membrane molecules (*37*). The interaction of cytochrome *c* with the membrane molecules would be analogous that with the surfactant molecules as described here. Thus, the intermediate conformations analogous to those detected in the presence of the surfactant (I'_s, I''_s) might be involved in the permeabilization of cytochrome *c* across the mitochrondrial membrane in the initial stages of apoptosis.

CONCLUSIONS

Interaction of sodium dodecyl sulfate with cytochrome *c* has been found to stabilize two partially folded intermediates. The first intermediate (I'_s) is formed in the presence of submicellar amounts of the surfactants in aqueous solution. This intermediate retained most of the secondary structure of the native structure but had no tertiary interactions as detected by the circular dichroism studies.

Partial unfolding of I'_s by urea at pH 7 leads to formation of I''_s which contains ∼50% of the secondary structure of the native protein with no tertiary structure. Kinetic studies on refolding of the urea induced unfolded cytochrome *c* suggested that I''_s is possibly analogous to the burst phase folding intermediate of the protein identified in aqueous solution (*6*).

Refolding of the protein at low pH shows that it forms a molten-globule intermediate ($I'_{s(lowpH)}$) in SDS solution, which is analogous to that formed during refolding of acid-unfolded protein (*35*). Studies on refolding of the acid-unfolded protein by the neutral surfactant LM indicated that the hydrophobic tail groups of the surfactant also interact with the unfolded protein leading to partial refolding of it which on further addition of sodium chloride forms the molten globule intermediate identical to $I'_{s(lowpH)}$. These studies thus showed that both hydrophobic and ionic interactions between SDS and cytochrome *c* play important roles in stabilization of the partially folded intermediate of the protein.

Interaction of the surfactant molecules with cytochrome *c* can be considered to mimic binding of the protein to the mitochondrial membrane during apoptotic release of cytochrome from mitochondria in vivo. The common folding intermediate between the apo-cytochrome *c* and the holoprotein could be analogous to the species formed on interaction of the surfactant with cytochrome *c*. The present results suggest that very small concentrations of surfactants probably are sufficient to form these partially folded forms of the protein and both electrostatic as well as hydrophobic interactions between the surfactant and the protein are involved in such conformation change.

ACKNOWLEDGMENT

The authors wish to thank Mr. Bharat T. Kansara for help in carrying out circular dichroism and stopped-flow experiments.

REFERENCES

1. Kim, P. S., and Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459−489.
2. Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 273−293.
3. Kuwajima, K. (1989) *Proteins 6*, 87−103.
4. Creighton, T. E. (1990) *Biochem. J. 270*, 1−16.
5. Baldwin, R. L. (2001) *Nat. Struct. Biol. 8*, 92−94.
6. Elöve, G. A., Chaffote, A. F., Roder, H., and Goldberg, M. E. (1992) *Biochemistry 31*, 6876−6883.
7. Yeh, S.-R., Han, S., and Rousseau, D. L. (1998) *Acc. Chem. Res. 31*, 727−736.
8. Xu, Y., Mayne, L., and Englander, S. W. (1998) *Nat. Struct. Biol. 5*, 774−778.
9. Shastry, M. C. R., and Roder, H. (1998) *Nat. Struct. Biol. 5*, 385−392.
10. Sosnick, T. R., Shtilerman, M. D., Mayne, L., and Englander, S. W. (1997) *Proc. Nat. Acad. Sci. U.S.A. 94*, 8545−8550.
11. Sosnick, T. R., Mayne, L., Hiller, R., and Englander, S. W. (1994) *Nature Struct. Biol. 1*, 149−156.
12. Takahashi, S., Yeh, S.-R, Das, T. K., Chan, C.-K., Gottfried, D. S., and Rousseau, D. L. (1997) *Nat. Struct. Biol. 4*, 44−50.
13. Elöve, G. A., Bhuyan, A. K., and Roder, H. (1994) *Biochemistry 33*, 6925−6935.
14. Akiyama, S., Takahashi, S., Ishimori, K., and Morishima, I. (2000) *Nat. Struct. Biol. 7*, 514−520.
15. Pettigrew, G. W., and Moore, G. R. (1987) *Cytochromes c. Biological Aspects*, Springer-Verlag, Berlin..
16. Fisher, W. R., Taniuchi, H., and Anfinsen, C. B. (1973) *J. Biol. Chem. 248*, 3188−3195.
17. Jordi, W., Li-Xin, Z., Pilon, M., Demel, R. A., and de Kruijff, B. (1989) *J. Biol. Chem. 264*, 2292−2301.
18. De Jongh, H. H. J., and de Kruijff, B. (1990) *Biochim. Biophys. Acta 1029*, 105−112.
19. De Jongh, H. H. J., Killian, J. A., and de Kruijff, B. (1992) *Biochemistry 31*, 1636−1643.
20. Sanghera N., and Pinheiro, T. J. T. (2000) *Protein Sci. 9*, 1194−1202.
21. Jemmerson, R., Liu, J., Hausauer, D., Lam, K., −P., Mondino, A., and Nelson, R. D. (1999) *Biochemistry 38*, 3599−3609.
22. Van der Goot, F. G., Gonzalez-Manas, J. M., Lakey, J. H., and Pattus, F. (1991) *Nat. Struct. Biol. 354*, 408−410.
23. Das, T. K., Mazumdar, S., and Mitra, S. (1998) *Eur. J. Biochem. 254*, 662−670.
24. Sutin, N., and Yandell, J. K. (1972) *J Biol. Chem. 247*, 6932−6936.
25. Bohm, G., Muhr, R., and Jaenicke, R. (1992) *Protein Eng. 5*, 191−195.
26. Rankin, S. E., Watts, A., Roder, H., and Pinheiro, T. J. T. (1999) *Prot. Sci. 8*, 381−393.
27. Urry, D. W. (1967) *J. Biol. Chem. 24*, 4441−4448.
28. Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113−175.
29. Davies, A. M., Guillemette, J. G., Smith, M., Greenwood, C., Thurgood, A. G. P., Mauk, A. G., and Moore, G. R. (1993) *Biochemistry 32*, 5431−5435.
30. Pinheiro, T. J. T., Elöve, G. A., Watts, A., and Roder, H. (1997) *Biochemistry 36*, 13122−13132.
31. Hamada, D., Kuroda, Y., Kataoka, M., Aimoto, S., Yoshimura, T., and Goto, Y. (1996) *J. Mol. Biol. 256*, 172−186.
32. Brems, D. N., and Stellwagen, E. (1983) *J. Biol. Chem. 258*, 3655−3660.
33. Ridge, J. A., Baldwin, R. L., and Labhardt, A. M. (1981) *Biochemistry 20*, 1622−1630.
34. Ramdas, L., and Nall, B. T. (1986). (1986) *Biochemistry 25*, 6959−6964.
35. Goto, Y., Calcino, L. J., and Fink, A. L. (1990) *Proc. Nat. Acad. Sci. U.S.A. 87*, 573−577.
36. Ptitsyn, O. B. (1992) in Protein Folding (Creighton, T. E., Ed.) pp 243−300, W. H. Freeman and Company, New York.
37. Cortese, J. D., Voglino, A. L., and Hackenbrock, C. R. (1998) *Biochemistry 37*, 6402−6409.
38. Majhi, P. R., and Blume, A. (2001) *Langmuir 17*, 3844−3851.
39. Bushnell, G. W., Louie, G. V., and Brayer, G. D. (1990) *J. Mol. Biol. 214*, 585−595.

BI0351662