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Characterization of molten globule state of cytochrome c at alkaline, native and acidic pH induced by butanol and SDS

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Abstract

In our earlier communications, we had studied the acid induced unfolding of stem bromelain, glucose oxidase and fetuin [Eur. J. Biochem. 269 (2002) 47; Biochem. Biophys. Res. Comm. 303 (2003) 685; Biochim. Biophys. Acta 1649 (2003) 164] and effect of salts and alcohols on the acid unfolded state of α-chymotrypsinogen and stem bromelain [Biochim. Biophy. Acta 1481 (2000) 229; Arch. Biochem. Biophys. 413 (2) (2003) 199]. Here, we report the presence of molten globule like equilibrium intermediate state under alkaline, native and acid conditions in the presence of SDS and butanol. A systematic investigation of sodium dodecyl sulphate and butanol induced conformational alterations in alkaline (U_1) and acidic (U_2) unfolded states of horse heart ferricytochrome c was examined by circular dichroism (CD), tryptophan fluorescence and 1-anilino-8-napthalene sulfonate (ANS) binding. The cytochrome c (cyt c) at pH 9 and 2 shows the loss of approximately 61% and 65% helical secondary structure. Addition of increasing concentrations of butanol (0–7.2 M) and sodium dodecyl sulphate (0–5 mM) led to an increase in ellipticity value at 208 and 222 nm, which is the characteristic of formation of α-helical structure. Cyt c is a heme protein in which the tryptophan fluorescence is quenched in the native state by resonance energy transfer to the heme group attached to cystines at positions 14 and 17. At alkaline and acidic pH protein shows enhancement in tryptophan fluorescence and quenched ANS fluorescence. Addition of increasing concentration of butanol and SDS to alkaline or acid unfolded state leads to decrease in tryptophan and increase in ANS fluorescence with a blue shift in λ_{max}, respectively. In the presence of 7.2 M butanol and 5 mM SDS two different intermediate states I_1 and I_2 were obtained at alkaline and acidic pH, respectively. States I_1 and I_2 have native like secondary structure with disordered side chains (loss of tertiary structure) as predicted from tryptophan fluorescence and high ANS binding. These results altogether imply that the butanol and SDS induced intermediate states at alkaline and acid pH lies between the unfolded and native state. At pH 6, in the presence of 7.2 M butanol or 5 mM SDS leads to the loss of CD bands at 208 and 222 nm with the appearance of trough at 228 nm also with increase in tryptophan and ANS fluorescence in contrast to native protein. This partially unfolded intermediate state obtained represents the folding pathway from native to unfolded structure. To summarize; the 7.2 M butanol and 5 mM SDS stabilizes the intermediate state (I_1 and I_2) obtained at low and alkaline pH. While the same destabilizes the native structure of protein at pH 6, suggesting a difference in the mechanism of conformational stability.

Keywords: Alcohol-induced state; Anion-induced state; Circular dichroism; Cytochrome c; Fluorescence; Molten globule

1. Introduction

To understand the factors governing the formation of three dimensional protein structure, it is important to elucidate the hierarchy of interactions that stabilize

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the native and molten globule state (Haq, Ahmed, & Khan, 2003). A molten globule exits as an intermediate between the native and denatured states, is defined as a compact conformation with a comparable amount of native like secondary structure but a high enhancement in the intramolecular motion, i.e. largely disordered tertiary structure (Ptitsyn, 1995a,b). A large barrier to crack the protein folding code is the high cooperativity of folding reactions at equilibrium (Naseem, Khan, Haq, & Naeem, 2003; Gupta, Khan, & Saleemuddin, 2003). A solvent perturbation study is a good approach to evaluate the stabilizing force of protein structure. The interesting co solvents in this context can be either polyols and sugars or alcohol and surfactants since they have potential protecting effects on various types of protein denaturation (Fink, Calciano, Goto, Kurotsv, & Palleros, 1994; Gekko & Timasheff, 1981a,b).

Cytochrome c (cyt c) is a small globular protein (104 amino acid residues, 12700 Da) carrying a large component of basic residues. Out of its 104 residues 24 are lysine, arginine and histidine. It is an essential redox protein found in the mitochondria of all eukaryotic as well as prokaryotic cells, functioning as an electron transporter in the energy yielding respiratory chain. It has been widely used as a model for both the kinetic and thermodynamic studies of protein folding (Marmorino & Pielak, 1995). Davis Searles, Morar, Saunderes, Eri, and Pielak (1998) have reported that sugars induce the MG state of cytochrome c and its driving force is an increased steric repulsion between the proteins and sugar solutions. Santucci et al. (2000) has reported the formation of molten globule like state of cytochrome c induced in the presence of high concentration of glycerol. The main driving force of protein stabilization is enhancement of hydrophobic interaction as studied by polyols and alcohols. The molten globule (A state) of ferriccytochrome c, a single-chain hemoprotein, in the presence of salts has been extensively characterized (Goto, Calciano, & Fink, 1990; Goto, Takahashi, & Fink, 1990; Marmorino, Lehti, & Pielak, 1998). At pH 2.2, ferricytochrome c is substantially unfolded at low ionic strength; upon addition of salts, the protein cooperatively folds to compact structure, the A state, stabilized by binding of anions to the positively charged groups on the protein surface (Goto, Takahashi, et al., 1990). The A state possesses α-helix structure comparable to that of the native state, but a fluctuating tertiary conformation. In particular, the hydrophobic core containing the two major helices (the N- and C-terminal helices) and the heme group is preserved in the A state, stabilized by non bonded interactions (Goto & Nishikiori, 1991; Jeng, Englander, Elove, Wand, & Roder, 1990; Jordan, Eads, & Spiro, 1995), while the loop regions are fluctuating and partly disordered (Davis Searles et al., 1998). Of the two native axial ligands of the heme-iron, only His18 is thought to remain coordinated to the heme iron in A state, while the Met80 axial bond is lost (Goto, Takahashi, et al., 1990; Haq, Rasheedi, & Khan, 2002; Jeng et al., 1990). Goto, Calciano, et al. (1990) have shown that the net charge of the anion is the main determinant for the stabilization of the A state; the higher the charge, the lower the ion concentration needed to induce formation of the molten globule. Santucci et al. (2000) have ascribed that the small anions induce formation of a compact, highly structured state, in which the native Met80-Fe(III) axial bond is recovered and the native like redox properties are restored (Goto, Takahashi, et al., 1990; Jeng et al., 1990; Jordan et al., 1995). The polyanion like poly(vinylsulfate) has been reported to induce a molten globule–like state of cytochrome c at acidic pH. In horse heart cytochrome c, bonds between the heme and four amino acids (Cys14, Cys17, His18 and Met80) anchor the heme and, together with an extensive array of non-covalent side-chain contacts, these bonds help to make the internal structure around the heme macrocycle relatively rigid (Antalik & Sedlak, 1999). The residues which are in Van der Waal's contact with heme are bulky hydrophobic groups that help to stabilize the structure and control the level of redox potential. Because of the support of the covalent thiol bondings to the heme, the proximal His bond is not cleaved under denaturing conditions normally employed in folding measurements that grossly perturb the tertiary structure around the heme and cleave off the Met80 ligation. Hence, Cyt c serves as a very good model for studying the unfolding/refolding phenomena of the polypeptide chain with the heme participating simultaneously in the process without bimolecular recombination (Pascher, Chesick, Winkler, & Grey, 1996).

The main driving force of protein stabilization by polyols or alcohol is enhancement of hydrophobic interaction (Khan, Khan, & Muzammil, 2000). On the

other hand, surfactants have a polar head and a nonpolar tail; the latter causes a hydrophobic effect, which arises primarily from the strong attractive forces between water molecules themselves. The forces involved between protein and surfactants are both electrostatics and hydrophobic. Surfactants are unique among protein denaturants: even as low as 1 mM SDS, for instance, can often alter protein conformation. The addition of a surfactant can either increase or decrease the helical conformation (Hiramatsu & Yang, 1983). This is unlike the unfolding of most proteins in 5–6 M guanidine hydrochloride or 8–10 M urea. Detergent extraction of integral proteins from the membranes often results in the disruption of protein subunits or even converts β forms into helices. Though a lot of prior work has been reported on the kinetics intermediates of cyt c at acidic pH in the presence of SDS and methanol (Das, Mazumdar, & Mitra, 1998; Goto, Calciano, et al., 1990; Goto, Takahashi, et al., 1990; Hiramatsu & Yang, 1983; Kamatari, Takashi, Kataoka, & Akasaka, 1996). Also, the kinetic approaches to elucidating the folding mechanism of cytochrome c took advantage of the presence of the covalently bound heme group, which serves as a useful optical marker, both because of its axial coordination changes and its efficient Trp fluorescence quenching (Gianni, Travaglini-Allocatelli, Brunori, Shastry, & Roderm, 2003). Novelty in our studies extends on the equilibrium intermediate lies between the alkaline unfolded and native state obtained in the presence of SDS and butanol which is not reported so far and for comparison equilibrium intermediate obtained at acidic pH and native state are also taken in consideration.

2. Materials and methods

2.1. Materials

Horse heart ferricytochrome c (CIIA O 46515) (type III) was purchased from Sigma, St. Louis, MO, USA and the purity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis. SDS (SRL, India) and tertiary butanol (Qualigens Fine Chemicals, India) were used without any further purification. All other chemicals used in this study were of analytical grade.

2.2. Methods

All the measurements were carried out at room temperature. Typically, cytochrome c stock solution (3 mg/ml) was prepared which had been dialysed against distilled water. The concentration of native cyt c was determined from the extinction coefficient of 106,000 M^{-1} cm^{-1} at 410 nm: pH 7. The alkaline and acid unfolded state of cyt c (0.314 mM) was prepared by dialyzing the protein independently against respective buffer. Buffers were 20 mM Gly NaOH, phosphate and Gly HCl adjusted to pH 9, 6 and 2. For studying the effect of butanol (0–7.2 M) and SDS (0–5 mM) on alkaline, native and acidic pH, the unfolded protein was incubated at room temperature for 1 h in the presence of different molarities of SDS and butanol to carry out spectroscopic measurements.

2.3. Optical spectroscopy

2.3.1. Soret absorption spectroscopy

The Soret absorption of the heme group was monitored with a Hitachi single beam spectrophotometer U-1500 using a 1 cm path length cell.

2.3.2. Circular dichroism measurements

Circular dichroism (CD) was measured with a JASCO J720 spectropolarimeter calibrated with ammonium d-10 camphorsulfonate. A cell of path length 0.1 was used for scanning between 190–250 nm. Protein concentrations of the samples were typically 20 μM for far ultraviolet CD studies. The results were expressed as the mean residue ellipticity (MRE in $^{\circ}$ cm^2/dmol), which is defined as:

$$\mathrm{MRE} = \frac{\theta_{\mathrm{obs}}\ (\mathrm{m}^{\circ})}{10 \times n \times \mathrm{Cp} \times l}$$

where θ_{obs}, is the observed ellipticity in degrees ($^{\circ}$), Cp the molar fraction, and 'l' the length of light path in cm. The α-helical content of cytochrome c was calculated from the MRE value at 222 nm using the following equation as described by Chen et al. (1972).

$$\%\,\alpha\text{-Helix} = \frac{\mathrm{MRE}_{222} - 2340}{30300 \times 100}$$

2.3.3. Fluorescence measurements

Fluorescence spectra were recorded with a Shimadzu RF 540 spectrofluorophotometer in a 10 mm path length quartz cell. Samples containing different concentrations of alcohol (butanol) and surfactant (SDS) were equilibrated at room temperature for 10 min before recording for tryptophan fluorescence measurements. The excitation wavelength was 280 nm and the emission was recorded from 300 to 500 nm. The final protein concentration was 5 μM for all experiments.

2.3.4. ANS-fluorescence measurements

ANS binding was measured by fluorescence emission with excitation at 380 nm and emission was recorded from 400 to 600 nm. Typically, ANS concentration was 100 molar excess of protein concentration and protein concentration was in the vicinity of 5 μM.

3. Results

3.1. Effect of butanol on alkaline and acidic unfolded states of cytochrome c

Fig. 1, shows the far UV-CD spectra of cyt c in the 200–250 nm region in presence of butanol. Fig. 1A shows the effect of butanol on alkaline-unfolded (U_1) state of cytochrome c at pH 9. At pH 9, there is decrease in negative molar ellipticity (curve 1 of Fig. 1A), as compare to native preparation (curve 1 of Fig. 1C) indicates the alkaline unfolded, U_1 state. This state though results in loss of 65% (approximately) secondary structure but some amount of helical content (approximately 13.5%) is retained as compared to native where overall 35% helix is present. As there is gradual formation of negative peak at 208 nm and plateau at 222 nm with increase in the concentration of butanol upto 7.2 M. This suggests that butanol

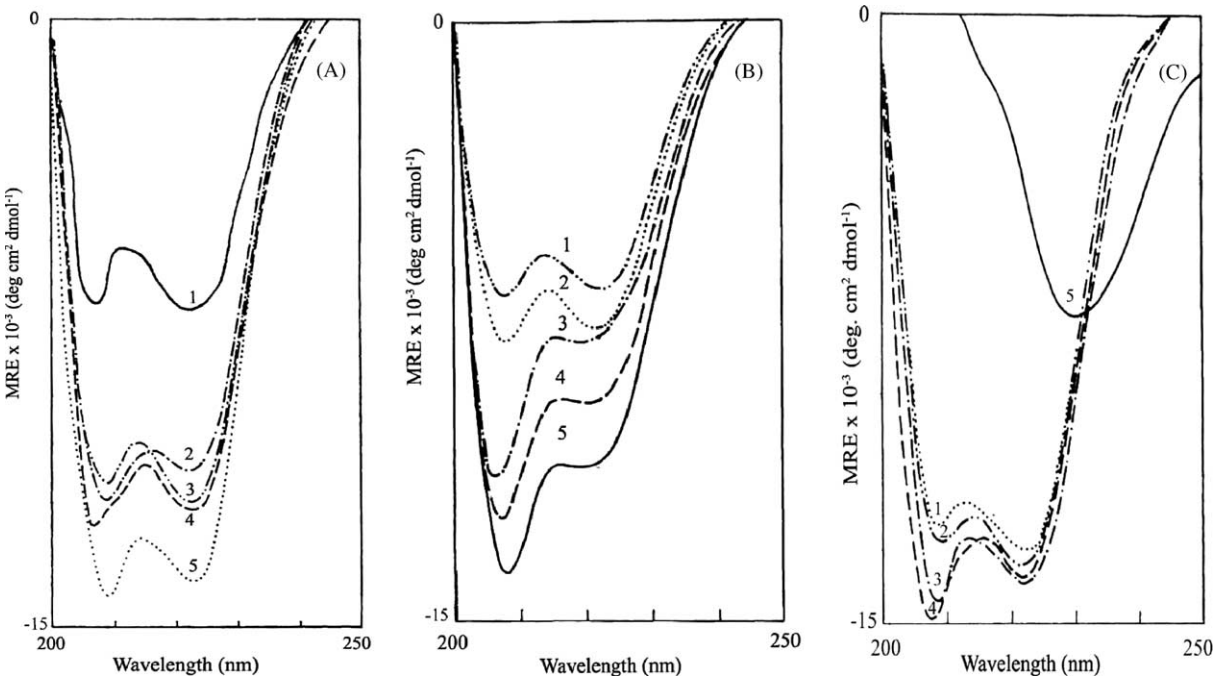


Fig. 1. (A) Far-UV CD spectra of cyt c in the presence of butanol (0–7.2 M) at pH 9 in 20 mM glycine NaOH. Curves 1–5 represent different molarities of butanol (0, 1.03, 3.09, 5.1, 7.2 M). Protein concentration was 20 μM. (B) Far-UV CD spectra of cyt c (20 μM) in the presence of butanol (0–7.2 M) at pH 2 in 20 mM glycine-HCl. Curves 1–5 represents 0, 1.03, 3.09, 5.1and 7.2 M butanol, respectively. (C) Far-UV CD spectra of cyt c in the presence of butanol (0–7.2 M) at pH 6 in 20 mM phosphate buffer. The molarities of butanol varies from curves 1 to 5 were 0, 1.03, 3.09, 5.1, and 7.2 M, respectively. Protein concentration was 20 μM.

induces the formation of ordered secondary structure in the alkaline unfolded state leading to the formation of an intermediate (I_1 state) with secondary structure close to native cytochrome c. Higher concentration of butanol decreases the polarity of the solvent as compared to alkaline solvent, leading to the transition of U_1 to I_1 (an intermediate obtained in the presence of 7.2 M butanol) characterized by the presence of minimum at 208 nm and a plateau at 219–222 nm. The curve 1 of Fig. 1B, indicates the acid unfolded state (U_2) of cyt c in the absence of butanol at pH 2.0, showing the loss of ordered secondary structure approximately 65% though some amount of helical content (approximately 12%) is retained as in native where overall 35% helix is present. Thus, the decrease of ellipticity at 222 nm indicates the break down of the intact secondary structure (curve 1). In the presence of increasing concentration of butanol, there is enhancement of negative peaks at 208 and 222 nm. The induction of secondary structure is evident from the formation of a prominent minimum at 208 nm and a plateau in the wavelength range 219–222 nm. In the presence of butanol, this transition leads to formation of I_2 state (an intermediate at 7.2 M concentration of butanol at low pH) from U_2 (the acid unfolded) state. At pH 2, in the presence of high concentration of butanol the hydrogen bonding responsible for protein secondary structure formation become dominant (due to hydrogen bonding between protein and butanol rich media). As the concentration of butanol is increased, the hydrogen bonding within the butanol molecules increases hence resulting in the intramolecular hydrogen bonding with in the protein molecule (Fig. 1B, curves 2–5).

As can be seen from Fig. 1C, at pH 6, addition of butanol upto 5.1 M lead to an increase in ellipticity at 208 and 222 nm. This is suggestive of an increase in secondary structural content (stabilization of α-helices). As alcohols can induce a higher helicity than in native state upto certain concentration (Hiramatsu & Yang, 1983). However higher concentration of butanol (7.2 M) leads to disruption of secondary structure as seen by the loss of CD bands at 208 and 222 nm with the appearance of a trough at 228 nm (curve 5). As butanol decreases, the polarity of the solvent as compared to the buffer initially surrounding, increasing the hydrophobicity it results in the disruption of α-helix indicating a different mechanism of stabilization. α summary of the respective maximum MREs and % α-helix in presence of butanol at pH 2, 6 and 9 are given in Table I. In addition, with *n*- or *s*-butanol although less soluble, there was induction of secondary structure same as that of *t*-butanol upto 2 M, beyond which, protein was precipitated. Hence, it was not feasible to carry on experiments with *n*- or *s*-butanol hence was replaced by *t*-butanol. 7.2 M butanol breaks down the conformation of cyt c at pH 6. But interestingly upon pH titration from 7 to 2 or from 7 to 11 there was induction of secondary structure, but there was no retention of tertiary structure. At pH 2 and 9, the retention of secondary structure was not significant. However, near UV CD studies (data not shown) did not show any major corresponding induction in tertiary structure. Hence, it may be considered that although the secondary structure increases gradually the protein continues to remain in a collapsed state for the entire concentration range of butanol. As there is formation of secondary structure, this intermediate state can be referred to as molten globule like state. Further increase in the butanol concentration beyond that at which maximum ellipticity was observed, leads to protein precipitation.

3.2. Tryptophan fluorescence and ANS binding in alkaline unfolded state of cytochrome c in the presence of butanol

The intrinsic fluorescence maximum (λ_{max}) is an excellent parameter to monitor the polarity of tryptophan environment in the protein, and is sensitive to the protein conformation (Santucci, Polizio, & Desideri, 1999). Horse cytochrome c has a single tryptophan residue at position 59; hence the most sensitive method of monitoring the gross conformational changes in this protein is to study the tryptophan fluorescence (Stryer, 1968). Fig. 3A summarizes the results of fluorescence studies on native cytochrome c at pH 6 and 9 in the absence and presence of (0–7.2 M) butanol. At pH 9, curve 1 in Fig. 2A shows the enhancement in tryptophan fluorescence. The increase in the tryptophan distance from the heme results in an increase in the fluorescence intensity and this can be used to detect subtle conformational changes occurring around the heme (Hamada, Hoshino, Kataoka, Fink, & Goto, 1993; Stryer, 1968). The unfolding of cytochrome c leads to transition to unfolded (U_1) state, at this state

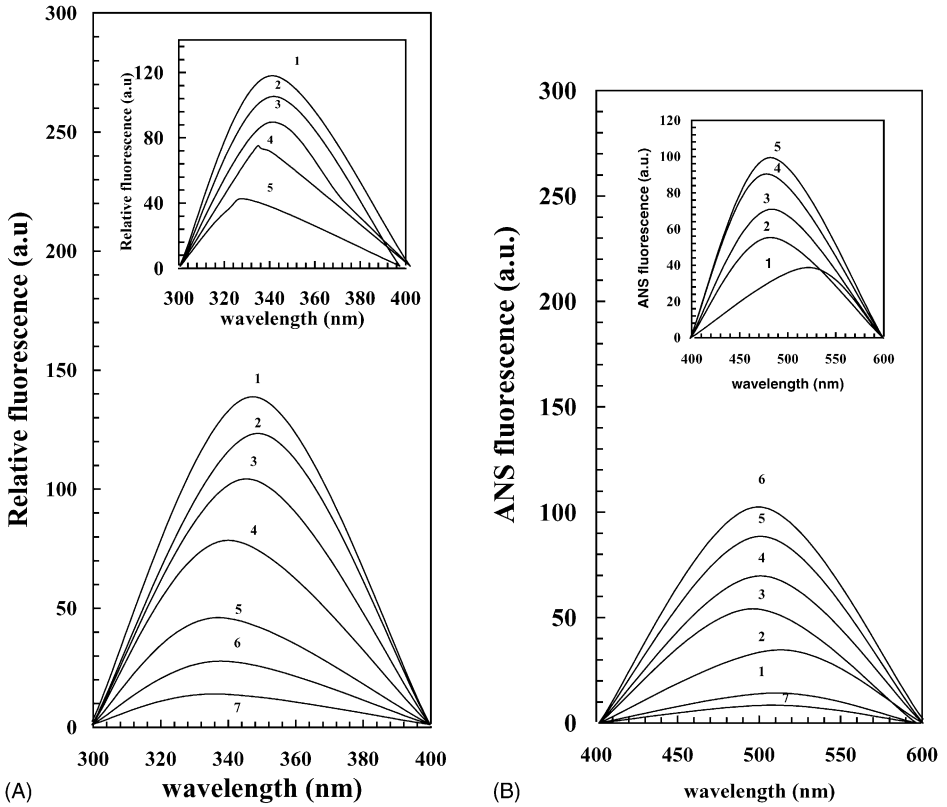


Fig. 2. (A) Intrinsic fluorescence emission spectra of cyt c at alkaline pH 9 and at pH 6 in the presence of increasing concentration of butanol (0–7.2 M). Excitation wavelength was 280 nm. Curve 1 represents alkaline unfolded state (U_1), curve 2 represents cytochrome c at pH 6 in the presence of 7.2 M butanol, curves 3–6 represents cyt c at pH 9 with 1.03, 3.09, 5.1 and 7.2 M butanol, respectively, and curve 7 represents native cytochrome c. Concentration of cyt c was 5 μM. (B) Effect of increasing concentration of butanol on alkaline pH 9 and at pH 6 in the presence of butanol by ANS fluorescence measurements. Excitation wavelength was 380 nm. Curve 1 represents cyt c at pH 9, curve 2 represents cyt c at pH 6 in the presence of 7.2 M butanol, curves 3–6 represents 0, 1.03, 3.09, 5.1 and 7.2 M butanol at pH 9, respectively and curve 7 represents native cytochrome c at pH 6. Protein concentration was 5 μM. (A) (inset) Fluorescence emission spectra of cyt c at pH 2 in absence and presence of increasing concentration of butanol (0–7.2 M). Excitation wavelength was 280 nm. Curves 1–5 represents 0, 1.03, 3.09, 5.1 and 7.2 M butanol, respectively. Protein concentration was 5 μM. (B) (inset) ANS fluorescence emission spectra of cyt c at pH 2 in the absence and presence of butanol. Excitation wavelength was 380 nm. Curve 1 represents acid unfolded state, curves 2–5 represent 1.03, 3.09, 5.1 and 7.2 M butanol, respectively. Protein concentration was 5 μM.

quenching is lost as heme and tryptophan are at a distance apart. Addition of increasing concentration of butanol to cytochrome c leads to the transition of U_1 (curve 1) to I_1 state (curves 3–6), which approaches to native state (curve 7) indicating the heme and tryptophan are again in close proximity. Increasing concentration of butanol has no effect on the fluorescence at pH 6.0 except at 7.2 M, which had resulted in the increase in the fluorescence intensity and red shift in λ_{max} of approximately 15 nm. In the presence of ANS, energy transfer donor/acceptor combination occurs due to overlap of tryptophan emission and ANS excitation. It is well known that ANS binds only to the solvent exposed hydrophobic clusters that are originally buried inside the protein in the native state. Binding of ANS to hydrophobic regions of protein results in an increase in fluorescence intensity, which has been widely used to detect the molten globule states of different proteins (Matulis, Baumann, Bloomfield, & Lovrien, 1999; Matulis & Lovrien, 1998; Pawar & Deshpande, 2000; Ptitsyn, Pain, Semisotnov, Zerovnik, & Razgulyaev, 1990; Semisotnov et al.,

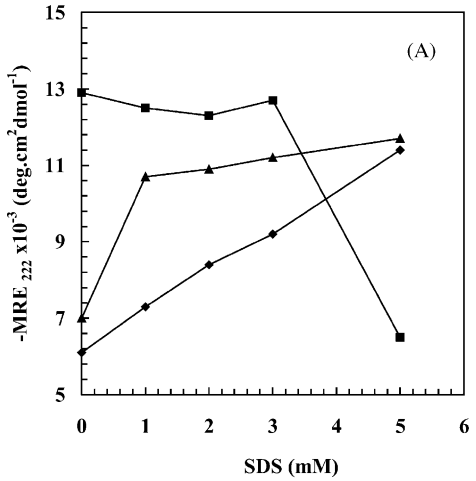
1991; Xie, Guot, & Zhou, 2004; Ray, Singh, & Balaram, 2001). Fig 2B shows the ANS fluorescence spectra of cytochrome c. At pH 6 (data not shown for clarity), upto 5.1 M butanol, no changes were observed but at 7.2 M butanol (curve 2 of Fig. 2B) induces the increase in ANS fluorescence spectra as compared to native protein at pH 6 (curve 7) with a red shift of 20 nm in λ_{max} less unfolded as compared to cyt c at pH 9 (curve 1 of Fig. 2B). At pH 9 (curve 1 of Fig. 2B), as the concentration of butanol is increase (curves 3–6) a blue shift in λ_{max} of approximately 20 nm is obtained (480 nm) with significant increase in ANS fluorescence intensity as compared to the spectra obtained at pH 9 in absence of butanol (curve 1 of Fig. 2B). The increase in ANS binding at 7.2 M butanol at pH 9 indicates that in this intermediate (I_1) state the hydrophobic clusters, which were originally buried inside, are now solvent exposed. As hydrophobic cluster are loosely pack, resulting in large surface area, are the characteristic of molten globule, hence I_1 is characterized as molten globule. By small angle X-ray scattering molten globule state of cytochrome c has also been reported (Kataoka, Hagihara, Mihara, & Goto, 1993). The CD and fluorescence results altogether suggests that at 7.2 M butanol the hydrogen bonds between butanol rich media and protein will become weaker, making the hydrogen bonds with in polypeptide chain comparatively stronger and thereby inducing helical structure in U_1 further leading to I_1 state. Thus, it can be concluded that butanol is responsible for making hydrogen bond within the polypeptide chains and weakens the hydrophobic interactions, which were responsible for tertiary structure resulting in retention of secondary with partial tertiary structure.

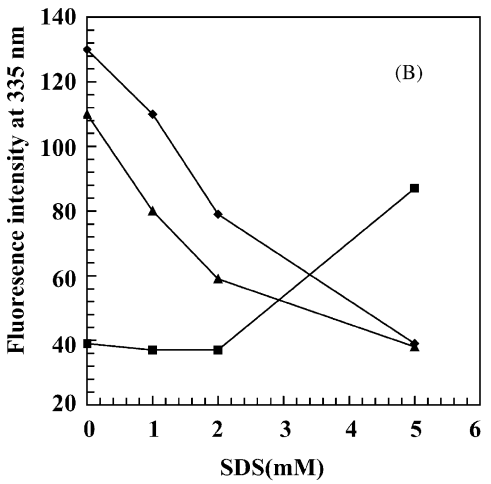
3.3. Solvent accessibility of tryptophan and ANS binding in acid unfolded state in the presence of butanol

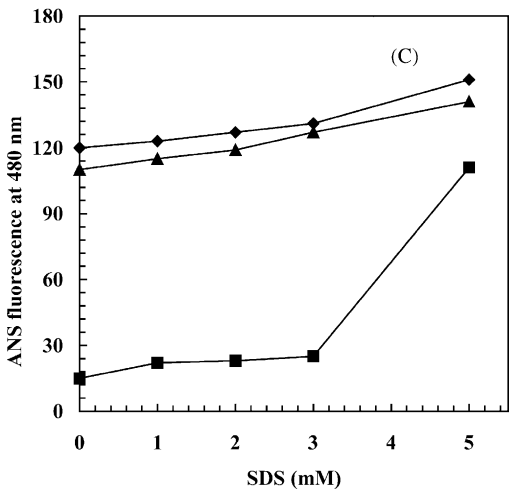
Curve 1 of Fig. 2A (inset) indicates acid unfolded (U_2) cytochrome c at pH 2. The quenching is lost on unfolding (U_2) as compared to native state. This state has the extended conformation having heme and tryptophan apart. But increasing concentration of butanol upto 7.2 M (curves 2–5) induces change in tryptophan environment with reoccurrence of quenching on going to the I_2 state, i.e. 7.2 M butanol. In the presence of 7.2 M butanol, the fluorescence intensity is quenched with a significant blue shift in the emission λmax from 351 to 335 nm resulting in I_2 state where the tryptophan and heme are again in close proximity. The solvent exposure of the hydrophobic surface in the acid unfolded state was studied by ANS binding. As can be seen from Fig. 2B at pH 2, in the absence of butanol (curve 1) the fluorescence maxima is obtained at 510 nm which is the characteristic of free ANS in water, which in the presence of increasing concentration of butanol from 1.3 to 7.2 M (curves 2–5) the fluorescence emission maximum is shifted to 480 nm, which is the characteristic of ANS-bound protein (Fig. 2B inset). This can be interpreted as hydrophobic interactions increases and hydrophobic patches are in proper orientation resulting an increase in ANS binding as usually occurs in molten globule state. Hence, this intermediate state obtained can be characterized as molten globule. Kamatari et al. (1996) have reported methanol-induced conformational transitions at acidic pH of cytochrome c; resulting in two non native states which lack specific tertiary structure but has secondary structural content and tryptophan environment similar to those of native state.

3.4. Effect of SDS on alkaline and acid unfolded states of cytochrome c

CD signal measured at 222 nm is a more selective probe for the helicity because interferences caused by other secondary structure elements are relatively weak at this wavelength. Fig. 3A shows the MRE value at 222 nm, respectively, in the presence of varying concentration of sodium dodecyl sulphate (0–5 mM) at different pH values, i.e. 2, 6 and 9, respectively. At pH 9 (Fig. 3A), there is loss of ordered secondary structure with 13.5% retention of α-helix (overall 35% α-helix is present in native state). As the concentration of SDS is increased upto 5 mM at pH 9, a compact state, retaining the substantial secondary structure is obtained as can be seen by increase in negative MRE value at 222 nm. The 12-carbon hydrophobic chain of SDS creates a non-polar environment that decreases the polarity of the solvent at pH 9 causing an increase in hydrophobicity and also interacting with hydrophobic cluster of the protein. This results in protein folding, for the protein to adopt a compact structure with decreased hydrophobic surface area. The unfavourable







repulsion might be shielded by anion rising from SDS can partially be interpreted with in the framework of Debye–Hückel screening effect. The sulphate anion exerts an "ionic atmosphere" effect around the protein. The counter ion presence around the charge groups will weaker the repulsion, thus permitting the other forces favoring folding to become relatively strengthened and resulting in folding of the protein (Battistuzzi, Borsari, Loschi, Martinelli, & Sola, 1999). α summary of the respective maximum MREs and % α-helix in presence of SDS at pH 2, 6 and 9 are given in Table 1. At pH 6 in the presence of 5 mM SDS, there is loss of secondary structure from helix to coil transition thus involving some other mechanism of protein stability at pH 6 with a trough at 228 nm (fig not shown for clarity). Momotani et al. (1981) has also reported the circular dichroic spectra with a trough near 230 nm in lectins observed on denaturation processes by sodium dodecyl sulphate. On contrary, at alkaline pH SDS induces destabilizing effect on native protein at pH 6. Das et al. (1998) have reported a partially unfolded kinetic intermediate of cytochrome c in the presence of SDS at pH 7 between completely unfolded and native folded state can support this result. As can be seen from Fig. 3A, at pH 2, in the absence of SDS (retention of α-helix is 12% while overall 35% is present in native) as compare to the presence of increasing concentration of SDS, there is increase in the negative MRE value at 222 nm. The increase in MRE value at 222 nm approaches the MRE value obtained for α-helix of native protein and regains up to 29% α-helix. The addition of sulphate anion to cytochrome c, leads to electrostatic interactions with the positively charged centers on the protein (binding of SO_4^{2-} anion), so as to effectively shield the repulsive forces, which favor unfolding, and consequently the intrinsic hydrophobic interactions of protein in an aqueous

Fig. 3. (A) MRE values at 222 nm of cyt c in presence of sodium dodecyl sulphate [0–5 mM] at pH 9 (▲), 6 (■) and 2 (◆), respectively. Protein concentration was 20 μM. (B) Fluorescence emission values of cyt c at 335 nm in absence and presence of increasing concentration of sodium dodecyl sulphate (0–5 mM) at three different stages, i.e. pH 9 (▲), 6 (■) and 2 (◆), respectively. Protein concentration was 5 μM. (C) ANS emission values of cyt c at 480 nm in the presence of increasing concentration of sodium dodecyl sulphate (0–5 mM) on alkaline [(pH 9 (▲)] induced state, native state of cytochrome c [pH 6 (■)] and acid [pH 2 (◆)]. Protein concentration was 5 μM.

Table 1
Structural propensities of intermediate states of cytochrome c as studied by CD

State	Conditions	$MRE_{222\,nm}$ ($^\circ$ cm^2 dmol^{-1})	helix[a] (%)
Native	pH 6.0, 25 °C	13,000	35.0
Acid unfolded (U_2)	pH 2.0, 25 °C	5,965	12.0
Butanol (I_1) intermediate	pH 2.0, 25 °C, 7.2 M	13,506	36.8
SDS intermediate (I_2)	pH 2.0,25 °C, 5 mM	11,100	28.0
Alkaline unfolded (U_1)	pH 9.0, 25 °C	6,418	13.5
Butanol intermediate (I_1)	pH 9.0, 25 °C, 7.2 M	13,706	37.8
SDS intermediate (I_2)	pH 9.0, 25 °C, 5 mM	11,150	29

[a] Calculated by the method of Chen et al. (1972).

environment manifest themselves (Goto, Takahashi, et al., 1990). This results in protein folding, for the protein to adopt a compact structure with increased hydrophobic surface area. Further increase in the SDS concentration beyond that which maximum ellipticity was observed leads to the transition of SDS monomer to the formation of micelles. The critical micellar concentration of SDS in water is reported to be 8.3 mM while it further decreases in buffer. Hiramatsu and Yang (1983) have reported a similar study on concanavalin A.

3.5. Intrinsic fluorescence of alkaline and acid unfolded state of cytochrome c in presence of SDS

The solvent accessibility of tryptophan residues in the alkaline (U_1) or acid unfolded (U_2) states were assessed using fluorescence emission spectroscopy data at 335 nm. At pH 9 (Fig. 3B), the alkaline unfolded state (U_1); there was an enhancement of fluorescence intensity at 335 nm as the heme and tryptophan were a part in extended conformation. As can be predicted from Fig. 3B, in the presence of increasing concentration of SDS there is gradual decrease in fluorescence intensity at 335 nm. The transition from U_1 to I_1 state (5 mM SDS) resulted in quenching of tryptophan fluorescence at 335 nm due to close proximity of heme and tryptophan. The electrostatic effects of the formation of ionic couples at the protein surface due to specific sulfate anion on the conformation of cytochrome c can be partially interpreted within the frame of Debye–Hückel theory with simple electrostatic consideration, or 12-carbon chain of SDS creates the hydrophobic environment which results in cytochrome c refolding. Thus, I_1 state can be considered as an intermediate between unfolded and native state. At low pH in the absence of SDS, there is increase in tryptophan fluorescence at 335 nm. This is due increase in the distance between heme and tryptophan in the unfolded protein (U_2). Sulfate anion of sodium dodecyl sulphate which follows electro selectivity series, shield the electrostatic repulsion which is the cause of protein unfolding at low pH, by directly binding to the positive charges, resulting in the manifestation of hydrophobic interactions, favoring folding of the unfolded protein. As the concentration of SDS (0–5 mM) is increased, it acts as source of sulphate anion which binds positively charged groups of the protein resulting in screening of the repulsive effect, thus overcoming charge-charge repulsion, causing a compact structure. The sulfate ion present in the electronegative series is found to be effective in inducing secondary and partial tertiary structure. The electronegative series given by Goto and collaborators is as follows (Goto & Nishikiori, 1991; Goto, Takahashi, et al., 1990):

$$Fe(CN)_6^{3-} > SO_4^{2-} > CCl_3COO \rightarrow SCN \rightarrow ClO^{-4} > I \rightarrow NO_3^- > CF_3COO^- > Br \rightarrow Cl^-$$

At pH 6, however no changes were observed on addition of SDS upto 2 mM but as the concentration is increase beyond 2 mM there was enhancement in fluorescence intensity at 335 nm, thus destabilizing the structure of cytochrome c. Das et al. (1998) can support this result.

3.6. ANS fluorescence of alkaline and acid unfolded states of cytochrome c in presence of SDS

Fig. 3C show the fluorescence emission values of ANS with cytochrome c at 480 nm as a function of

increasing SDS concentration (0–5 mM) at pH 2, 6 and 9, respectively. At alkaline pH 9, there was a sharp increase in ANS fluorescence as compared to native folded state, pH 6. As the concentration of SDS is increased upto 5 mM, there is a further increase in ANS fluorescence (Fig. 3C) with a blue shift in λ_{max} of 20 nm (data not shown). The increase in ANS fluorescence is due to large solvent exposed surface area forming a compact state to which hydrophobic dye binds and partially due to increase in hydrophobic interaction as there is partial retention of tertiary structure. At pH 2 in the absence of SDS, there is sharp increase in ANS fluorescence but as the concentration of SDS is increase there is further increase in ANS fluorescence (Fig. 3C) with a blue shift in λ_{max} of 20 nm (data not shown), which is the characteristic of ANS bound protein. At pH 6, the fluorescence intensity remains quenched indicating the absence of any exposed hydrophobic surface area between ANS molecule and cytochrome c under native conditions. In the presence of 5 mM SDS at pH 6, there is enhancement in ANS fluorescence intensity indicating the exposure of hydrophobic residues to which ANS binds and hence resulting in disruption of tertiary structure of protein (Fig. 3C).

4. Discussion

After proteins are synthesized on ribosome they are effectively and efficiently folded into their biological active form. The three-dimensional structure of a protein encoded in its linear sequence of amino acids has been referred to as the second half of the genetic code. Many fully unfolded proteins, with randomly coiled polypeptide chains, can spontaneously refold into their native structure with high efficiency and fidelity. The mechanism by which such an intricate process takes place still eludes protein chemists and molecular biologists (Ptitsyn, 1995c). Cyt c is one of the most studied protein with respect to its intermediates which are stabilized during acid denaturation. Upon HCl titration of cytochrome c starting at neutral pH, the protein first unfolds in the vicinity of pH 3–4. It is thus substantially unfolded at pH 2 (Goto, Calciano, et al., 1990; Goto & Nishikiori, 1991; Goto, Takahashi, et al., 1990). Hagihara et al. (1994) has studied the molten globule state of cyt c on acetylation, thermal denaturation, in the presence of urea and Gn–HCl. But our studies extend on the presence of intermediate at three different pH in presence of alcohol and surfactant. In our present study, there are five conformational states: an intermediate state obtained at pH 6 in the presence of 7.2 M butanol and 5 mM SDS, alkaline induced unfolded (U_1), acidic induced unfolded (U_2) state, I_1, i.e. an intermediate state obtained at alkaline pH in the presence of 7.2 M butanol and 5 mM SDS and I_2, i.e. an intermediate state obtained at acidic pH in the presence of 7.2 M butanol and 5 mM SDS. The alkaline and acidic unfolded states, have many similar properties: (1) their tryptophan fluorescence emission spectra have maxima at 340 nm, indicating complete exposure of the tryptophan residues to the solvent. (2) There is also loss of some tertiary as well as secondary structure as depicted by near and far UV CD spectroscopy. Altogether the earlier studies have shown that the productive folding pathway of horse cytochrome c, starting from the unfolded polypeptide, proceeds through a compact intermediate, I_c^{HW}, in which the native His18 ligand and a water molecule are coordinated to the heme iron, followed by formation of the native His18-Fe-Met80 heme coordinated form, $N^{(HM)}$, during the final stages of folding (Ahmad, Madhusudana, & Bhakuni, 2000; Rumbley, Hoang, & Mayne, 2001; Shastry & Roder, 1998; Telford, Teczan, Gray, & Winkler, 1999; Yeh & Rousseau, 1998). Moreover unfolding experiments on different cytochromes c indicated that the Fe-Met80 deligation step is delimited by a native-like transition state (Dyson & Beattie, 1982; Gianni et al., 2003; Goto & Fink, 1994; Goto, Hagihara, Hamada, Hoshino, & Nishii, 1993) involving a high-energy intermediate (M). Therefore a general scheme that describes the folding of cytochrome c is:

$$U \leftrightarrow I_c{}^{(HW)} \leftrightarrow M(HW) \leftrightarrow N^{(HM)}$$

In the presence of alcohols, a parallel pathway in protein folding has been also reported by kinetic studies on cytochrome c_{551} (Gianni et al., 2003) from *Pseudomonas aeruginosa*. Goto, Calciano, et al. (1990) have reported an intermediate state (A) that retains 31% α-helix while intermediate obtained in our studies at acidic pH in the presence of butanol and SDS retains 29.8 and 28% α-helix, respectively. At the two extremes of pH, in presence of butanol (7.2 M) and sodium dodecyl sulphate (5 mM) the

states I_1 and I_2 though different also have properties in common. These include the following: (i) The far UV CD spectra shows that the secondary structure content of states I_1 and I_2 is similar to that of the native state, although the retention of the helical content is not same as that of native. (ii) The tryptophan fluorescence spectra are similar with a maximum at 335 nm, blue shifted from unfolded to native state, indicating an internalization of tryptophan. The fluorescence emission of state I_1 and I_2 is substantially quenched compared to that of the unfolded protein, as cytochrome c is a heme protein. (iii) Both I_1 and I_2 bind ANS strongly, as compared to unfolded states whereas the hydrophobic dye does not bind to the native protein.

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