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8-Anilino-1-naphthalene Sulfonic Acid (ANS) Induces Folding of Acid Unfolded Cytochrome *c* to Molten Globule State as a Result of Electrostatic Interactions[†,‡]

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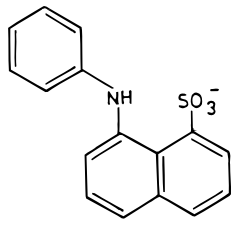
ABSTRACT: Hydrophobic interaction of 8-anilino-1-naphthalene sulfonic acid (ANS) with proteins is one of the widely used methods for characterizing/detecting partially folded states of proteins. We have carried out a systematic investigation on the effect of ANS, a charged hydrophobic fluorescent dye, on structural properties of acid-unfolded horse heart cytochrome *c* at pH 2.0 by a combination of optical methods and electrospray ionization mass spectroscopy (ESI MS). ANS was found to induce, a secondary structure similar to native protein and quenching of fluorescence of tryptophan residue, in the acid-unfolded protein. However, the tertiary structure was found to be disrupted thus indicating that ANS stabilizes a molten globule state in acid-unfolded protein. To understand the mechanism of ANS-induced folding of acid-unfolded cytochrome *c*, comparative ESI MS, soret absorption, and tryptophan fluorescence studies using nile red, a neutral hydrophobic dye, and ANS were carried out. These studies suggested that, at low pH, electrostatic interactions between negatively charged ANS molecules and positively charged amino acid residues present in acid-unfolded cytochrome *c* are probably responsible for ANS-induced folding of acid-unfolded protein to partially folded compact state or molten globule state. This is the first experimental demonstration of ANS induced folding of unfolded protein and puts to question the usefulness of ANS for characterization/determination of partially folded intermediates of proteins observed under low pH conditions.

8-Anilino-1-naphthalene sulfonic acid (ANS),[1] a charged



8-Anilio-1-naphthalene sulfonate

(1)

hydrophobic fluorescent molecule of molecular mass 673 Da, and its dimeric form bis-ANS have become almost standard for diagnosing the population of compact partially folded intermediates of proteins (*1*, *2*). The "preferable interaction" of this dye with equilibrium and kinetic compact intermediates in relation to native and completely unfolded proteins is well established. ANS and bis-ANS are minimally fluorescent in polar environments, such as aqueous solutions (quantum yield about 0.004), but their fluorescence emission is dramatically increased in nonpolar environments (*3*, *4*), like on binding to apomyoglobin (quantum yield as high as 0.98). The partially folded states of proteins have clusters of hydrophobic side chains that are not yet fully occluded in the native core structure and provide binding sites for ANS (*1*, *2*). Although ANS and bis-ANS have frequently been used in identification of partially folded intermediates, however, possible effects of dye on the conformation and stability of proteins have largely been overlooked. Some recent equilibrium studies have shown that relatively higher concentrations of ANS and bis-ANS significantly perturb the protein conformation. ANS binding to the nucleotide-binding site on DnaK resulting into conformational change in protein has been reported (*5*). Photoincorporation of bis-ANS into the N-terminal and apical domain of $\alpha\beta$-crystalline and GroEL, respectively, resulting in diminishing of their chaperone-like activity has been observed (*6*, *7*). Hence, the considerations for ANS induced structural changes in proteins is of significant relevance when use of this dye to detect partially folded intermediate of proteins is considered.

The importance of electrostatic interactions in determining the stability of proteins has long been recognized (*8*). Proteins on acid denaturation are unfolded at low pH because of the presence of destabilizing repulsive interactions between like charges in the protein (*9*, *10*). Salts have been known to affect the physicochemical properties of the proteins such as their solubility (*11*), stability (*12*) and pK_a (*13*) to a great extent. Salts affect mainly the electrostatic interactions in the protein molecules. In presence of salts, a conformation transition at acidic or alkaline pH from a largely unfolded state to an

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[1] Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; SDS−PAGE, sodium dodecylsulfate−polyacrylamide gel electrophoresis; far-UV CD, far-ultraviolet circular dichroism; ESI, electrospray ionization; MS, mass spectroscopy.

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intermediate conformational state have been reported for several small globular and even multimeric proteins (*14*−*17*). Solvent-dependent changes of charge repulsion has been suggested to be responsible for this change (*16*, *17*).

Cytochrome *c* carries a large component of basic residues; 24 of its 104 residues are lysine, arginine, or histidine. At acidic pH, the compensating negatively charged residues are neutralized, thus enhancing the global-scale electrostatic repulsion which drives the compact globular protein toward a more expanded form, as under these conditions, many tertiary structural interactions that stabilize the native protein are lost. Horse ferricytochrome *c* is substantially unfolded under conditions of low salt at pH 2.0. In acidic solution containing high salt concentration (*17*−*19*) or when the charges are neutralized by acetylation (*20*), the molten globule state of cytochrome *c* had been observed.

We have studied the structural changes associated with binding of ANS molecules to horse cytochrome *c* at pH 2.0. Cytochrome *c* was chosen for the study as it offers a large number of optical probes that allow a detail characterization of the protein conformation. Furthermore, several partially folded intermediates of this protein have been reported to be stabilized at acidic pH, depending on the salt concentration and temperature (*19*, *21*, *22*).

MATERIALS AND METHODS

Materials. All the chemicals used in the study were purchased from Sigma Chemical Co., St. Louis, and were of the highest purity grade. The purity of horse cytochrome *c* (type IV) was checked by SDS−PAGE followed by silver staining and found to be >95% pure.

Methods. All the measurements were carried out at 27 °C. Typically, cytochrome *c* stock solution (1 mg/mL), which had been dialyzed against deionized water was mixed with appropriate HCl concentration and incubated for 1 h before addition of ANS. After addition of ANS, the pH of the solution was again checked and maintained at 2.0. For various studies in presence of ANS, the samples were incubated for 2 h in presence of ANS before carrying out the measurements. The concentration of native cytochrome *c* was determined from the extinction coefficient of 1.06×10^5 M^{-1} cm^{-1} at 410 nm at pH 7.0 (*20*). For other protein samples, the concentration was measured by the method of Lowry et al. (*23*).

The Soret absorption of heme group was monitored with Shimadzu double beam spectrophotometer UV-240, using 2 mm light path.

CD was measured with JASCO J500 spectropolarimeter calibrated with ammonium d-10-camphorsulfonate. A 0.1 cm path-length cell was used, and the spectra were recorded in the wavelength range 190−250 nm. Protein concentrations were about 0.35 mg/mL. The results are expressed as the mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100 \times \theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of light path in centimeters. All the spectra were analyzed by subtracting the solvent baseline from the recorded spectra.

Fluorescence spectra were recorded with Perkin-Elmer LS 5B spectroluminescencemeter in a 5 mm path-length quartz cell. Samples containing different concentrations of GdmCl were equilibrated for 3 h at 27 °C before recording. For tryptophan fluorescence measurements the excitation wavelength was 290 nm and the emission was recorded from 300 to 500 nm. Protein concentration was 7 μM for all experiments. All the spectra were normalized for the solvent contribution to fluorescence.

Fluorescence energy transfer studies were carried out using Perkin-Elmer LS 5B spectroluminescencemeter in a 5 mm path-length quartz cell. Samples containing different concentrations of ANS were equilibrated for 3 h at 27 °C before recording. The excitation wavelength was 290 nm, and the emission was recorded from 305 to 550 nm. Protein concentration was 7 μM for all experiments. All the spectra were normalized for the solvent contribution to fluorescence.

ESI MS Measurements. The mass spectra were recorded on a MICROMASS QUATTRO II mass spectrometer (Micrpmass, Altrimcham, U.K.) equipped with an electrospray ionization ion source. The sample solution (20 μM of protein was required to obtain a good spectra) was introduced into the ion source using a Harvard Apparatus model 11 Syringe pump. In order to improve the sensitivity and stability of the spectra of the ion beam, the source was operated at 80 °C; however, the spectra of the samples were also recorded at 30 °C and were found to be similar. The electrospray capillary was set at 3.5 kV and the cone voltage at 20 V. Nitrogen was used as the neubalizing and drying gas at a flow rate of 15 and 300 L/h, respectively. The signal stability and intensity for ESI MS were significantly increased by adding a small amount of methanol (3%) to the aqueous solution of cytochrome *c*. The spectra were acquired and processed using MassLynx software supplied with the instrument. The mass spectrometer was scanned from *m*/*z* 500 to 2200 in 6 s, and the spectra presented are average spectra of seven to eight scans. The charge states were determined by MassLynx software, and the deconvolution of the spectra was carried out using MaxEnt software.

RESULTS

Effect of ANS on the structural features of acid-unfolded cytochrome *c* at pH 2.0 were studied by monitoring the changes in tryptophan fluorescence, Soret absorption, far-UV CD, and ESI mass spectroscopy of the protein in the presence of increasing ANS concentrations.

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. In the native protein, the fluorescence of tryptophan is completely quenched due to resonance energy transfer to the adjacent heme group attached to cystines at position 14 and 17 (*18*, *24*). In the salt-induced molten globule of cytochrome *c* at pH 2.0 also, quenching of tryptophan fluorescence similar to that of native protein has been reported (*18*, *24*). However, acid-induced unfolding of the protein at low pH (2.0), leads to an increase in tryptophan fluorescence due to decrease in quenching efficiency as a result of increased distance between heme and tryptophan in the unfolded protein. Furthermore, the fluorescence emission maxima is observed at 345 nm, indicating the exposure of tryptophan residue to the solvent in the acid-unfolded protein (*18*).

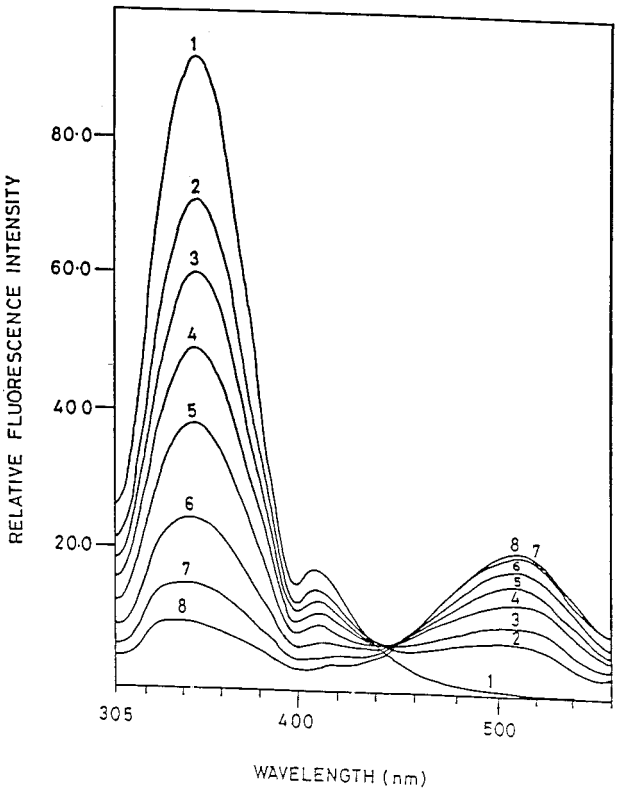


FIGURE 1: Fluorescence energy transfer from acid-unfolded cytochrome *c* to ANS at pH 2.0. Fluorescence emission spectra of samples with 7 μM cytochrome *c* in the presence of (1) 0, (2), 2.5, (3) 5, (4) 7.5, (5) 10, (6) 15, (7) 20, and (8) 25 ANS:protein (mol:mol) in 20 mM HCl scanned at 27 °C. The excitation wavelength was 290 nm. All measurements were carried out after the samples had been equilibrated for 3 h at 27 °C.

As the tryptophan residue in the acid-unfolded cytochrome *c* is exposed to the solvent, the quenching of tryptophan fluorescence in the presence of ANS can be due to two possibilities: (i) fluorescence energy transfer between tryptophan residue of the protein and ANS molecules present in the solvent, as tryptophan and ANS make a good fluorescence energy-transfer donor/acceptor combination due to overlap of tryptophan emission and ANS excitation (*25*), and (ii) ANS-induced folding of acid-unfolded protein leading to burial of exposed tryptophan moiety as is the case in native protein. To decipher between these possibilities, fluorescence energy-transfer studies were carried out.

Figure 1 summarizes the results of fluorescence energy-transfer studies on cytochrome *c* at pH 2.0 in the presence of increasing ANS concentration. Addition of increasing concentration of ANS resulted in a significant quenching of tryptophan fluorescence and at ANS to protein ratio of $\geq$20:1 (mol/mol), almost complete loss of fluorescence signal was observed. However, no significant change in wavelength of emission of maximum fluorescence (510 nm) of ANS was observed under these conditions. Furthermore, the changes in fluorescence intensity of ANS at 510 nm with increasing concentration of dye in the presence of protein as observed in Figure 1 was found to be similar to that observed for free dye (in absence of protein) at similar ANS concentrations (data not shown), indicating the absence of any hydrophobic interaction between the ANS molecules and protein under these conditions. The above presented observations suggest that the quenching of tryptophan fluorescence of acid-unfolded cytochrome *c* in the presence of increasing ANS concentration is due to burial of tryptophan residue of protein as a result of folding of the unfolded protein.

Soret absorption of cytochrome *c* reflects the spin state of iron and can be used to monitor changes in the ligation and spin state of the heme iron and, hence, in gaining information about the tertiary structure around the heme iron (*26*, *27*). In the native state, heme iron is ligated by His18 and Met80 (*26*, *27*). Coordination of the iron with these strong-field protein ligands produces a low-spin complex with Soret absorption maximum at 410 nm. The acid-unfolding of the protein leads to displacement of both protein ligands from the heme iron, which are consequently replaced by two weak-field ligands from the solvent, such as water, thus producing a high-spin complex with Soret absorption maximum at 394 nm and a maximum ϵ value. In the molten globule state (cytochrome *c* at pH 2.0 in the presence of 500 mM NaCl), a mixed-spin state is observed with Soret absorption maximum at 397 nm and an almost similar ϵ as that of native protein but significantly lesser than that of the unfolded protein. Hence, Soret absorption seems to be the only optical method which can clearly distinguish the three states namely, native, unfolded, and molten globule of cytochrome *c*.

Figure 2 shows the effect of addition of increasing ANS concentration on the Soret absorption of acid-unfolded cytochrome *c*. For cytochrome *c* at pH 2.0, the Soret absorption maxima at 394 nm was observed. Addition of increasing concentration of ANS resulted in decrease in Soret absorption along with a slight red shift in the wavelength maxima of emission. The effect of ANS on the Soret absorption of acid-unfolded cytochrome *c* can be classified in two stages. In the initial stage, at low ANS concentration (0$-$10 ANS:protein mol:mol), there is a significant decrease in intensity of absorption with a slight red shift in the wavelength maximum of absorption. However, in the final stage, at ANS concentration range $>$15:1 mol:mol, ANS: protein, the decrease in Soret band absorption intensity is accompanied with a significant red shift in the wavelength maxima (maximum of 396 nm at ANS:protein 25:1 mol:mol) of Soret absorption. These results indicate that in the presence of higher ANS concentration the acid-unfolded cytochrome *c* undergoes partial folding.

The effect of ANS on the secondary structure of acid-unfolded cytochrome *c* was studied by CD measurements. The far-UV region of the CD spectrum monitors changes in the secondary structure of protein (*28*). Figure 3 shows the far-UV CD spectra of acid-unfolded cytochrome *c* and that in the presence of ANS. For acid-unfolded cytochrome *c* at pH 2.0, a far-UV CD spectra similar to that observed for protein in the presence of 6 M guanidine hydrochloride was observed, suggesting an absence of defined secondary structure under these conditions. However, in the presence of ANS (ANS:protein, 20:1 mol:mol) two distinct bands around 208 and 222 nm are observed indicative of presence of high degree of helicity in protein under these conditions (*28*). In fact, the far-UV CD spectra of ANS-induced partially folded intermediate observed at ANS:protein ratio of 20:1 (mol:mol) was very similar to that of native protein. These observations suggest that ANS induces significant secondary structure in acid-unfolded cytochrome *c*.

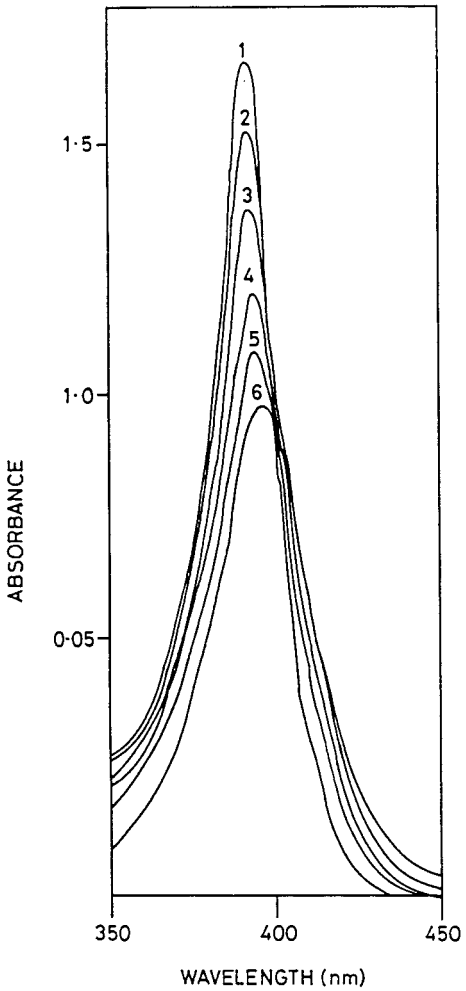
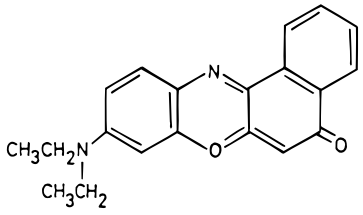


FIGURE 2: Soret absorption spectra of cytochrome *c* at pH 2.0 as a function of increasing ANS concentration at 27 °C. Soret absorption spectra of samples with 7 μM cytochrome *c* in the presence of (1) 0, (2), 5, (3) 10, (4) 15, (5) 20, and (6) 25 ANS: protein (mol:mol) in 20 mM HCl scanned at 27 °C. All measurements were carried out after the samples had been equilibrated for 3 h at 27 °C.

Figure 4 shows the changes in Soret absorption and tryptophan fluorescence of acid-unfolded cytochrome *c* in the presence of nile red at pH 2.0. No significant changes in



Nile Red
(2)

the Soret absorption or tryptophan fluorescence spectra of acid-unfolded cytochrome *c* on addition of even high concentration of dye (nile red: protein 15:1 mol/mol) were observed. These observations indicate that even high concentrations of neutral hydrophobic dye, nile red, does not induce any significant structural changes in the acid-unfolded cytochrome *c*.

Figure 5 shows the ESI mass spectra of cytochrome *c*, its acid-unfolded state at pH 2.0, and that in the presence of

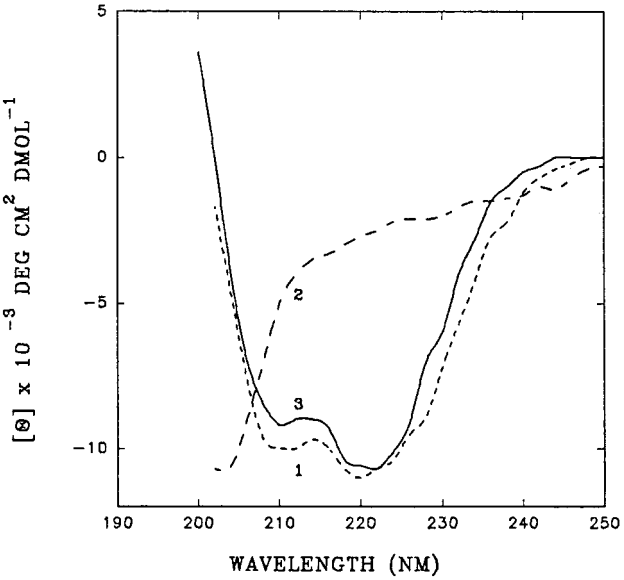


FIGURE 3: Far-UV CD spectra of native and acid denatured cytochrome *c* at pH 2.0 in the presence and absence of ANS. The various curves are (1) cytochrome *c* (pH 7.0), (2) acid denatured cytochrome *c* at pH 2.0 and (3) acid denatured cytochrome *c* in presence of ANS: protein ratio of 20:1 mol:mol, at pH 2.0. All measurements were carried out after the samples had been equilibrated for 3 h at 27 °C.

ANS and nile red. For native protein (pH 6.4), a relatively narrow distribution of charge states with 8^+ being the most intense peak was observed (Figure 5A). Lowering the pH to 2.0 by formic acid resulted in shift of the maximum of charge state distribution to 15^+ (Figure 5B). Addition of nile red (nile red:protein 8:1 mol:mol) under these conditions did not bring about any change in the spectra of acid-denatured protein (pH 2.0) as again a maximum of charge state distribution to 15^+ was observed (Figure 5C). However, for addition of ANS (ANS:protein 8:1 mol:mol) to acid-denatured protein at pH 2.0, a complex spectra with maximum of charge state distribution shifted to 14^+ was observed. Earlier reports on ESI MS studies on RNase A (*29*) and acid-induced unfolding of cytochrome *c* (*30*) have demonstrated that charge state distribution generated during ESI is not sensitive to the changes in the secondary structure of protein during denaturation but highly sensitive to changes in tertiary structure. So the above-presented results suggest that addition of nile red does not bring any significant change in tertiary structure of acid-unfolded cytochrome *c* at pH 2.0; however, a significant change in tertiary structure of protein is observed in the presence of ANS under similar conditions.

Figure 6 shows the transformed ESI mass spectra of cytochrome *c* at pH 2.0 and that in the presence of nile red and ANS (dye:protein 8:1 mol:mol). For acid-denatured protein at pH 2.0 and in the presence of nile red, a single population of the molecular species of 12 360 Da, corresponding to the molecular mass of protein was observed (Figure 6, panels A and B). However, in the presence of ANS under similar conditions, apart from the peak of parent protein, peaks corresponding to the ANS−protein complexes of various stoichiometry ($n = 1-7$ where n = number of ANS molecules per protein molecule) were observed. Furthermore, the relative population of ANS−protein complexes was far greater as compared to that corresponding to

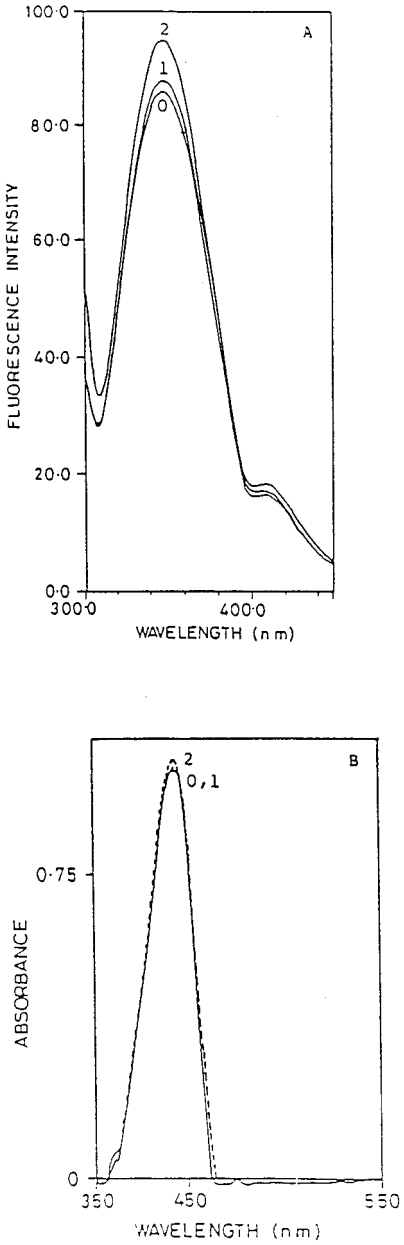


FIGURE 4: (A) Fluorescence emission spectra of samples with 7 μM cytochrome *c* in the presence of (1) 0, (2) 7, and (3) 15 nile red:protein (mol:mol) in 20 mM HCl scanned at 27 °C. The excitation wavelength was 290 nm. All measurements were carried out after the samples had been equilibrated for 3 h at 27 °C. (B) Soret absorption spectra of samples with 7 μM cytochrome *c* in the presence of (1) 0, (2) 7, and (3) 15 nile red: protein (mol:mol) in 20 mM HCl scanned at 27 °C. All measurements were carried out after the samples had been equilibrated for 3 h at 27 °C.

the native protein. At higher ANS concentration (ANS:protein 10:1 mol:mol), no peak corresponding to protein was observed as all the peaks were of ANS−protein complexes of different stoichiometry. These observations demonstrate that ANS binds strongly with acid-unfolded cytochrome *c* at pH 2.0, whereas, no binding of nile red with protein was observed under similar conditions.

DISCUSSION

For cytochrome *c*, a variety of optical methods are available to monitor the conformational changes in solution. Changes in the heme absorption and tryptophan fluorescence specifically monitor changes in the tertiary structure of the protein whereas the far-UV CD spectrum is a probe for changes in secondary structure. For cytochrome *c* at pH 2.0, a significant tryptophan fluorescence with emission maxima at 345 nm, a Soret band absorption at 394 nm, and a far-UV CD spectra similar to that reported for unfolded protein was observed. These observations suggest that acid unfolding of cytochrome *c* at pH 2.0 leads to a breakdown of both the protein tertiary and secondary structure. Addition of increasing concentration of ANS to acid-denatured cytochrome *c* at pH 2.0 resulted in significant changes in the heme absorption, tryptophan fluorescence, and far-UV CD properties. In presence of higher ANS concentrations, the tryptophan fluorescence was found to be significantly quenched ($\sim$ 85% as compared to acid-unfolded protein), suggesting that under these conditions the exposed tryptophan moiety present in the unfolded protein comes close to the heme moiety as is the case in native protein. Soret absorption showed a red shift in absorption maxima, and a significant loss in the absorption intensity as compared to acid-unfolded protein again indicated folding of acid-unfolded protein. The far-UV CD spectra of acid-unfolded cytochrome *c* showed induction of a high degree of α-helical structure by ANS and at higher ANS concentrations a far-UV CD spectra similar to the native protein was observed. However, no changes in tertiary structure as compared to acid-unfolded protein were observed in the presence of ANS, as studied by ESI mass spectroscopy. These changes in the structural properties of acid-unfolded cytochrome *c* in the presence of ANS collectively suggest that ANS induces folding of acid-unfolded protein to molten globule state, as similar changes have been reported for the salt-induced transition from acid-unfolded protein to molten globule intermediate of cytochrome *c* (*21*). In contrast to this, with increasing concentrations of nile red, a neutral hydrophobic dye, no change in tryptophan fluorescence, Soret absorption, or ESI mass spectra of acid-unfolded protein were observed, suggesting that nile red does not bring about folding of acid-unfolded cytochrome *c*.

The main forces that are important for protein structure are the hydrophobic interactions, the valence forces, the dispersion forces and the repulsive forces (*31*). It is well-known that on decreasing the pH from neutrality to approximately 2.0, proteins become maximally positively charged, since the p*K*s of most carboxylic groups are $\geq$3. The resulting intramolecular repulsion between the positively charged groups is the driving force for acid unfolding of proteins as they fail to overcome the interactions such as hydrophobic forces, salt bridges, and metal ion−protein interactions that favor folding. Anions reduce this electrostatic repulsion by Debye−Huckel screening effect and by the interaction with positive charges by ion-pair formation (or anion binding) These may result in the manifestation of the intrinsic hydrophobic interactions of proteins which favor folding. Anion-dependent conformational transitions from unfolded to partially folded compact state under acidic conditions have been observed for several proteins (*32*−*35*), suggesting the importance of charge repulsion in the stability of acidic molten globules. Various strong acids such as sulfuric acid, perchloric acid, nitric acid, trichloracetic acid, etc., and their neutral salts have been reported to refold the

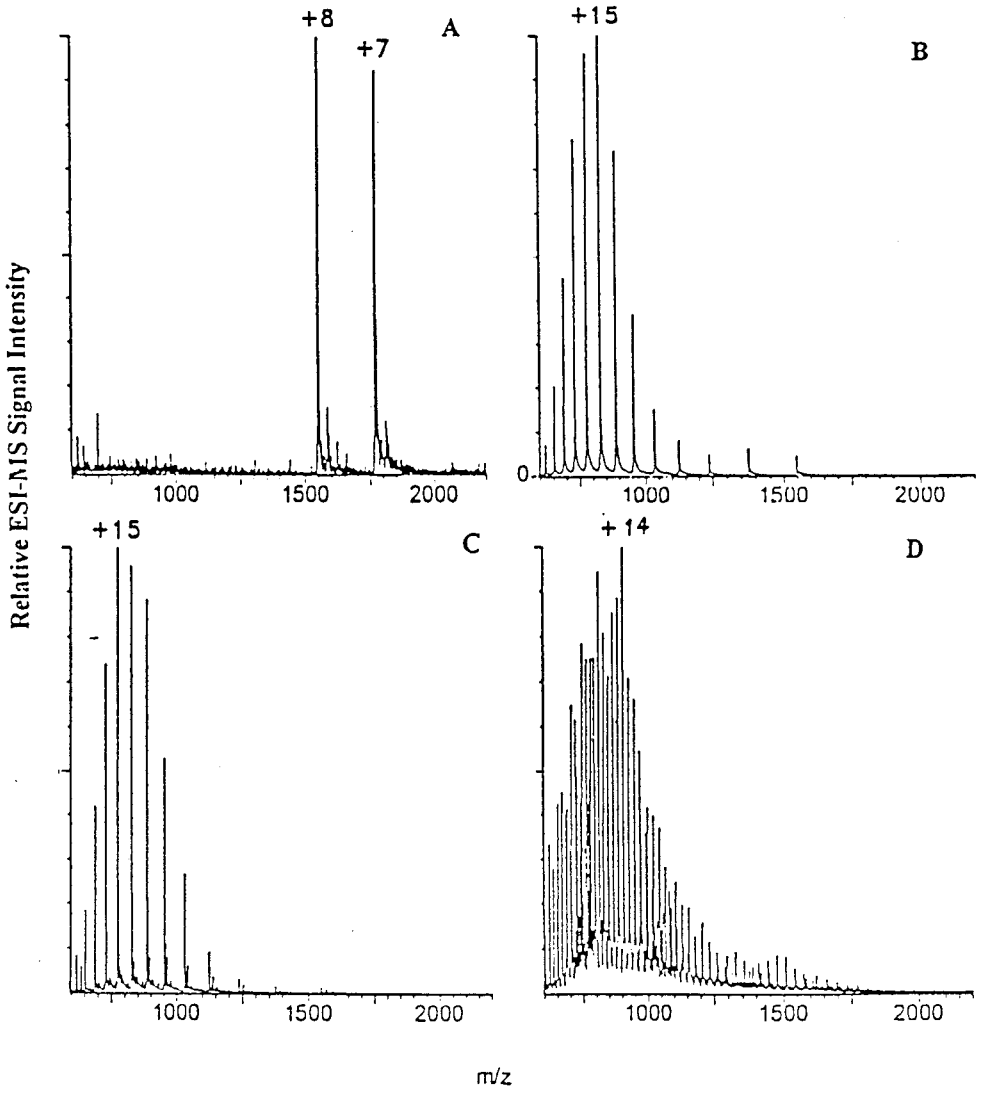


FIGURE 5: ESI mass spectra of cytochrome *c* in water containing (A) 0.5 mM ammonium acetate, pH 6.4, (B) pH 2.0, (C) nile red (8:1 nile red:protein mol:mol) at pH 2.0 (D) ANS (8:1 ANS:protein mol:mol) at pH 2.0. The pH of the samples to 2.0 was adjusted by formic acid.

acid-unfolded cytochrome *c* to a partially folded compact intermediate state, thus confirming that the key factor in the anion-induced folding of acid-denatured cytochrome *c* is the affinity of the anions for the ammonium ions of proteins (*36*). The effectiveness of different anions in promoting structure in the acid-unfolded cytochrome *c* has been reported to vary dramatically (K_ds varying from micromolar to molar) and follows the electroselectivity series rather than Hofmenister series (*36*).

The fluorescence energy-transfer studies of cytochrome *c* in the presence of ANS at pH 2.0 as reported in this paper; showed no change in ANS fluorescence intensity or wavelength of emission of the free dye as compared to that in the presence of acid-unfolded protein. As the fluorescence emission of ANS is known to increase with a red shift in emission maxima on binding to hydrophobic clusters of a protein (*3*, *4*) these observations suggest that there is no hydrophobic interaction between ANS and acid-unfolded cytochrome *c* at low pH. The absence of hydrophobic interactions between ANS and acid-unfolded cytochrome *c* is further strengthened by the ESI MS studies using nile red. The transformed ESI mass spectra of acid-unfolded cytochrome *c* in the presence of ANS at pH 2.0 showed a large relative population of molecular species corresponding to ANS−protein complexes of various stoichiometry. However, under similar conditions, no complex formation of acid-unfolded protein with nile red was observed, as only single population of the molecular species corresponding to the protein was observed. These observations demonstrate that nile red does not interact with acid-unfolded protein whereas under similar conditions a very strong interaction between ANS and acid-unfolded protein was observed. Nile red (*2*) is a neutral hydrophobic fluorescent dye which can interact with unfolded protein only by hydrophobic interactions whereas ANS is a charged hydrophobic dye (*1*) having negative sulfonate anion at pH $\leq$3.6 (*37*) and can interact both by hydrophobic as well as electrostatic interactions depending on the pH studied. Such an electrostatic interaction between ANS and charged amino acid residues protein at low pH has been reported for ANS−α-chymotrypsin complex at pH 3.6 by X-ray studies (*37*). Recently, Matulis and Lovrien (*38*) have reported that the ANS^- anion can bind dominantly and strongly to cationic groups of water-soluble proteins and polyamino acids through ion-pair formation. Using titration calorimetry they demonstrated that ANS binding to a number of proteins depends on protein cationic charges and solution pH and occurs largely through ANS sulfonate group. At pH 2.0, cytochrome *c* has a large net

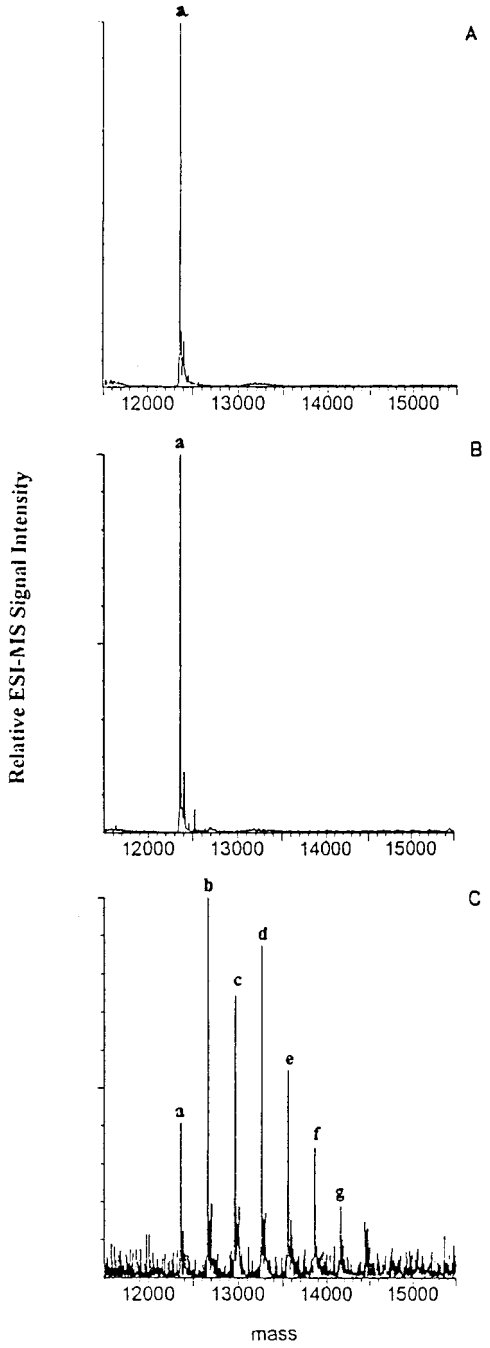


FIGURE 6: Transformed ESI mass spectra of cytochrome *c* in water containing (A) formic acid, pH 2.0 (B) nile red (8:1 nile red:protein mol:mol) at pH 2.0 (C) ANS (8:1 ANS:protein mol:mol) at pH 2.0. The pH of the samples to 2.0 was adjusted by formic acid. In the figures "a" denotes the signal corresponding to the native protein and "b to g" corresponding to that observed for ANS−protein complexes with increasing number of ANS molecules bound, i.e., 1 to 7 ANS−protein, respectively.

positive charge of +24, and ANS a negative sulfonate ion, hence, the two molecules can interact by ion-pair binding resulting in stabilization of ANS−protein complex. The presence of such a complex at pH 2.0 was confirmed by the ESI MS studies as presented above. The ion-pair binding between the sulfonate anion and the cationic groups of acid-unfolded cytochrome *c* would lead to effective shielding of repulsive forces responsible for unfolding of protein at low pH, resulting in folding of acid-unfolded protein to molten globule state.

ANS-induced stabilization of molten globule intermediate of acid-unfolded cytochrome *c* as reported in this paper may be a general property of many proteins at low pH however, the ANS concentration required for this will depend on the particular protein. The observations presented in this paper strongly put to question the validity of use of ANS in detection of partially folded intermediates especially those observed under low pH conditions.

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