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Effects of Guanidine Hydrochloride on the Conformation and Enzyme Activity of Streptomycin Adenylyltransferase Monitored by Circular Dichroism and Fluorescence Spectroscopy

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Abstract—Equilibrium denaturation of streptomycin adenylyltransferase (SMATase) has been studied by CD spectroscopy, fluorescence emission spectroscopy, and binding of the hydrophobic dye 1-anilino-8-naphthalene sulfonic acid (ANS). Far-UV CD spectra show retention of 90% native-like secondary structure at 0.5 M guanidine hydrochloride (GdnHCl). The mean residue ellipticities at 222 nm and enzyme activity plotted against GdnHCl concentration showed loss of about 50 and 75% of secondary structure and 35 and 60% of activity at 0.75 and 1.5 M GdnHCl, respectively. At 6 M GdnHCl, there was loss of secondary structure and activity leading to the formation of GdnHCl-induced unfolded state as evidenced by CD and fluorescence spectroscopy as well as by measuring enzymatic activity. The denaturant-mediated decrease in fluorescence intensity and 5 nm red shift of λ_{max} point to gradual unfolding of SMATase when GdnHCl is added up from 0.5 M to a maximum of 6 M. Decreasing of ANS binding and red shift (~5 nm) were observed in this state compared to the native folded state, indicating the partial destruction of surface hydrophobic patches of the protein molecule on denaturation. Disruption of disulfide bonds in the protein resulted in sharp decrease in surface hydrophobicity of the protein, indicating that the surface hydrophobic patches are held by disulfide bonds even in the GdnHCl denatured state. Acrylamide and potassium iodide quenching of the intrinsic tryptophan fluorescence of SMATase showed that the native protein is in folded conformation with majority of the tryptophan residues exposed to the solvent, and about 20% of them are in negatively charged environment.

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Protein folding is one of the most intensely investigated areas of structural biology. Although the acquisition of a native, biologically functional conformation by a linear polypeptide chain in the absence of cellular factors and without input of energy has been the central dogma of protein folding, participation of additional factors such as peptidyl disulfide or prolyl isomerase [1] and molecular chaperones such as GroEL [2] and crystallins [3] that enhance protein folding efficiencies have been demonstrated. Protein misfolding has been shown to be a cause of serious diseases [4].

Virtually all studies of the protein-folding reaction add heat, acid, or a chemical denaturant to an aqueous protein solution in order to perturb the protein structure. When chemical denaturants are used, very high concentrations are usually necessary to observe any change in protein structure. In a solution with such high denaturant concentrations, both the structure of the protein and the structure of the solvent around the protein can be altered. Despite their routine use, our understanding of the molecular mechanism by which chemical denaturants like urea or guanidine hydrochloride (GdnHCl) cause a protein to unfold is still rather limited. While it does seem clear that chemical denaturants interact directly with the protein [5], it is also clear that at high concentrations these molecules can cause substantial changes to the behavior of the solvent itself [6]. The relative importance of these two

Abbreviations: ANS) 1-anilino-8-naphthalene sulfonic acid; DTT) dithiothreitol; GdnHCl) guanidine hydrochloride; MRE) molar residue ellipticity.

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effects in protein unfolding is still ambiguous. The location of the denaturant-binding sites is also unclear.

Suggestions have been made that denaturants unfold proteins by migrating into the interior of the protein and forming hydrogen bonds to atoms in the backbone [7]. In support of such a model, the exchange rates of some peptide NH protons, which are found on both the surface and the interior of a protein, are decreased in the presence of GdnHCl [8]. In addition, crystallographic studies of diketopiperazine and urea show hydrogen bonding between the amide groups and the urea [9]. Other models have suggested that denaturants act by decreasing the hydrophobic effect [10, 11], which, in this instance, is usually postulated to be the dominant force stabilizing protein structure.

Equilibrium denaturation studies, to date, have focused primarily on monomeric globular proteins. These studies demonstrate that equilibrium denaturation can provide a sensitive measure of structural phenomena that may not be readily apparent, even in high-resolution crystallographic structures [12, 13]. GdnHCl-induced denaturation studies of streptomycin adenylyltransferase (SMATase) were undertaken with the purpose of defining structural characteristics necessary for studying structure/function relationship of SMATase through spectroscopic analysis.

Streptomycin is the most active aminoglycoside against *Mycobacterium tuberculosis* including many multidrug resistant strains [14]. The bactericidal activity of streptomycin is due to inhibition of bacterial protein synthesis through binding to prokaryotic 16S rRNA and disruption of the integrity of the bacterial cell membrane [15]. The major problem in the use of this broad-spectrum antibiotic is the acquisition of resistance to it. One of the mechanisms of resistance is inactivation of streptomycin via ATP-dependent O-adenylation catalyzed by SMATase [16]. SMATase catalyzes the transfer of the adenyl group from ATP into hydroxyl groups at 3′ and 6′ position of the streptomycin molecule [17]. SMATase is of considerable interest because of its role in the problem of streptomycin resistance, which has led to discontinuation of use of this broad-spectrum antibiotic against tuberculosis [18, 19]. SMATase is one of the important aminoglycoside-modifying enzymes under the category of nucleotidyltransferases. Although it is 35 years since microbial resistance to streptomycin was reported, no detailed spectroscopic analysis of SMATase has been described to date.

In this paper, we present a detailed analysis of the native and denatured states of SMATase in terms of their spectroscopic properties by far-UV CD spectroscopy, intrinsic tryptophan (Trp) fluorescence measurement, 1-anilino-8-naphthalene sulfonic acid (ANS) binding fluorescence measurement, and potassium iodide and acrylamide quenching of the intrinsic tryptophan fluorescence. To the best of our knowledge, this is the first report on the structural and functional properties of SMATase by CD and fluorescence spectroscopy.

MATERIALS AND METHODS

Chemicals. ANS and streptomycin sulfate were obtained from Sigma (USA). High purity grade GdnHCl, acrylamide, potassium iodide, and sodium thiosulfate were purchased from Merck (India). [α-^{32}P]ATP (3000 mCi/mol) was from BRAC (India). All other reagents used were of analytical grade.

Buffers and solutions. The buffers used for the spectroscopic measurements were 20 mM Tris-HCl (pH 7.8) and 20 mM sodium cacodylate (pH 7.8). Unfolding conditions were provided by stock of GdnHCl (6 M). ANS concentration was calculated spectrophotometrically using extinction coefficient 5000 $M^{-1}\cdot cm^{-1}$ at 350 nm. All the solutions were prepared in deionized water and filtered through a 0.45-μm filter.

Protein purification and quantification. SMATase was purified as previously described [20]. After purification, the recovered protein was dialyzed (in spectropore tubing of 15 kD cut off) against two changes of 500 volumes of 20 mM sodium cacodylate buffer, pH 7.8. Protein purity was assessed by SDS-PAGE followed by Coomassie brilliant blue R-250 staining. All protein preparations showed greater than 99% purity when assessed by these methods. The protein concentration was determined by the protein–dye binding method of Bradford using bovine serum albumin as the standard [21].

Enzyme assay. Protein samples (7 μM) were incubated with various concentrations of GdnHCl (0.5-6.0 M) at 37°C for 30 min prior to enzyme assay. The enzyme activities of denatured protein samples were determined by the method of Hass and Dowding [22]. The assay mixture contained 10 μl of assay buffer (20 mM Tris-HCl, pH 7.8, 1.25 mM $MgCl_2$, 0.5 mM NH_4Cl, 1 mM EDTA), 10 μl of [α-^{32}P]ATP (3000 mCi/mol), 10 μl of the denatured protein sample, and 2 μl of streptomycin (1 mg/ml). The reaction mixtures were incubated at 37°C for 20 min. The samples withdrawn from assay mixtures were chilled and pipetted onto a membrane (1 cm^2) of Whatman P-81 phosphocellulose paper. After 5 min at room temperature, the papers were placed in 100 ml of hot distilled water (70-80°C) for 5 min. The liquid was then poured off and the operation repeated three times. The papers were rinsed four times with 100 ml of distilled water and dried at room temperature. These were then placed in 8-ml scintillation vials containing 4 ml of scintillation cocktail-O (contents per liter: 6 g PPO and 0.2 g POPOP in toluene) and counted using a Beckman LS 9800 liquid scintillation counter (Beckman, USA). The activity was expressed in terms of counts per minute (cpm).

GdnHCl-induced denaturation of SMATase. Denaturation of SMATase at pH 7.8 and room temperature in the

presence of GdnHCl was studied by far-UV CD spectroscopy. Increasing amounts of GdnHCl were added to a fixed concentration (7 μM) of protein and allowed to equilibrate for 30 min at 37°C before taking fluorescence and CD measurements. Molar residue ellipticity (MRE) values were calculated according to Chaudhuri et al. [23] and plotted against denaturant concentration.

The fraction of SMATase in denatured state (f_d) was defined as:

$$f_d = (RF_c - RF_0)/(RF_d - RF_0), \quad (1)$$

where RF_c is relative fluorescence intensity at any concentration of GdnHCl and RF_0 and RF_d are the fluorescence intensities in the native and denatured states, respectively.

CD measurements. CD spectra were recorded using a JASCO J-810 CD polarimeter (JASCO, Japan). Each spectrum represented the average of three accumulations recorded between wavelengths of 200 and 250 nm, with a 0.2-nm resolution, bandwidth of 0.5 nm, response time of 4 sec, sensitivity of 100 mdeg, and a scan speed of 20 nm/min using a cuvette of 0.1 cm pathlength. All spectra were corrected for background by the subtraction of the buffer blank. All measurements were made at 37°C. The molar residue ellipticity (θ) was calculated using the formula: $[\theta] = \theta_{obs}$ (in mdeg)/(molar concentration of the protein × path length (in mm) × number of amino acid residues in the protein) [23]. The CD intensities were expressed as molar residue ellipticity (MRE) with the unit (deg·cm^2·dmol^{-1}). The percentages of the different secondary structures (α-helix, β-sheet, β-turn, and random coil) were estimated (prediction errors in the range of 200-250 nm were 5%) according to Sreerama and Woody [24] using the Selcon 3 program.

Fluorescence studies. Steady-state fluorescence was recorded on a Perkin-Elmer LS55 spectrofluorimeter. Intrinsic tryptophan fluorescence spectra were recorded by exciting the samples at 280 nm with excitation and emission slit widths set at 5 nm. The emission spectra were recorded in the range of 300-400 nm. Baseline corrections were carried out with buffer without protein in all cases.

Binding of ANS. Binding of ANS to SMATase was studied by excitation of the dye at 350 nm and the relative emission spectra were recorded from 400 to 600 nm. The excitation and emission slit widths were set at 5 and 8 nm, respectively. The fluorescence emission spectra of two solutions (one with ANS alone, one with both ANS and the protein) were taken with excitation at 350 nm. The first solution contained 20 mM sodium cacodylate (pH 7.8) and 100 μM ANS. The second solution was prepared in the same way except that it contained 7 μM (final concentration) of SMATase. The protein solution was equilibrated at 37°C without ANS and then left for 30 min in the presence of ANS before measurements.

Denaturation of SMATase in the presence of ANS. Samples of SMATase (7 μM) were prepared in various concentrations of GdnHCl (0-6 M) and left for 30 min to equilibrate in the presence of ANS. Using the same conditions described in the previous section, the fluorescence intensity of ANS was recorded for each sample at 37°C.

Quenching studies. Fluorescence quenching experiments were carried out by the addition of a small aliquot of acrylamide and potassium iodide stock solutions (4 M) to the protein solution (7 μM) previously incubated at pH 7.8 at 37°C for 1 h, and the intrinsic tryptophan fluorescence intensities were determined (λ_{ex}, 280 nm; λ_{em}, 344 nm). To prevent I_3^- formation, 5-μl aliquots of 1 mM

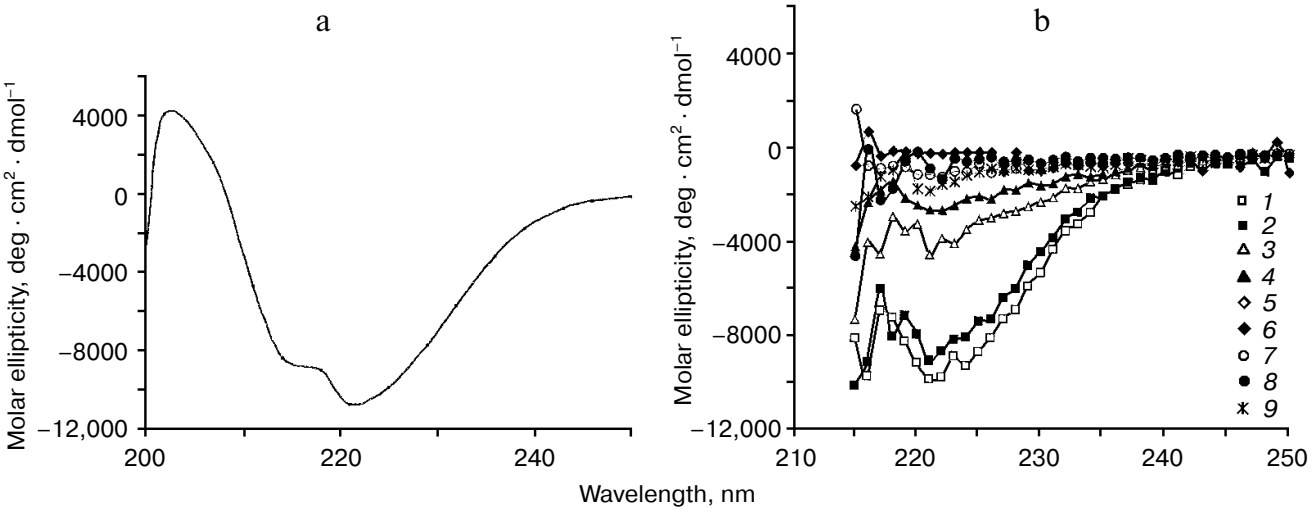

Fig. 1. Far-UV CD spectrum of native (a) and GdnHCl-denatured SMATase (7 μM) (b) in 20 mM sodium cacodylate, pH 7.8. b: *1-9*) 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 M GdnHCl, respectively. All spectra were corrected against the respective buffers-only spectra and averaged from three consecutive scans.

Secondary structure content of native and denatured SMATase by circular dichroism

SMATase	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
Native	65.7	8.4	4.0	21.9
Denaturated (6 M GdnHCl)	0.0	21.5	18.8	59.7

sodium thiosulfate were added to each 1 ml protein solution. Correction for the inner filter effect due to quencher was made using the formula:

$$F_{corr} = F_{obs} - \text{antilog}(A_{ex} + A_{em}/2),$$

where A_{ex} is the absorption at the excitation wavelength and A_{em} is the absorption at the emission wavelength. The quenching data were analyzed according to the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q], \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher [Q] and K_{SV} is the collisional quenching constant. For proteins containing more than one fluorescing tryptophan residue differentially exposed to the quencher, the Stern–Volmer plot will be nonlinear, and hence a modified Stern–Volmer equation has been applied:

$$F_0/(F_0 - F) = 1/K_{SV}[Q]{:}f_a + 1/f_a, \quad (3)$$

where F_0 is the fluorescence intensity in the absence of quencher, $(F_0 - F)$ is the difference in the fluorescence intensities in the absence and presence of the quencher [Q], and f_a is the fraction of tryptophan residues accessible to the quencher.

RESULTS AND DISCUSSION

CD spectra of the native and denatured SMATase. The CD spectra of the SMATase were recorded at 37°C in the far-UV (200-250 nm) region. Figure 1a shows the far-UV CD curve for the native state with two minima, at 212 and 222 nm, which is characteristic of α-helical proteins. The secondary structural component of the protein in the native and unfolded states was calculated from CD data using the Selcon 3 program [24]. The table shows the different fractions of secondary structure elements for this protein.

GdnHCl, which is a strong chemical denaturant, was utilized to determine whether this compound could effect complete unfolding, and the spectral changes observed clearly reflect loss of secondary structure. CD spectra were collected at 37°C as a function of different GdnHCl concentrations (0.5-6.0 M) to assess the folded state of SMATase protein. The stability of SMATase monomer is reflected in the low level of structure disruption (~10%) observed at 0.5 M GdnHCl (Fig. 1b). At 0.5-1.5 M GdnHCl concentrations, a considerable reduction of the regular conformations takes place suggesting that SMATase does not preserve its structure at these denaturant concentrations. Native (N) to unfolded (U) state transition takes place between 0.5 to 1.5 M GdnHCl, with the midpoint of transition (C_m) being approximately 0.75 M GdnHCl. At 1.0 M GdnHCl, the protein lost 72% of secondary structure and was completely unfolded at 4.0-6.0 M GdnHCl. At 1.5 M GdnHCl, SMATase retains 15% of the secondary structure. The denaturation data indicates that the protein undergoes complete loss of its α-helical component and changes in other secondary structural elements in 6 M GdnHCl, and disulfide bonds cannot prevent the destruction of α-helix in the protein.

Enzyme activity versus secondary structural elements. In far-UV CD studies, we have shown the loss of second-

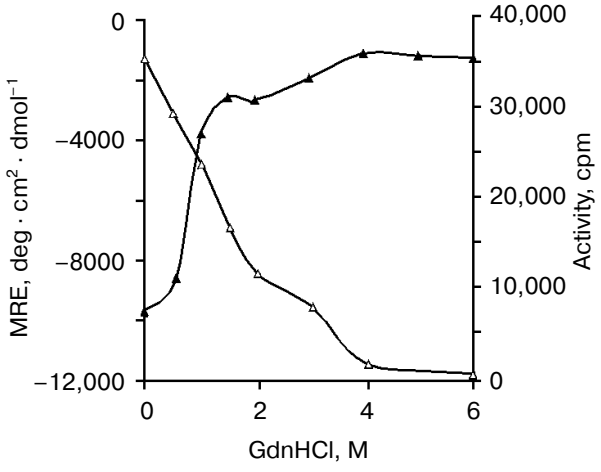

Fig. 2. Molar residue ellipticity (MRE) and enzyme activity of SMATase against denaturant (GdnHCl) concentration. Increasing amounts of GdnHCl were added to a fixed amount of protein (7 μM). Closed symbols, MRE at 222 nm; open symbols, activity. The data shown here were the averages from three independent experiments.

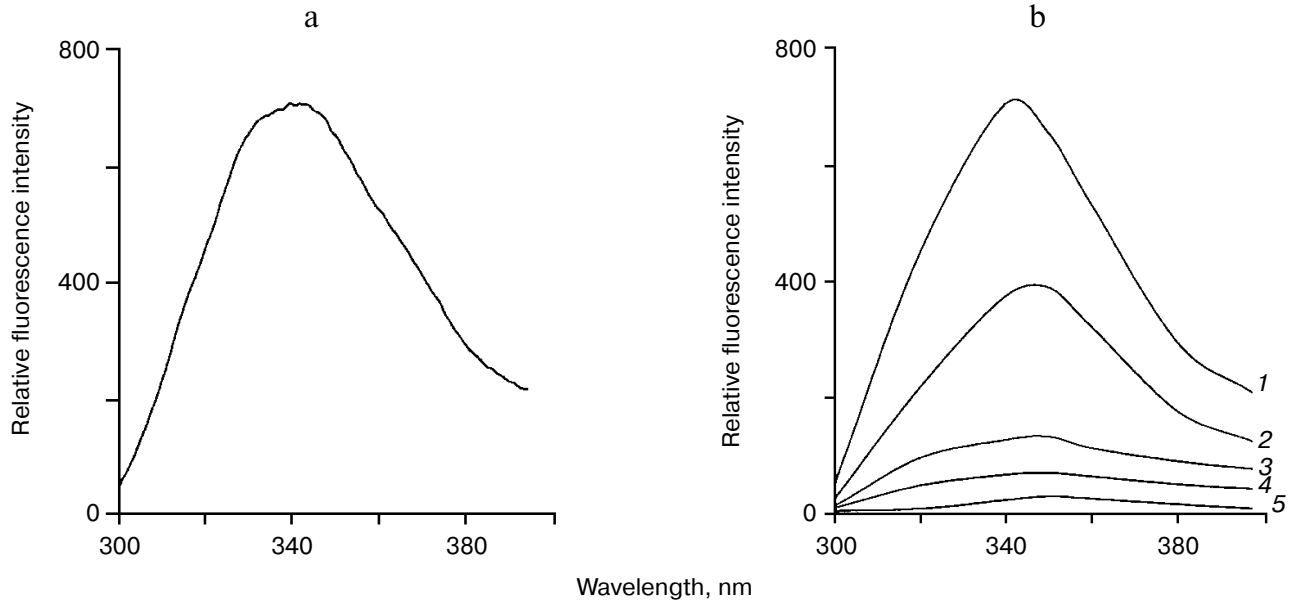

Fig. 3. a) Fluorescence emission spectrum of SMATase excited at 280 nm. b) Changes of emission spectra of SMATase (excited at 280 nm) at various concentration of GdnHCl. *1-5*) 0, 1, 2, 4, and 6 M GdnHCl, respectively.

ary structure of SMATase induced by denaturation with GdnHCl, and it was dependent on concentration of the denaturant. To investigate the effects of structural changes induced by GdnHCl on the SMATase activity, the enzyme solution (7 μM) was incubated with various concentrations of GdnHCl (0.5, 1, 1.5, 2, 3, 4, 5, and 6 M) for 30 min at 37°C. Enzyme activity was then assessed by the radioactive assay method of Hass and Dowding [22]. Figure 2 shows that the structural changes induced by change in the GdnHCl concentration within the range 0.5-6.0 M, as monitored by CD spectroscopy, are reflected in the loss of enzyme activity of the SMATase. It was observed that there was a concomitant loss of enzyme activity with the loss of secondary structure (Fig. 2). At ~0.75 M GdnHCl, there was loss of 50% secondary structure and 25% activity, respectively. Hence, the loss of secondary structure is not linearly dependent on destruction of catalytic site. Interestingly, at 0.5 M GdnHCl SMATase showed spectral features close to the native protein with 90% α-helical content and retained almost 85% enzyme activity.

Intrinsic tryptophan fluorescence. The spectral parameters of tryptophan fluorescence such as position, shape, and intensity are dependent on the dynamic and electronic properties of the chromophore environment. Hence, steady-state Trp fluorescence has been extensively used to obtain information on the structural and dynamic properties of proteins [25]. The modification of the microenvironment of Trp residues of SMATase has been monitored by studying the changes in the intensity and wavelength of emission (λ_{max}) of Trp fluorescence as a function of GdnHCl concentration. SMATase contains four tryptophan and three cysteine residues.

The tryptophan emission spectrum of SMATase is shown in Fig. 3a. The most intense emission spectrum with a single peak at 343 nm was obtained when excited at 280 nm. Figure 3b shows the comparative fluorescence emission spectra of SMATase at four different GdnHCl concentrations, i.e. 1, 2, 4, and 6 M. With progressive

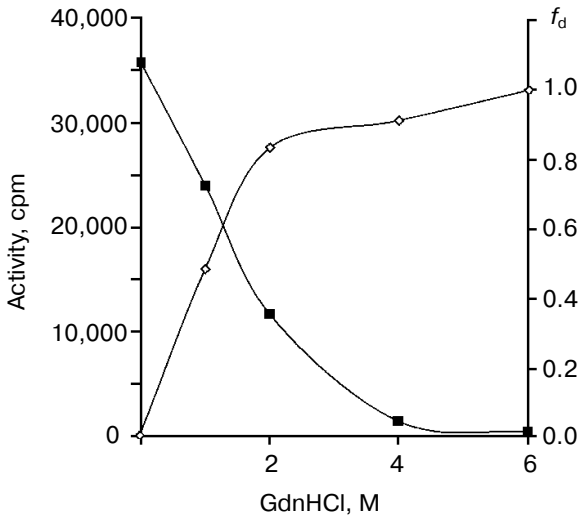

Fig. 4. Enzyme activity (closed symbols) and fraction of SMATase molecules in the denatured state (f_d) (open symbols) versus GdnHCl concentration.

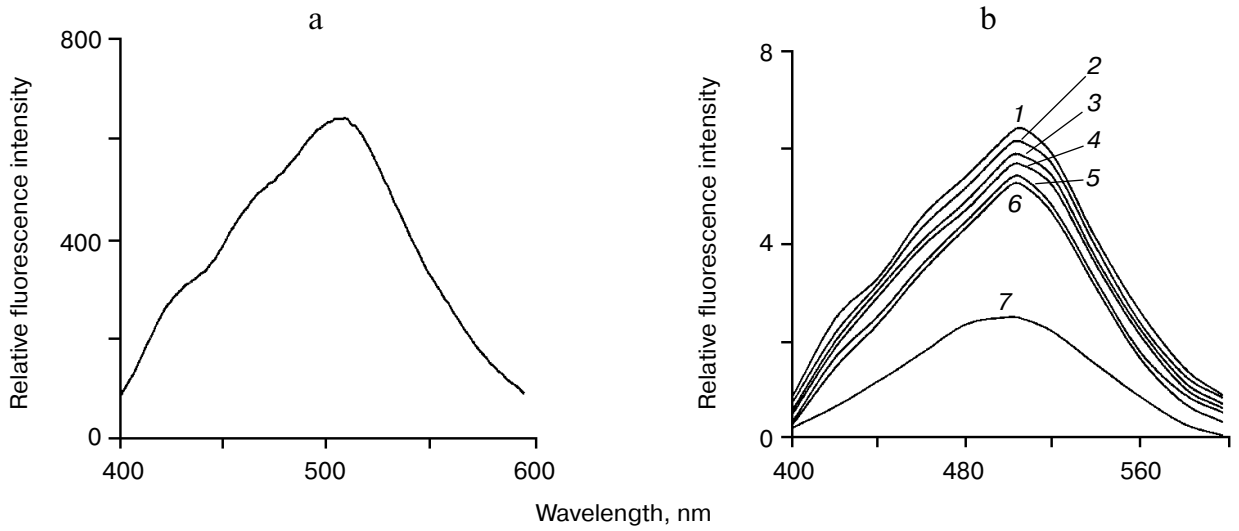

Fig. 5. Fluorescence emission spectra of ANS bound to SMATase (a) and interaction of ANS with various forms of SMATase (b). The excitation wavelength was 350 nm. b: *1-6*) 0, 1, 2, 3, 4, and 6 M GdnHCl, respectively; *7*) 6 M GdnHCl and 2 mM DTT. Protein/ANS ratio was 1 : 100.

increase in GdnHCl concentration up to 6 M, the relative fluorescence intensity gradually decreases, indicating that the protein conformation is getting altered. Figure 3b also shows that the emission maximum (λ_{max}) is red shifted (343 to 348 nm) by 5 nm as the protein encounters high denaturant concentration, which indicates that the microenvironment of aromatic amino acids (Trp residues) is getting more polar. This result indicates that at 6 M GdnHCl, SMATase exists in a conformation that is different from the native conformation.

Figure 4 shows the relation between fraction denatured state (f_d) calculated from Eq. (1) using fluorescence intensity and enzyme activity versus GdnHCl. We observed that there was positive correlation between fraction of denatured SMATase molecules and its enzymatic activity. With increase in fraction denatured, the activity gradually decreases with increasing concentration of GdnHCl. Here we observed that the catalytic site of the enzyme is more resistant to denaturant than the overall conformation around Trp residues, because at 2 M GdnHCl concentration, the maximum change of Trp fluorescence occurs, but the protein still retains 30% of the catalytic activity. The loss of intrinsic tryptophan fluorescence intensity might also arise due to the aggregation of the protein, which in turn removes denatured protein from the mixture. However, this possibility can be ignored during GdnHCl induced denaturation because the aggregated protein remains soluble in the presence of the denaturing agent.

ANS binding and surface property of SMATase. Figure 5 shows the fluorescence spectra of ANS in the 400-600 nm wavelength range in the presence of native (Fig. 5a) and GdnHCl-denatured protein (Fig. 5b). ANS fluorescence intensity decreased gradually on increasing concentration of GdnHCl from 1 to 6 M, whereas significant change in ANS fluorescence was observed in presence of 2 mM dithiothreitol (DTT) and 6 M GdnHCl (Fig. 5b). In addition to this, emission maxima (λ_{max}) showed no shift of the maxima at 506 nm on increasing the GdnHCl concentration. As can be seen, binding of ANS to the GdnHCl (6 M)-denatured state of SMATase at 2 mM DTT produced a large (threefold compared to

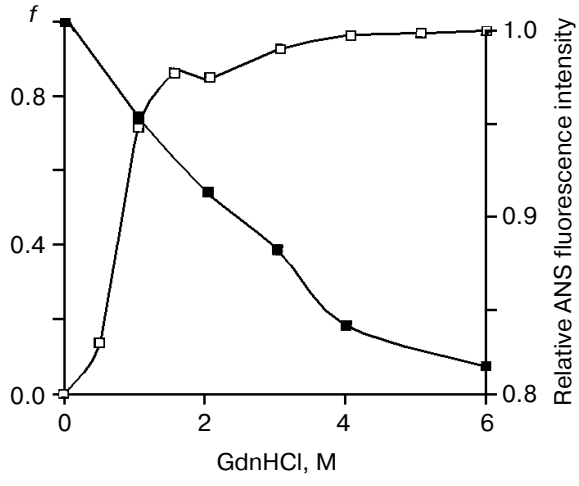

Fig. 6. Fraction of protein denatured (*f*) at 222 nm (open symbols) and relative ANS fluorescence intensity (closed symbols) versus GdnHCl concentration. Molar residue ellipticity (MRE) was monitored at 222 nm and 37°C and the ANS fluorescence intensity at 506 nm with excitation at 350 nm. MRE is reported as a fraction of denatured SMATase molecules (*f*) relative to MRE observed in the absence of denaturant. Data from triplicate experiments for GdnHCl are shown.

the native SMATase) decrease in fluorescence intensity accompanied by no shift in spectral maximum of 506 nm, indicating minimum exposure of hydrophobic regions of the protein molecule on denaturation with GdnHCl. However, the substantial change in ANS fluorescence on binding with disulfide ruptured denatured protein indicates that part of the hydrophobic regions contributing to the surface hydrophobicity of SMATase is shielded by disulfide bonds.

Figure 6 shows the effect of GdnHCl on the molar residue ellipticity measurements at 222 nm and protein bound ANS fluorescence intensity. These results also suggest that on denaturation a fraction of the surface hydrophobic region is lost, although most of it still remains intact, probably due to intact disulfide bonds.

Potassium iodide and acrylamide quenching of intrinsic tryptophan fluorescence. The fluorescence properties of Trp residues can be used to obtain topological information on proteins. Fluorescence quenching of the tryptophan residues by different types of quenchers has been shown to be useful in getting information about the solvent accessibility of these residues in proteins and the polarity of their microenvironment [26]. Relative fluorescence spectra of SMATase in the presence of potassium iodide and acrylamide quenchers in the 300-400 nm range are shown in Figs. 7a and 7b, respectively.

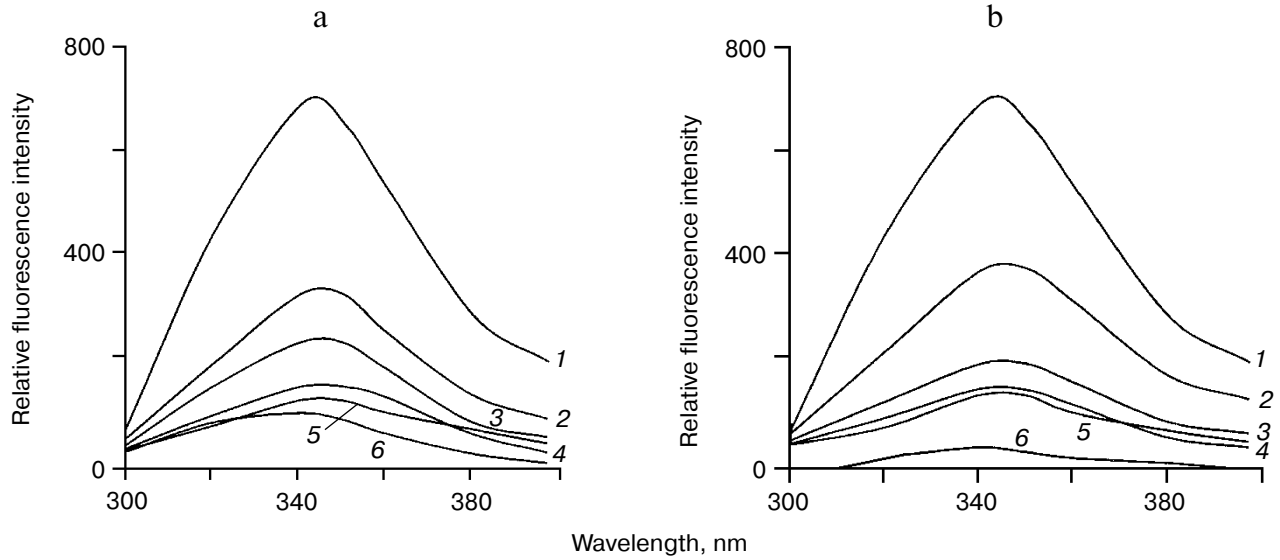

Fig. 7. Quenching of SMATase intrinsic fluorescence spectra by potassium iodide (a) and acrylamide (b). Excitation wavelength was at 280 nm. Concentrations of quenchers: *1-6*) 0, 0.2, 0.4, 0.6, 0.8, and 1.0 M, respectively.

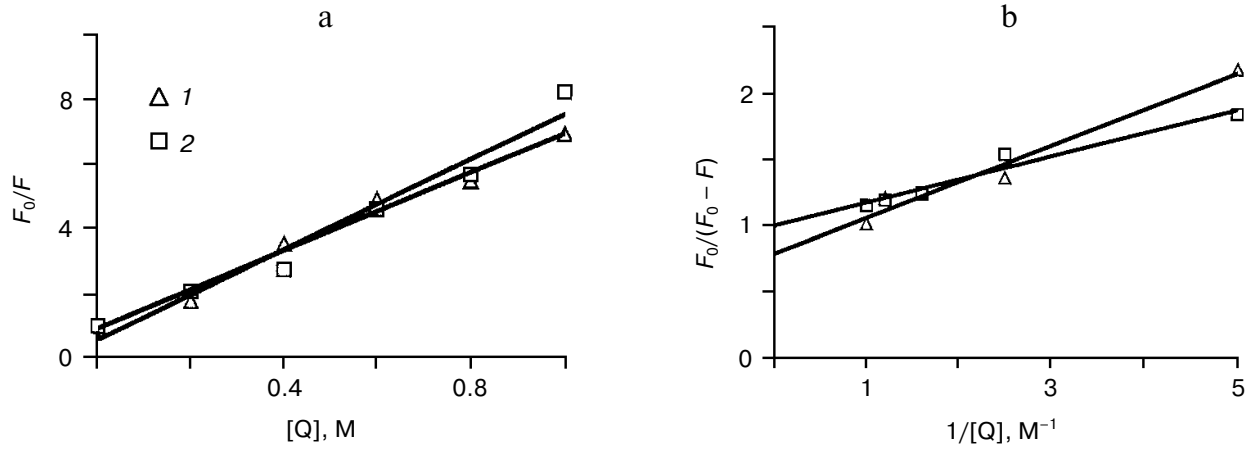

Fig. 8. Stern–Volmer plot (a) and modified Stern–Volmer plot (b) of potassium iodide (*1*) and acrylamide (*2*) quenching. Values shown are the ratio of fluorescence in the absence of quencher (F_0) to the fluorescence at that concentration of quencher (F).

The Stern–Volmer plot (Fig. 8a) and the modified Stern–Volmer plot (Fig. 8b) for quenching of intrinsic SMATase fluorescence by potassium iodide and acrylamide are shown. The quenching constants (K_{SV} values) calculated for potassium iodide and acrylamide were 4.56 and 5.52 M^{-1}, respectively. Following Eq. (3), f_a of acrylamide was determined to be 0.99, which meant that 99% of the fluorescence of tryptophan residues in SMATase could be quenched by acrylamide. Similarly, f_a value of KI quencher was 0.78. The Stern–Volmer plot indicates that the aromatic amino acids in the protein are more exposed to the solvent in the native folded conformation at pH 7.8 and tryptophan fluorescence is quenched more than 99% in case of acrylamide. Thus, it can be used to assess the degree of exposure of Trp residues of the protein. The close approach of fluorophore and quencher can be prevented by steric factors as well as charged factors. Since a charged and hydrated quencher such as iodide is sensitive to local charge effects, quenching by this molecule can reveal the charged state of the local environment around Trp fluorophores [27-31]. From the results above, it was concluded that nearly all Trp residues of SMATase were on the surface of SMATase molecules, and about 20% of them located in negatively charged areas, where iodide could not approach.

The present study provides for the first time a structure–function relationship of SMATase probed by far-UV CD and fluorescence spectroscopy. Conformational stability experiments under native and GdnHCl-induced denatured state of SMATase proved that α-helices are essential for the enzyme activity. The conformational changes monitored by CD and fluorescence spectroscopy correlate the different conformations of SMATase with its catalytic property. The surface topology of this protein is greatly protected by disulfide bridges in the protein. The 3-D structure of SMATase by X-ray crystallography can provide detailed structural information.

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