Exposure of hydrophobic surfaces of protein on electrostatic interactions with 8-anilino-1-naphthalene sulfonic acid (ANS)
7.1 INTRODUCTION

The fluorescent dye ANS has been widely used since long for detecting the dynamical conformational ensembles stabilized during equilibrium and kinetic folding/unfolding of proteins (Ptitsyn 1991; Engelhard and Evans 1995; Ptitsyn 1995). ANS is non-fluorescent in water but fluoresces brilliantly on binding to solvent accessible non polar hydrophobic clusters on the proteins (Lakowicz 1999). This peculiar feature of ANS and its derivatives makes them a sensitive tool for detecting folding/unfolding intermediates, hydrophobic site exposure on conformational changes in protein, binding pockets of several carrier proteins/enzymes, misfolded conformations as those accumulated during protein aggregation and fibril formation and many other processes that modify the exposure of buried hydrophobic residues of the protein to water (Cardamone and Puri 1992; Shi et al. 1994; Arighi et al. 1998; Ory and Banaszak 1999; Chapeaurouge et al. 2002; Maki et al. 2004; Scheibel 2005; Gasymov et al. 2008).

ANS is an amphipathic molecule consisting of a hydrophobic anilinonaphthalene group and a charged sulfonate group (Figure 3). Although the majority of the available information in literature emphasizes that hydrophobic character of ANS is responsible for its interaction with the proteins however, the contribution of electrostatic interactions between negatively charged sulfonate group of ANS and positively charged amino acid side chains present in protein has also been well documented (Matulis and Lovrien 1998). Structural analysis and binding energetic studies of ANS-protein complexes reveal that for some proteins, binding of ANS to protein is primarily occurs via ion pair formation with positively charged side chains (Matulis and Lovrien 1998; Ory and Banaszak 1999; Schonbrunn et al. 2000; Lartigue et al. 2003). Moreover, we and others have previously demonstrated that addition of ANS to the acid denatured states induces partial folding in the acid-unfolded protein due to the ionic interactions between ANS anion and cationic groups present in the protein (Ali et al. 1999; Kamen and Woody 2001). ANS has also been reported to act as conformational tightening agent i.e. reduces the conformational flexibility of acid expanded states of proteins (Matulis et al. 1999). Besides these, enhancement of thermal stability of BSA in presence of ANS has also been reported (Celej et al. 2005). To the best of our knowledge the denaturing action of ANS on proteins has not been reported till date.

pH is known to influence the structure and stability of the proteins by altering the net charges on it. The balance of complex network of electrostatic interactions in the native
conformation of a protein is affected by changing the pH of the solution. Most of the proteins denature at extremes of pH because of the presence of destabilizing repulsive interactions between similar charges in the native conformations (Goto 1990; Tan et al. 1995). The exact behavior of the proteins at particular pH is a complex interplay between a variety of stabilizing and destabilizing forces, some of which are sensitive to the environment. Neutral salts influence the physicochemical properties of proteins by affecting mainly the electrostatic and hydrophobic interactions (Von Hipple 1969; Goto 1989).

TgFNR is a monomeric nuclear encoded apicoplast localized protein that operates as a general electron switch at the bifurcation step of the many different electron transfer pathways yet to be identified (Vollmer et al. 2001; Pandini et al. 2002b). TgFNR carries a large number of basic (59) and acidic (49) residues and has an isoelectric point about 8.58. Structurally, TgFNR consists of two structural domains that interact strongly with each other under physiological conditions. Our studies with TgFNR have demonstrated that ionic interactions play an important role in maintenance of conformational stability of this protein. Modulation of ionic interactions by alterations in environmental pH (especially pH about 4.0) leads to selective modification in NADP⁺-binding domain resulting in induction of noncooperativity in otherwise cooperative TgFNR molecule (Singh and Bhakuni 2008).

Structurally TgFNR is significantly stable over pH range 4 to 8. Comparative studies using a charged and neutral hydrophobic dye ANS and NR respectively (Figure 3), demonstrated that under acidic conditions (pH about 4) only ANS binds to TgFNR but no binding of NR to protein was observed. Detailed investigations were undertaken to understand the underlying mechanism of differential binding of the two hydrophobic dyes to TgFNR at pH 4.0.

7.2 RESULTS AND DISCUSSION

Usually during folding/unfolding of the proteins, partially unfolded intermediates are stabilized in the solution of low pH, high salts or in the presence of moderate concentrations of chaotropic agents. The exposed hydrophobic clusters are unique features of several of these stabilized intermediates. The hydrophobic dye ANS is immensely useful in identification of such intermediates as the fluorescence quantum yield of this molecule increases dramatically along with blue shift of fluorescence maxima when the dye binds to the exposed hydrophobic clusters present on partially unfolded intermediates of protein (Lakowicz 1999).
Our recent studies have demonstrated that electrostatic interactions play a pivotal role in the structural, functional and stability properties of TgFNR. Under physiological conditions the enzyme is stabilized in an open conformation which is an absolute requirement for optimum functioning of the enzyme. Induction of compaction/rigidity in the enzyme conformation by modulation of environmental pH leads to loss of functional activity and structural cooperativity existing in the native protein domain (Singh and Bhakuni 2008). No significant structural alterations in the enzyme were observed between pH 7.5 to 4.0 however, bringing down the pH to below 4.0 leads to significant unfolding of the enzyme but complete unfolding was not observed even at pH 2.5 (Figure 1).

![Figure 1: Changes in the spectral properties of TgFNR (3µM) under various pH conditions at 25°C.](image)

(A) Far UV CD spectra (B) FAD fluorescence spectra (C) Tryptophan fluorescence spectra (D) Near UV CD spectra. In all profiles solid, dotted and dashed lines are representative of protein samples incubated at pH 7.5, 4.0 and 2.5 respectively.
In order to evaluate the stabilization of intermediates, having exposed hydrophobic clusters, during the pH-induced unfolding of the enzyme, we studied the binding of ANS to TgFNR under various pH conditions.

Figure 2 summarises the changes in the fluorescence characteristics of ANS on incubation with TgFNR under various pH conditions. No significant binding of ANS was observed between pH 7.5 and 5.5. However, as the pH of the solution was lowered below 5.0, the fluorescence intensity of the added ANS increased about 120 times along with blue shift in emission maxima to 465nm (Figure 2 inset). The maximum enhancement in the fluorescent intensity was observed at pH of about 4.0 indicating significant solvent exposure of buried hydrophobic clusters of protein under these conditions. This observation was surprising because evidences from CD and fluorescence (tryptophan and FAD) experiments (Figure 1) indicated no significant alterations in the tertiary or secondary structure of the enzyme under these conditions.

**Figure 2:** pH dependence of fluorescence intensity of ANS (closed circle) and NR (open circle) in the presence of TgFNR at 25 °C. Inset shows the normalized fluorescence spectra of ANS, profiles 1-3 are representative of fluorescence spectra obtained at pH 7.5, 4.0 and 2.5 respectively.
In order to confirm that the binding of ANS to TgFNR at pH below 5.0 is preferentially due to solvent exposure of hydrophobic clusters of protein, we carried out similar studies with another fluorescent dye, NR. NR is a neutral fluorescent dye that is sensitive to local polarity (Dutta 1996; Ghoneim 2000). In water NR has low fluorescence quantum yield (~0.02) with emission maxima centred at 665 nm whereas, in more hydrophobic environment its quantum yield increases and emission maxima shows blue shift to about 610 nm (Sackett and Wolff 1987; Hendriks et al. 2002). Because of neutral character, NR is used as reference to discriminate between electrostatic and hydrophobic nature of ANS binding to proteins (Ali et al. 1999).

Figure 3: Chemical structure of ANS and NR.

The interaction between NR and TgFNR were studied by monitoring the changes in the NR fluorescence intensity under varying pH conditions (Figure 2). No significant alterations in the NR fluorescence characteristics were observed between pH 7.5 and 2.5 which suggested the absence of binding of NR to the protein under these conditions. This indicates that the buried hydrophobic clusters of TgFNR are not exposed in the pH range 2.5 to 8. In other words no intermediate conformation of the protein having exposed hydrophobic clusters is stabilized during pH-induced unfolding of TgFNR, which is in contrast to the results obtained by ANS.

For understanding the genesis of the differential binding of ANS and NR to the protein at pH 4.0 we carried out detailed comparative studies using the two dyes. Time and concentration dependent binding of ANS to the native and pH 4.0 stabilized conformation of TgFNR was studied to optimize the condition for maximum binding of dye to protein. Figure 4A shows the time kinetics of binding of ANS to protein as studied by monitoring the changes in the fluorescence intensity of ANS as a function of time. ANS is virtually non-fluorescent in the entire time range of investigation in the protein solution at pH 7.5. However, at pH 4.0 addition of protein to ANS resulted in a rapid increase in the fluorescence
intensity (maximum of about 400 times) between 100 and 250 seconds suggesting significant binding of the fluorophore to the protein in this time period. Hence, 300 seconds was taken as ideal time of incubation of ANS with protein for achieving maximum binding.

Figure 4: Binding of ANS to TgFNR at 25 °C. (A) Time kinetics of increase of ANS fluorescence at 465nm in the presence of TgFNR at pH 7.5 (profile 1) and 4.0 (profile 2). Arrow in the figure indicates the time at with ANS was added to the reaction mixture. (B) Plot of fluorescence intensity at 465nm verses ANS concentrations at pH 7.5 (solid circle) and pH 4.0 (solid square) at 25 °C. Inset shows the plot of fluorescence intensity of ANS in presence of TgFNR at pH 4.0 plotted against concentration up to 25 μM ANS.

Figure 4B summarizes the concentration dependent changes in fluorescence intensity of ANS upon binding with a fixed concentration TgFNR. Titration of TgFNR with ANS shows two phases of interactions at pH 4.0. In the first phase, up to ANS concentration of about 25μM, a small linear enhancement in fluorescence intensity was observed however, thereafter a significantly large augmentation in fluorescence intensity was observed with higher concentration of ANS. Though, molar concentrations of ANS compared to that of protein (~10:1) used in the first phase was sufficient enough in most of the cases reported in literature, to induce significant enhancement in its fluorescence intensity if hydrophobic clusters were presents on proteins for binding with dye (Kirk et al. 1996) Increased fluorescence intensity although to lower extent and blue shift in the emission maxima are also expected on ANS binding to the external ionic binding sites in the protein (Gasymov and Glasgow 2007). Investigation of polyamino acid and ANS complex have shown that the enhancement in fluorescence intensity and blue shift in the emission maxima are governed by photophysical processes that include –NH group of protein and SO₃⁻ groups of ANS (Gasymov and Glasgow 2007). A small enhancement in the fluorescence intensity observed at low concentration of ANS (Figure 4B inset) may probably be due to electrostatic interaction between ANS and protein under these conditions. This enhancement is
significantly lower as compared to that observed for binding of ANS to hydrophobic patches. For NR no binding of dye to protein was observed both at pH 4.0 and 7.5 (Figure 4B).

Although both the fluorescent probes used in study have same basic nuclei i.e. naphthalene, but ANS has a charged sulphonate group where as NR is neutral (Figure 3). Studies on ANS-protein complexes have shown that ANS binding depends primarily on ion pairing with positively charged side chains of proteins (Matulis and Lovrien 1998). This electrostatic mechanism of ANS-protein binding has been demonstrated for buried as well as external sites (Gasymov and Glasgow 2007). Based on these difference in the structure characteristics of the two dyes it seems that ANS probably first binds to TgFNR via electrostatic interactions which leads to partial unfolding of enzyme. As a result of this ANS-induced partial unfolding of TgFNR the buried hydrophobic clusters of the enzyme get exposed to solvent and become accessible for hydrophobic binding to ANS.

To test this hypothesis we studied the binding of the neutral hydrophobic dye NR to TgFNR in the presence of NaCl at pH 7.5 and 4.0. NaCl has been used as charge neutralizing agent to mimic the effect of ANS sulfonate group however it is required in higher concentration to bring about similar changes as ANS (Kamen and Woody 2001). Figure 5A and B compares the fluorescence intensity and emission spectra respectively, of NR and protein in buffer alone and in presence of increasing concentration of NaCl at pH 7.5 and 4.0.

**Figure 5:** Effect of increasing concentrations of NaCl on fluorescence properties NR in presence of TgFNR at 25°C. (A) Changes in NR fluorescence intensity at 620 nm with increasing concentrations of NaCl at pH 7.5 (open circle) and 4.0 (solid circle). (B) Changes in NR fluorescence properties of TgFNR with increasing concentrations of NaCl at pH 4.0. Profiles 1-8 represent profile of NR alone, NR incubated with TgFNR at pH 4.0, NR incubated with TgFNR at pH 4.0 in presence of 20, 60, 100, 140, 180 and 220 mM NaCl respectively.
At pH 4.0 in presence of increasing concentrations of NaCl, the fluorescence intensity shows a substantial increase and a remarkable blue shift with maximum located at 620 nm (Figure 5 A & B). It was not possible to saturate the binding of NR to the protein at pH 4.0 with increasing concentration of NaCl because precipitation of reaction mixture was observed above 260 mM NaCl concentration. The enhancement in fluorescence intensity and spectral blue shift of the emission of the NR unequivocally showed that at pH 4.0 in presence of NaCl the dye binds to the exposed hydrophobic clusters on the protein surface and also support the absence of such exposed hydrophobic clusters in native protein under similar concentration of NaCl. No alteration in the spectral behavior of NR was observed for TgFNR at pH 7.5 in presence of NaCl.

Salts and ANS both have been known to influence the physicochemical properties of proteins by modulating the electrostatic as well as hydrophobic interactions in the protein molecule (Von Hipple 1969; Kamen and Woody 2001). In the presence of increasing concentrations of NaCl, NR binding was observed like ANS however it requires in milli molar range, whereas ANS needed in micromolar range. To further gain insight in the relative contribution of ionic and hydrophobic effect, we used another chemical compound DSS which has been previously used for such studies (Kamen and Woody 2001). DSS is nonfluorescent in aqueous or organic solvents. DSS is a very simple molecule containing a sulfonate group, like ANS and a short aliphatic chain (Figure 6A). Effect of increasing concentrations of DSS on fluorescence properties of NR in presence of TgFNR at pH 4.0 was shown in Figure 6 B & C. An almost linear enhancement in NR fluorescence intensity and blue shift in emission maxima was observed. DSS is capable to bring about similar spectral changes like NaCl but it also requires in milli molar range. However, the concentration of DSS needed for similar spectra changes was about 40 times less compared to that of NaCl. It was also not possible to saturate the binding of NR to the protein at pH 4.0 with increasing concentrations of DSS because irreversible aggregation of the reaction mixture was occurred at higher concentration of DSS.
The above presented results suggest that alterations in protein conformation at pH 4.0 by modulation of ionic interactions seems mandatory for the exposure of buried hydrophobic clusters and subsequent binding of hydrophobic dye to TgFNR. Furthermore, a combination of hydrophobic as well as electrostatic interactions both is responsible for inducing structural alterations although hydrophobic contribution in more prominent than electrostatic effect.

The binding of TgFNR to the octyl-sepharose matrix permits the assessment of relative hydrophobic exposure in the absence and presence of increasing concentrations of ANS, DSS and NaCl. Octyl-Sepharose is a cross linked beaded agarose that contain octyl group (~50 μmol per mL of swollen sepharose) attached by ether linkage. Binding of TgFNR to the octyl-Sepharose have been carried as detailed in materials and methods section. The quantity of protein bound to the octyl sepharose resin was SDS PAGE and densitometric analysis of protein bands were shown in Figure 7. BSA was used as a positive control which has known to have exposed hydrophobic clusters under similar conditions (Daniel and Weber 1966; Matulis and Lovrien 1998). A sequential enhancement in the amount of bounded
protein was observed with increasing concentrations of ANS, DSS and NaCl. The results obtained from octyl-Sepharose binding assay were consistent with the spectral changes

![Figure 7](image)

**Figure 7:** The Binding of TgFNR to octyl-sepharose in presence of increasing concentrations of ANS, NaCl and DSS at pH 4.0, 25 °C. Panel A- SDS PAGE profile of matrix bound TgFNR in absence and presence of increasing concentrations of ANS. Panel B shows densitometric analysis of protein bands shown in panel A, Bar 1-8 represent profile of BSA, TgFNR incubated in presence of 0, 12.5, 25, 50, 75, 100 and 125 μM ANS respectively. Panel C- SDS PAGE profile of matrix bound TgFNR in absence and presence of increasing concentrations of ANS. Panel D is densitometric analysis of protein bands shown in panel C, Bar 1-7 represent profile of BSA, TgFNR incubated in presence of 0, 5, 10, 50, 100 and 150 mM NaCl respectively. Panel E- SDS PAGE profile of matrix bound TgFNR in absence and presence of increasing concentrations of DSS. Panel F shows densitometric analysis of protein bands shown in panel E, Bar 1- represent profile of BSA, TgFNR incubated in presence of 0, 1, 2, 3, 4 and 5 mM DSS respectively.

observed for ANS and NR under similar conditions and further support our finding of ANS induced hydrophobic exposure of TgFNR at pH 4.0.
One remote possibility is that spectral changes such as those observed for TgFNR in presence of ANS, NaCl or DSS might be as a result of intermolecular association of protein molecules under these conditions. For addressing this issue we carried out light scattering experiments. Figure 8 summarizes increase in light scattering of TgFNR under various conditions where fluorophore binds to the protein. No significant enhancement in fluorescence signal was observed for TgFNR (3 μM) in presence of saturating concentration of ANS (150 μM) indicating absence of aggregation of protein under these conditions. Similarly for NaCl (250 mM) and DSS (5 mM), no significant light scattering was observed. However, further increase in NaCl (500 mM) and DSS (5 mM) concentrations resulted in sharp increase in signal due to aggregation of protein. Overall, the light scattering studies suggest that under the conditions of study reported in this chapter no aggregation of TgFNR occurs.

Figure 8: Light scattering of TgFNR at pH 4.0, 25 °C. (A) Light scattering measurements in presence of TgFNR (3 μM) in absence (solid line) and presence of saturating concentration of 150 μM ANS (dashed line). (B) Light scattering measurements of TgFNR (5 μM) in absence (dotted line) and presence of NR with 250 mM NaCl (dashed line), and 500 mM NaCl (solid line). (C) Light scattering measurements of TgFNR (5 μM) in absence (dotted line) and presence of NR with 5 mM DSS (dashed line), and 10 mM DSS (solid line).
In order to confirm that alteration in ionic strength of the solution at pH 4.0 does induce partial unfolding of the TgFNR, we carried out structural studies with protein in presence of ANS, DSS and NaCl. Figure 9 shows the changes in secondary and tertiary structure of TgFNR at pH 4.0 in presence of increasing concentration of ANS, NaCl and DSS as studied by far- and near-UV CD. No significant change in far-UV CD spectra of TgFNR at pH 4.0 was observed in presence of up to 100 μM ANS, 200 mM NaCl and 5 mM DSS (Figure 9 A B & C). The contribution of aromatic side chains to the near UV CD spectra of protein depend on conformations, environments and nearest (<10 Å) spatial arrangement neighboring residues (Strickland 1974; Kelly et al. 2005). Therefore the near UV spectra of proteins are unique for individual proteins and provide reflection of its tertiary structure. For TgFNR, the near UV CD spectrum is mainly influenced by contribution of 18 aromatic residues (phenylalanine-7, tryptophan- 4 and tyrosine-7). A significant loss of near UV CD signal was observed with increasing of NaCl and DSS concentrations (Figure 9 D, E). This indicates that aromatic moieties present in TgFNR were buried in the protein core at pH 4.0 due to which they show restricted movement as observed by CD signals. We could not perform near UV experiments in the presence of ANS because ANS is a molecule which is optically in active in solution; but on binding to the protein can give rise to CD band. Furthermore the loss in near UV CD signal obtained from ANS binding would be compensated by the signals obtained by binding of ANS to the protein molecule. Presence of increasing concentrations of DSS and NaCl under these conditions lead to significant loss of near UV signal indicating the solvent exposure of these buried hydrophobic residues of the protein due to partial unfolding of the protein under these conditions.
Figure 9: Changes in structural properties of TgFNR on incubation with increasing concentration of ANS, NaCl and DSS at pH 4.0, 25°C. Panel A, B and C shows changes in the CD ellipticity at 222nm of TgFNR with increasing concentrations of ANS, NaCl and DSS respectively. Panel D represents near UV CD spectra of TgFNR in absence and presence of increasing concentrations of NaCl. Profiles 1-4 are representative of TgFNR in absence and presence of 50, 100 and 150 mM NaCl concentration respectively. Panel E represents near UV CD spectra of TgFNR in absence and presence of increasing concentrations of DSS. Profiles 1-6 are representative of TgFNR in absence and presence of 1, 2, 3, 4 and 5 mM DSS concentrations respectively.
Sequential binding of NR to TgFNR at pH 4.0 in the presence of increasing concentrations of NaCl and DSS unequivocally supports the notion that neutralization of the pre-existing positively charged side chains of the amino acids is an absolute requirement for the exposure of hydrophobic clusters in TgFNR. The data is directly correlated with the ANS titration of the protein at pH 4.0 where two phases of ANS binding were observed. In the first phase, the binding of the ANS to the protein is mainly electrostatic in nature and a certain load of ANS concentration (about 10 times of protein concentration) was necessary to bring about exposure of hydrophobic clusters in protein. Ionic nature of binding of the ANS to the pH 4.0 stabilized conformation of TgFNR also corroborates with our previous hypothesis where we proposed that the open conformation of the enzyme was due to electrostatic interactions between similar charged polar amino acids in the interior of the protein under physiological conditions. At acidic pH, deionization of these polar amino acid residues present in the interior of the protein occurs which abolishes the electrostatic repulsion existing under physiological conditions, leads to exposure of charged residues which indeed allowed the protein to fold in more compact fashion. Interaction of NR to the proteins under similar condition does not occur due to absence of negatively charged sulfonate ion which is indeed present in ANS molecule.

7.3 SUMMARY

Fluorescent dyes 8-anilino-1-naphthalene sulfonic acid (ANS) and Nile Red (NR) have been widely used probes for detection of hydrophobic clusters on the protein surfaces. pH dependent binding studies of these dyes with Toxoplasma gondii TgFNR showed that ANS alone and not NR binds to the protein at pH below 5.0 with maximum binding being observed at pH about 4.0. In order to understand the genesis of this differential binding of two fluorescent dyes to the protein under these conditions comparative structural studies on protein in presence of these dyes were carried out. ANS was found to interact via electrostatic as well as hydrophobic interactions with the protein depending on the concentrations of the dye used. NR did not bind to protein under any conditions. However, in presence of NaCl at pH 4.0, NR showed strong hydrophobic binding with the protein. Structural studies on TgFNR with NaCl showed that salt induced partial unfolding of protein leading to exposure of buried hydrophobic clusters due to which NR binding takes place. The studies presented in this paper demonstrate that ANS first interacts with the protein via electrostatic interactions leading to partial unfolding of protein, similar to that brought about
with NaCl, resulting in subsequently hydrophobic binding of dye to protein. This is the first report on the ANS induced partial unfolding of protein and puts question to the usefulness of this dye in detection of exposed hydrophobic clusters under various conditions in proteins.