Perturbation of Protein Tertiary Structure in Frozen Solutions Revealed by 1-Anilino-8-Naphthalene Sulfonate Fluorescence

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ABSTRACT Although freeze-induced perturbations of the protein native fold are common, the underlying mechanism is poorly understood owing to the difficulty of monitoring their structure in ice. In this report we propose that binding of the fluorescence probe 1-anilino-8-naphthalene sulfonate (ANS) to proteins in ice can provide a useful monitor of ice-induced strains on the native fold. Experiments conducted with copper-free azurin from *Pseudomonas aeruginosa*, as a model protein system, demonstrate that in frozen solutions the fluorescence of ANS is enhanced several fold and becomes blue shifted relative free ANS. From the enhancement factor it is estimated that, at −13°C, on average at least 1.6 ANS molecules become immobilized within hydrophobic sites of apo-azurin, sites that are destroyed when the structure is largely unfolded by guanidinium hydrochloride. The extent of ANS binding is influenced by temperature of ice as well as by conditions that affect the stability of the globular structure. Lowering the temperature from −4°C to −18°C leads to an apparent increase in the number of binding sites, an indication that low temperature and /or a reduced amount of liquid water augment the strain on the protein tertiary structure. It is significant that ANS binding is practically abolished when the native fold is stabilized upon formation of the Cd^{2+} complex or on addition of glycerol to the solution but is further enhanced in the presence of NaSCN, a known destabilizing agent. The results of the present study suggest that the ANS binding method may find practical utility in testing the effectiveness of various additives employed in protein formulations as well as to devise safer freeze-drying protocols of pharmaceutical proteins.

INTRODUCTION

The formation of ice can cause injury and death to living organisms and one of the causes is the cold lability of protein molecules (Franks, 1985). Freezing of protein solutions may result in irreversible protein aggregation and severe loss of catalytic activity of enzymes, reasons for which many proteins cannot be stored in ice or lyophilized from it without partial inhibition of their function (Franks, 1985). Notwithstanding the relevance of the phenomenon and the commercial importance for the growing polypeptide based pharmaceutical industry, little is known of the structure of proteins in ice. To date various hypotheses have been advanced concerning the cause of freeze damage and the underlying perturbation: 1), instability of the folded state at low temperature (cold denaturation); 2), the low activity and peculiar properties of the liquid water in equilibrium with ice; 3), protein-solutes and protein-protein interactions (leading to protein aggregation during thawing), elicited by the large freeze-induced concentration of solutes; and 4), interactions between proteins and the surface of ice. The dearth of structural information on proteins in ice, which represents the limiting factor in understanding the phenomenon of freeze damage, is owed primarily to the inapplicability or the poor sensitivity of usual spectroscopic techniques in this highly scattering and anisotropic medium. For instance, Fourier transform infrared spectroscopy experiments have not detected significant changes in the secondary structure of freeze labile proteins (Allison et al., 1996). However, these measurements require relatively large protein concentrations and under these conditions the perturbation is attenuated as if the protein itself acted as a stabilizer. A recent study by Trp phosphorescence provided direct evidence that the solidification of water leads to important perturbations of the globular fold in all proteins examined (Strambini and Gabellieri, 1996). From the often drastic shortening of the phosphorescence lifetime of Trp residues buried in the rigid core of these macromolecules it was inferred that in ice the internal flexibility is greatly enhanced as is characteristic of partially unfolded polypeptides or of the loss of tertiary structure as in the formation of molten globule states. The inverse correlation observed between the extent of the perturbation and the fraction of liquid water in equilibrium with ice suggested that the perturbation may derive from the adsorption of the macromolecule to the liquid/solid interface.

Protein dynamics provides only indirect information on the structure of the macromolecule and, further, this is limited to specific sites of the protein interior, as Trp phosphorescence probes exclusively the immediate environment of the chromophore (Strambini and Gonnelli, 1995; Gonnelli and Strambini, 1995). In this report we examine the possibility of monitoring alterations of the protein tertiary structure in ice by observing the extent of 1-anilino-8-naphthalene sulfonate (ANS) binding. This chromophore is feebly fluorescent in water, but its spectrum is blue-shifted and its intensity is dramatically increased in nonpolar solvents or when it binds to apolar sites of proteins (Stryer, 1965; Daniel and Weber, 1966). In the last four decades, ANS has been widely used as a hydrophobic probe to study biological membranes and apolar sites in proteins (for a general review, see Slavik,

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1982). More recently, strong binding of ANS to molten globule states of proteins has been linked to the loss of tertiary structure (Semisotonov et al., 1991) and the method has since then been used to characterize transient states in protein denaturation (Das et al., 1995; Guha and Bhattacharyya, 1995; Uversky et al., 1996; Bismuto et al., 2001). Presently, hydrophobicity is no longer considered the only determinant of the fluorescence enhancement or the sole driving force of ANS binding to proteins. The importance of electrostatic interactions between the sulfonate group of ANS and positive charges on the polypeptide has been clearly demonstrated (Matulis and Lovrien, 1998). Kirk et al. (1996) have pointed out that whereas hydrophobicity affects principally the blue shift of the spectrum, other factors, such as geometrical constrictions imposed on ANS by the binding site and the exclusion of mobile water are mainly responsible for the enhancement of the quantum yield.

In this exploratory study we report ice-induced binding of ANS to azurin from *Pseudomonas aeruginosa*, a small (14 kDa), monomeric copper protein well characterized in terms of its structure (Nar et al., 1991, 1992) and thermodynamic stability (Engeseth and McMillin, 1986; Mei et al., 1999). The copper-free protein (apoazurin, Az) exhibits strong and homogeneous phosphorescence in solution at ambient temperature. Upon the formation of ice, its long lifetime ($\approx$1 s) becomes remarkably shorter and more heterogeneous, disclosing a wide distribution of partially unfolded conformations of the macromolecule (Strambini and Gabellieri, 1996). The results of the present study indicate that the formation of ice does lead to ANS binding to the protein and, further, that the extent of the process is significantly modulated by stabilizing/destabilizing conditions. In fact, binding is practically abolished by the addition of glycerol to the solution or by introducing Cd^{2+} in the metal binding site, both of which are known to stabilize the folded state. On the contrary, ANS binding is enhanced by addition of destabilizing NaSCN as well as by lowering the temperature. It is therefore concluded that the solidification of water affects the tertiary structure of azurin and ANS fluorescence represents an alternative and complementary approach to intrinsic phosphorescence to monitor deleterious effects of freezing on the native fold of proteins.

MATERIALS AND METHODS

Materials

All chemicals were of the highest purity grade available from commercial sources and were used without further purification. NaCl suprapur and glycerol for fluorescence microscopy were purchased from Merck (Darmstadt, Germany). NaSCN and ANS were from Sigma (St. Louis, MO). Copper-free azurin (Az) from *P. aeruginosa* was a gift of Prof. A. Desideri, University of Rome Tor Vergata (Rome, Italy). CdAz was prepared by the addition of $CdCl_2$ (0.1 mM) to a solution of apo-azurin as previously described (Strambini and Gabellieri, 1991). Doubly distilled MilliQ water (Millipore, Bedford, MA) was used throughout.

Determination of the freeze concentration factor of solutes

Above the eutectic temperature, solutes in the liquid phase of ice are concentrated by a factor $f_c = 1/V_L$, where V_L is the fraction of liquid water in equilibrium with ice. For a 27-mM NaCl solution, which is roughly equivalent in colligative properties to 25 mM NaCl plus 2 mM buffering salts (Hepes or NaP), $V_L = (0.027)/[NaCl]_{ice}$, where $[NaCl]_{ice}$ is the concentration of NaCl in equilibrium with ice. The latter was determined experimentally to be between -4°C and -18°C by measuring the freezing temperature of progressively more concentrated NaCl solutions under conditions, sample volume, and cuvette analogous to those adopted in fluorescence measurements. NaCl solutions (0.5 mL volume placed in cylindrical cells, 4 mm i.d.) were first frozen and afterward were gradually warmed until all ice melted. To assure full equilibration the temperature was raised by 0.5°C every 6 h. Slower temperature profiles yielded the same results.

Sample preparation

Samples were prepared from ANS and azurin stocks dissolved in 2 mM Na-phosphate buffer, pH 7.5, containing 25 mM NaCl and 0.5 mM EDTA (to prevent binding of divalent metal cations to Az). In some experiments Na-phosphate was replaced by Hepes. In the case of CdAz, EDTA was omitted to avoid scavenging of protein-bound Cd^{2+}. The concentration of ANS and azurin was determined by absorbance, using $\varepsilon_{370} = 6800\ M^{-1}\ cm^{-1}$ (in methanol) and $\varepsilon_{280} = 8800\ M^{-1}\ cm^{-1}$, respectively. Unless otherwise stated, the protein concentration was maintained constant at 2.5 μM. For fluorescence measurements in ice 0.5 mL samples were placed in cylindrical spectrosil cuvettes, 4 mm i.d., and rapidly frozen in liquid nitrogen. Subsequently, the ice was melted leaving a small ice seed that was used for controlled freezing at -10°C. The frozen samples were then allowed to anneal for 12 h at -5°C, followed by a final equilibration of 2 h at the temperature selected for fluorescence measurements (from -4°C to -18°C). As mentioned in the Results section, after this procedure reproducible (<20% variability) and stable results, if not equilibrium conditions, were obtained.

Fluorescence measurements

To test for potentially weak binding of ANS to Az in solution the ANS concentration was raised up to 500 μM and small, 3 $\times$ 3-mm^2 cells were employed to reduce inner filter attenuation of the intensity by these optically dense samples. Correction for this artifact was, however, not required since only relative intensities between equally absorbing samples, ANS $\pm$ Az, were important. To reduce the variability of fluorescence intensity in frozen samples due to the poor homogeneity of medium the sample was rotated (3 Hz) during intensity measurements and the signal integrated for 10 s.

Fluorescence measurements were conducted on a homemade fluorometer. The excitation light, provided by a Cermax xenon lamp (LX150UV, ILC Technology Sunnyvale,CA), is selected ($\lambda_{ex} = 350$ nm, 6-nm bandpass) by a 0.23-m double-grating monochromator (SPEX 1680, Spex Industries, Edison, NJ) and modulated by a light chopper. Fluorescence emission collected at 90° from the excitation, is passed through a 420-nm cutoff filter and then dispersed by a 0.25-m grating monochromator (Jobin Yvon, H-25, Lille, France) set to a bandpass of 3 nm. A low-noise current preamplifier (SR570, Stanford, Sunnyvale, CA) followed by a lock-in amplifier (ITHACO 393, Ithaca, NY) operating at the chopper frequency are used to amplify the photomultiplier (EMI 9635QB, Rockaway, NJ) current. The output signal is digitized and stored by a multifunction board (PCI-20428W, Intelligent Instrumentation, Tucson, AZ) utilizing visual Designer software (PCI-20901S Ver.3.0, Intelligent Instrumentation).

RESULTS AND DISCUSSION

Ice-induced enhancement of ANS fluorescence by apo-azurin

The addition of apo-azurin (Az) to a solution of ANS has no effect on the fluorescence emission of the latter when in liquid water, but causes a large enhancement of its intensity upon the formation of ice. For a quantitative analysis of the phenomenon it is important to point out that fluorescence intensities measured promptly on frozen samples were poorly reproducible. The intensity was not uniform across the sample and differed particularly with the temperature at which ice is initially formed. Moreover, the signal was unstable in time and continuous variations were observed for days when ice was kept between −10°C and −20°C. Evidently, the rapid growth of ice crystals introduces heterogeneity in the solute composition of the small liquid water pools formed at intergranular spaces of ice, and afterwards hindered diffusion of ANS among water pools is probably responsible for very slow and incomplete equilibration. Ultimately, reproducible (<20% variability) and stable results, if not equilibrium conditions, were obtained upon freezing at −10°C in the presence of a small ice seed, followed by 12 h annealing at −5°C and final equilibration of the sample (2 h) at the temperature selected for fluorescence measurements (from −4°C to −18°C).

Fig. 1 shows the change in ANS (75 μM) fluorescence in ice (2 mM NaP, pH 7.5, and 25 mM NaCl) at −13°C, obtained by the addition of 2.5 μM Az. The same results were obtained with Hepes as buffering salt to indicate that they are independent of the buffer and of possible differences in pH change upon freezing. The intensity is enhanced about sevenfold and the spectrum is blue-shifted by 13 nm. This indicates that in ice ANS has accessibility to internal, hydrophobic protein sites apparently not available when the protein is in solution. Before concluding that the creation of ANS binding sites is owed to ice-induced partial disruption of the protein tertiary structure other possibilities, peculiar to the frozen medium, must be ruled out. For instance, one is the occupation of very weak binding sites reachable in ice thanks to the ~120-fold freeze-induced concentration of ANS ($[ANS]_{ice} = 9$ mM) in the liquid pool. Another possibility is that a higher ANS fluorescence is due to superficial, aspecific association of the probe to the polypeptide, although ANS molecules exposed to the aqueous phase are expected to remain weak emitters. Diverse evidence tends to rule out both these alternative explanations. First, in liquid supercooled solutions (−13°C) no fluorescence enhancement was observed up to an ANS concentration of 500 μM and an Az concentration of 20 μM (much larger ANS concentrations are not practical, as at very small Az /ANS molar ratios the weak emission from the largely unbound fraction would mask any enhancement from the few bound molecules). In ice, however, for the same average ANS concentration in the water pool as in supercooled solutions the intensity

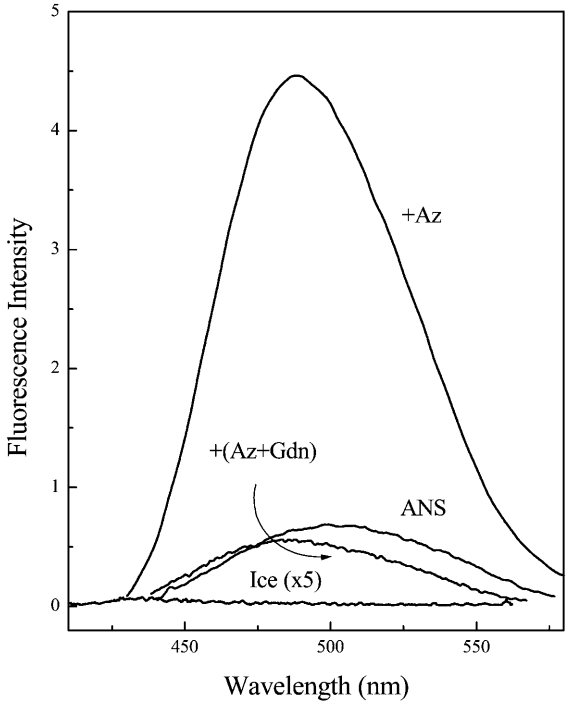

FIGURE 1 Enhancement of ANS (75 μM) fluorescence emission in ice (2 mM Na-phosphate, pH 7.5, plus 25 mM NaCl) at –13°C by the addition of Az (2.5 μM). The lack of Az enhancement when NaCl is replaced by 43 mM guanidinium hydrochloride (Gdn) and the low background luminescence from ice are also shown. $\lambda_{ex} = 350$ nm.

enhancement due to 10 μM azurin is quite distinct. Further, fluorescence changes due to aspecific ANS association to azurin can also be ruled out as under denaturing conditions the fluorescence enhancement is totally abolished. Experiments conducted at −13°C in the presence of 45 mM guanidine hydrochloride, an amount sufficient to largely or totally denature the protein, according to the complete quenching of Trp phosphorescence, demonstrate that the fluorescence of ANS is not affected by Az (Fig. 1). This behavior is normally observed in solution when ANS binding sites are destroyed upon major denaturation of the folded structure. Lastly, the propensity to form ANS binding sites in a protein is generally correlated to the loss of its tertiary structure, as exemplified by experimental conditions leading to the formation of the molten globule state (Semisotonov et al., 1991). Binding of Cd^{2+} to Az maintains its structure unaltered but remarkably increases the thermal stability of the protein, raising the melting temperature by ~30°C (Engeseth and McMillin, 1986). Based on this data, the effect of Cd^{2+} complexation on the thermodynamic stability is expected to be similar to that of native Cu^{2+} (increase in ΔG_D of 3 kcal/mol, at 20°C, and of Tm by ≈30°C) (Mei et al., 1999). The advantage of CdAz over CuAz is that in the former holoprotein ANS fluorescence is not quenched by intramolecular energy transfer to the metal center. Freezing experiments conducted with CdAz show that practically no ANS fluorescence enhancement is observed with the holoprotein (spectrum not shown).

The net contrast in ANS binding capacity between apo and holo forms does suggest that the disruption of the tertiary structure in ice, inferred from ANS binding, is to be ascribed to the greater structural plasticity of the apoform. Certainly, the lack of fluorescence enhancement in the case of CdAz rules out the possibility that the effects observed with Az be ascribed to a superficial, aspecific association of ANS to the polypeptide.

Effects of ANS concentration

The increment of ANS fluorescence in ice caused by 2.5 μM Az, at -13°C, is shown in Fig. 2 as a function of the starting ANS concentration in solution (10–200 μM). The intensity profile is reminiscent of a binding curve and shows that the enhancement tends to saturate above 75 μM, as at larger concentrations the intensity change is analogous to that of the protein-free control. However, unlike a simple hyperbolic binding curve, the fluorescence increases more gradually in the low concentration range, conferring to the profile a slightly sigmoidal shape. The profile recalls cooperative binding reactions but, of course, in a heterogeneous biphasic medium like ice (solid/liquid water), where solute diffusion is sluggish if not effectively hindered, several other possibilities can account for it. For example, at low ANS concentrations not all water pools containing the protein will necessarily have ANS molecules in them and, if diffusion in ice is slow, the extent of binding will initially be smaller. Further, there may be a variety of binding sites in the protein and the one of greatest affinity may be less fluorescent. Another important aspect to take into account is the broad distribution of protein conformations in the frozen medium, inferred from the wide distribution of phosphorescence lifetimes (Strambini and Gabellieri, 1996), as it may entail ample variability in the affinity to ANS within the protein population. For all these reasons plus the uncertainty that ANS equilibration among water pools is complete, we caution that fluorescence profiles such as those of Fig. 2 can at best provide a rough indication of the thermodynamic parameters governing ANS binding to the protein. Their main utility is to compare relative changes in the ANS binding propensity brought about by varying experimental conditions such as the addition of solutes, freezing protocols, and temperature.

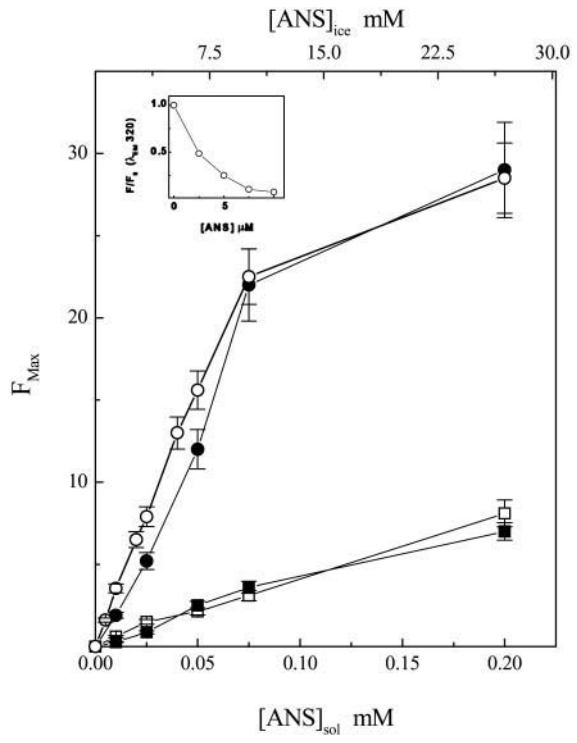

FIGURE 2 ANS fluorescence intensity, F_{max}, profiles in ice at –13°C as a function of its concentration. Direct excitation at 350 nm: ●, 2.5 μM Az; ■, 2.5 μM CdAz; and □, ANS control. Excitation at 280 nm: ○, 2.5 μM Az. The latter profile was normalized for the change in absorbance of ANS between 280 and 350 nm. The inset shows the Trp fluorescence intensity of Az (λ_{ex} 280 nm), measured at 320 nm with a 305-nm longpass filter. Indicated on the top horizontal axis is the concentration of ANS in the water pools of ice, $[ANS]_{ice}$, estimated from a freeze concentration factor, f_c = 120. Other conditions are as in Fig. 1.

The observation of Trp (W48) to ANS fluorescence energy transfer provides independent support for the formation of protein-ANS complexes. When the sample is excited at 280 nm, where both Trp and ANS absorb, the fluorescence intensity of W48 decreases sharply at increasing [ANS] (Fig. 2, *inset*) while, at the same time, the ANS fluorescence enhancement becomes significantly larger as compared to direct excitation of the probe at 350 nm (Fig. 2). This finding demonstrates that the strongly fluorescent ANS molecules are within the range of energy transfer of W48 ($r \leq R_0 = 2.3$ nm) (Slavik,1982) as would be expected upon complex formation. The comparison between quenching and enhancement profiles, however, indicates that W48 is also quenched by weakly fluorescent ANS molecules, either bound to the protein or lying on its surface, which in some parts is separated by only 0.8 nm from the chromophore. In particular, the early quenching of protein fluorescence, below 10 μM [ANS], is consistent with the existence of weakly fluorescent ANS binding sites of an affinity higher than that of those responsible for the fluorescence enhancement, whereas the attenuation of the enhancement above 50 μM [ANS] is indicative of an effective competition by energy transfer to external/superficial ANS molecules.

Having stressed that the data of Fig. 2 should not be considered as a usual binding curve, it may still be instructive to estimate an approximate affinity of ANS for Az. By assuming equilibrium conditions and an ANS activity in ice equal to the concentration in solution multiplied by the freeze concentration factor (120 at -13°C) one derives ANS binding constants in the 2–10 mM range. Compared to affinities of 0.1–0.4 mM observed for molten globule states in solution (Semisotonov et al., 1991), this estimate suggests that the binding sites created by the ice perturbation are intrinsically weaker. Whether the low affinity of ANS for

the protein reflects the decreased strength of hydrophobic interactions at low temperature or the peculiarly high solute concentration (5 M) and ionic strength of the water pool is not known. An estimate of the number of binding sites from the fluorescence profile is not possible as it would require knowledge of the average fluorescence quantum yield (φ_F) of ANS bound to Az. One can at best calculate the minimum average number of binding sites by assuming the largest possible fluorescence yield of ANS, $\varphi_F = 0.9$, found in some native proteins (Slavik, 1982). In aqueous solutions φ_F(water) = 0.004 (Stryer, 1965), but in ice at −13°C we have determined that the yield is twice as large. Hence, the maximum expected increment between free and bound ANS is ~100-fold. Near the saturation point (75 μM ANS) the ANS/Az molar ratio is 30. Thus, the minimum number of bound ANS molecules (n), with $\varphi_F = 0.9$, needed to give a sevenfold increase in fluorescence intensity is $1.6([(30 - n) \times 0.008 + n \times 0.9]/30 \times 0.008 = 7)$. The true number is probably larger because flexible sites are generally less fluorescent (Kirk et al., 1996).

Effect of temperature on ANS binding to Az

The Trp phosphorescence lifetime of azurin drops steeply as the temperature of ice is lowered down to −20°C, showing that the internal structure gains considerable flexibility on cooling of ice (Strambini and Gabellieri, 1996). The interpretation given to the lifetime/temperature profile is that the progressive freezing out of liquid water increases the strain/deformation of the compact native fold. To test whether these temperature-dependent structural alterations are also reflected in the ANS binding affinity/number of binding sites the fluorescence profiles were obtained at four different temperatures between −4°C and −18°C. The results are plotted in Fig. 3 as a function of the $[ANS]_{ice}$ calculated from the freeze-concentration factor. The data refer to the same set of samples measured at different temperatures following the sequence −13°C, −18°C, −8°C, and −4°C. At each new temperature, the samples were equilibrated for 2 h, even if after 20–30 min the fluorescence signal was apparently stable. Two features are worth noting in the ''binding'' curves of Fig. 3. First, as the temperature is lowered the fluorescence enhancement begins at progressively higher ANS concentrations (Fig. 3, *inset*), suggesting that the apparent affinity of the binding sites becomes smaller. The change in average binding constant is smooth and about a factor of 3. The trend need not necessarily point to a change in the nature of the Az binding sites because considering the drastic increase in solute concentration with freezing temperature the apparent decrease in affinity is likely to reflect large deviations of activity coefficients form ideality and/or colder temperature itself. Second, the maximum fluorescence enhancement factor (the plateau value) increases at lower temperature, a sign that the number of binding sites is larger. The tendency is not a smooth one as most of the overall twofold change in intensity is found between −8°C and −13°C. Presumably, nonequilibrium conditions may in part be responsible for the stepwise response, and the measured enhancement may not be a strict indicator of the number of binding sites. For instance, below −13°C, ANS diffusion among increasingly smaller water pools may be severely blocked and no further binding may occur at lower temperatures. At warmer temperatures, a sort of compensation between a decreased number of binding sites and a larger diffusion coefficient of ANS may lead to similar plateau values for the curves at −8°C and −4°C. The importance of slow binding kinetics in ice and sample history (ice structure) has not been examined in detail and will be the subject of future investigations. The stepwise behavior was not observed in the phosphorescence properties of Az. The main conclusion to be drawn from the temperature dependence of the ANS fluorescence enhancement is the net increase in the number of binding sites upon lowering the freezing temperature, a finding that is consistent with a greater perturbation of the protein tertiary structure.

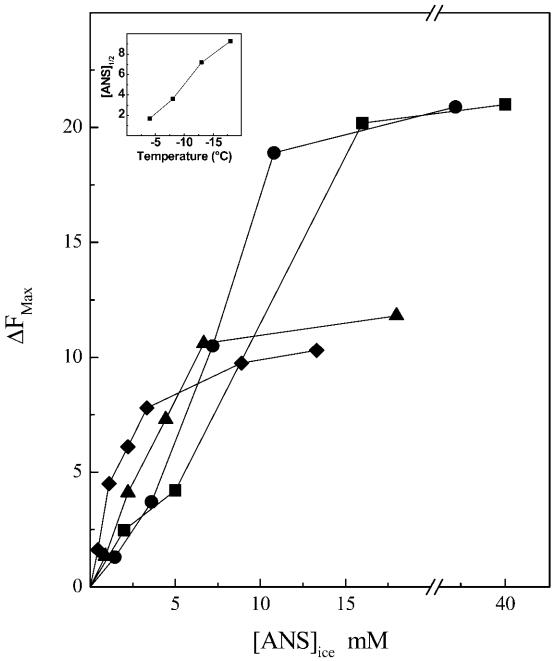

FIGURE 3 ANS fluorescence enhancement, $\Delta F_{max} = F_{max}(Az) - F_{max}$(control), profiles as a function of the ice temperature: ■, −18°C; ●, −13°C; ▲, −8°C; and ♦, −4°C. The $[Az]_{sol}$ is 2.5 μM. Other conditions are as in Fig. 1. The $[ANS]_{ice}$ was calculated from f_c = 185 (−18°C), 120 (−13°C), 82 (−8°C), and 41 (−4°C). The inset shows the dependence of the midpoint ANS concentration, $[ANS]_{1/2}$, on the ice temperature.

Effect of stabilizing and destabilizing agents

Glycerol is a classic additive that stabilizes the folded structure of proteins in solution and is a protective agent against freeze-thaw denaturation. Phosphorescence lifetimes of proteins in ice emphasize the ability of glycerol to pre-

serve a more native-like tertiary structure (Strambini and Gabellieri, 1996). The anion SCN^-, on the contrary, is a mild destabilizing agent both in solution and in ice (Allison et al., 1996), and is used in preference to strong denaturants when loosening rather than complete unfolding of the globular structure is required. Fig. 4 reports ANS binding curves obtained in the presence of 20 mM glycerol and 5 mM NaSCN, at −13°C. The fraction of liquid water was maintained constant by adjusting the NaCl concentration to give the solution the same colligative properties. The results show that glycerol, even in the millimolar concentration range (Fig. 4, *inset*), completely abolishes ANS binding to Az, whereas NaSCN more than doubles the fluorescence enhancement. The main effect of the destabilizer is not to increase the apparent affinity of ANS for Az but to create additional binding sites with equal and also lower affinity. In the case of NaSCN a fluorescence plateau is not reached within the ANS concentration range explored. The opposite effect of the two additives on the ANS fluorescence enhancement is nicely correlated to their modulation of the protein stability to unfolding.

In summary, binding of ANS to Az reveals a stressed condition of the native fold in ice that is consistent with partial loss of its tertiary structure. Preliminary tests with other proteins, such as alcohol dehydrogenase (horse liver) and glyceraldehyde-3-phosphate dehydrogenase (yeast), indicate that the phenomenon is not limited to Az and corroborate phosphorescence data that the solidification of water imposes a strain on the native fold of proteins in general. The response to stabilizing/destabilizing agents and Cd^{2+} binding to the apo-protein suggests that the extent of binding is inversely correlated to the thermodynamic stability of the globular fold. The parallel observation that these additives also modulate the degree of protection against damage from freeze-thawing or lyophilization processes strengthens the hypothesis that the initial instability of the system is caused by the solidification of water and that, subsequently, these imperfectly unfolded structures may proceed to form aggregates during thawing or are retained, and further distorted, in the successive drying of the sample (Prestrelski et al., 1993). This sensitivity of the ANS binding method to the tertiary structure of protein in ice and to the action of stabilizing/destabilizing agents should find practical utility in testing the effectiveness of various additives employed in protein formulations as well as devising safer freeze-drying protocols of pharmaceutical proteins.

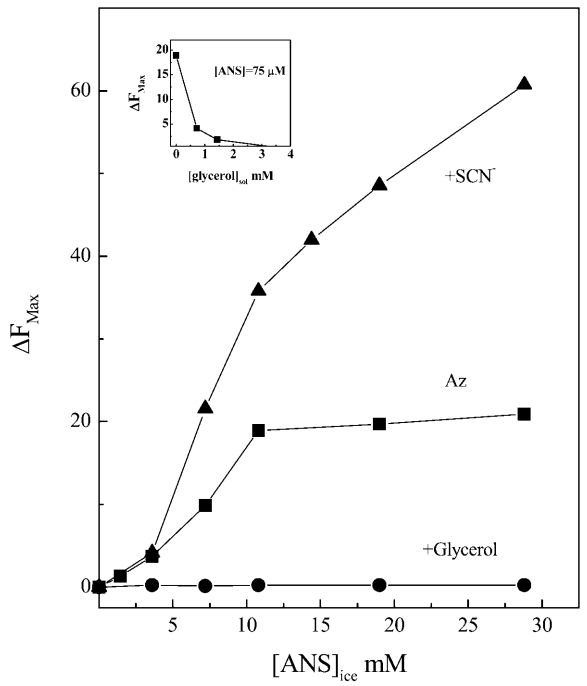

FIGURE 4 ANS fluorescence profiles in the presence of glycerol (●, 20 mM glycerol plus 15 mM NaCl) and of SCN^- (▲, 5 mM NaSCN plus 20 mM NaCl), in ice at –13°C. The data are compared to that obtained in the absence of additives (■). The $[Az]_{sol}$ is 2.5 μM. Other conditions are as in Fig. 1. The inset shows the dependence of ΔF_{max} on the glycerol concentration.

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