Protein Stability in Ice

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ABSTRACT This study presents an experimental approach, based on the change of Trp fluorescence between native and denatured states of proteins, which permits to monitor unfolding equilibria and the thermodynamic stability (ΔG°) of these macromolecules in frozen aqueous solutions. The results obtained by guanidinium chloride denaturation of the azurin mutant C112S from *Pseudomonas aeruginosa*, in the temperature range from -8 to -16°C, demonstrate that the stability of the native fold may be significantly perturbed in ice depending mainly on the size of the liquid water pool (V_L) in equilibrium with the solid phase. The data establish a threshold, around $V_L = 1.5\%$, below which in ice ΔG° decreases progressively relative to liquid state, up to 3 kcal/mole for $V_L = 0.285\%$. The sharp dependence of ΔG° on V_L is consistent with a mechanism based on adsorption of the protein to the ice surface. The reduction in ΔG° is accompanied by a corresponding decrease in *m*-value indicating that protein-ice interactions increase the solvent accessible surface area of the native fold or reduce that of the denatured state, or both. The method opens the possibility for examining in a more quantitative fashion the influence of various experimental conditions on the ice perturbation and in particular to test the effectiveness of numerous additives used in formulations to preserve labile pharmaco 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 (1). 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. Further, knowledge of the stability and structure of proteins below water-freezing temperatures is fundamental toward understanding structure-function relationships of psychrophilic organisms that replicate and function in water channels of ice (2,3). Despite the relevance of the phenomenon and the commercial value for the growing polypeptide-based pharmaceutical industry, little is known of the structure and thermodynamic stability of proteins in ice. Indeed, for the stabilization of pharmaco proteins during the industrial freeze-drying processes, formulations and protocols are still largely worked out empirically, case by case, drawing mostly on longtime experience and guided by thermodynamic data pertaining to stabilizing additives in liquid solutions (4–6).

Progress toward a quantitative description of protein stability in ice, capable of distinguishing between thermodynamic and kinetic control of degradation processes, has been hampered primarily by the inapplicability or poor sensitivity of usual spectroscopic techniques for determining unfolding equilibria in this medium. Frozen samples are highly scattering and heterogeneous with a spatially uneven concentration of solutes. It follows that the intensity of optical signals is characterized by large site-to-site variations and is generally combined to a conspicuous and unsteady background not readily accounted for.

The aim of this investigation is to develop an experimental approach that by overcoming the above limitations permits to determine protein unfolding equilibria in ice and assess the thermodynamic stability (ΔG°) of the macromolecule in this medium. The method exploits the change in Trp fluorescence spectrum and yield between native (N) and denatured (D) states of a protein to monitor the relative concentration of N and D in a chemically induced denaturation equilibrium. The main innovations with respect to standard fluorimetry are represented by: 1), replacement of lamp with pulsed laser excitation, with consequent drastic reduction in spurious background signals and; 2), simultaneous acquisition of the fluorescence spectrum by means of a CCD camera, combined to data analysis based on intensities ratio, a procedure that overcomes problems connected with site-to-site and sample-to-sample signal instability.

The model protein system selected for this study is azurin from *Pseudomonas aeruginosa*, a small (14 kDa), monomeric copper-binding protein well characterized in terms of its structure (7,8) and thermodynamic stability (9–11). Other suitable features of azurin are 1), it exhibits the most blue-shifted Trp fluorescence spectrum known in proteins (12), and, consequently, unfolding causes a very large spectral red shift that is convenient for accurate determination of the equilibrium composition of the mixture. 2), The macromolecule has a single Trp residue located within the rigid inner core of the globular structure wrapped up by a tight β-barrel motif. Exposure of W48 to the solvent, which causes the fluorescence change, requires no less than global unfolding as confirmed by the superposition of fluorescence and circular dichroism denaturation profiles (11). 3), The denaturation

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of Cu-free azurin by guanidinium chloride (Gdn) is a fully reversible process, and, even if there is evidence of transient intermediates, the equilibrium is well represented by a two-state model (11,13). Preliminary Gdn denaturation experiments in ice conducted with wild-type copper-free azurin have pointed out, however, that contrary to fluid solutions in ice recovery of the N-state was slow (several hours) and incomplete. Rapid equilibration and full, prompt recovery of the N-state was eventually discovered with the azurin mutant C112S, in which the substitution of Cys-112, a ligand of the metal binding site, with Ser prevents both the formation of the metal complex by trace metal impurities (11) and potential irreversible cross-linking reactions owed to disulfide formation. Here, we monitor Gdn-induced denaturation of C112S azurin (Caz) in solution and in ice at subzero temperatures. The results show that, under certain conditions, ice decreases significantly the stability ($\Delta G°$) of the native fold. The perturbation is predominantly modulated by the size of the liquid water pool in equilibrium with ice, becoming negligible above a threshold of 1.5%.

MATERIALS AND METHODS

All chemicals were of the highest purity grade available from commercial sources and were used without further purification. Tris (hydroxymethyl) aminomethane (Tris), and NaCl Suprapur® were from Merck (Darmstadt, Germany). Gdn was from Sigma-Aldrich (Deisenhofen, Germany). The concentration of Gdn was determined by refractive index (14) and confirmed by density measurements (15). Water, doubly distilled over quartz, was purified by a Milli-Q Plus system (Millipore, Bedford, MA).

Protein expression and purification

The preparation of C112S azurin was done following a procedure analogous to that described by Karlsson et al. (16) for the wild-type protein except for the omission of any $CuSO_4$ addition in both growth and purification medium (17). The plasmid carrying the wild-type sequence was a generous gift of Prof. A. Desideri (Università di Roma, ''Tor Vergata''). The C112A mutant was constructed using the QuikChange kit (Stratagene, La Jolla, CA) and confirmed by sequencing.

Sample preparation

Frozen samples are characterized by a solid phase of pure water (ice) in equilibrium with a liquid phase (liquidus) formed by liquid water and practically all the solutes (protein and salts) at relatively high concentration. The solutes concentration in the liquidus is determined exclusively by the freezing temperature (T_f) and the solutes composition, in our case the salt mixture employed in denaturation experiments, and is independent of the initial salt concentration in the starting liquid solution (1). This implies that by decreasing the salt concentration in the starting solution a larger fraction of water will become ice and the volume of the liquidus will shrink proportionally. Thus, for a given salt composition and T_f, the fraction of liquid water in equilibrium with ice (V_L) is simply $V_L = [salt]_{solution}/[salt]_{ice}$ (from $1 \times [salt]_{solution} = V_L \times [salt]_{ice}$).

Protein denaturation in solution and in ice was carried out in samples containing a Gdn-NaCl salt mixture, of constant total salt concentration and increasing Gdn content, prepared starting from a common stock of concentrated Gdn-NaCl solutions, varying from 30% to 90% in Gdn content (4 M total salt in 1.2 mM Tris pH7.5), referred to as mother mixtures. Mother mixtures were prepared from stock Gdn (6.60 M) and NaCl (5M) solutions. More dilute salt solutions, used for protein denaturation experiments in ice at various V_L, were all prepared from these same mother mixtures simply by dilution with pure water, a procedure that for a given salt composition assures exactly the same concentration of Gdn in ice ($[Gdn]_{ice}$) at all V_L values examined. The samples employed in denaturation experiments (6 μM in protein, 150 μL total volume) were prepared by adding 20 μL of protein stock (45 μM, in 0.1 mM Tris pH 7.5) to 130 μL of salt solution of the desired concentration and composition. The total salt concentration (at 20°C) of protein samples to be frozen ranged from 0.010 to 1.0 M, which at −14°C corresponds to a liquid fraction (V_L) ranging approximately from 0.28 to 29%.

Determination of [Gdn] in ice from freezing temperature versus salt concentration plots

A critical step in the derivation of denaturation profiles in ice is the correct evaluation of the denaturant concentration in the liquid phase of frozen samples, $[Gdn]_{ice}$. The latter was calculated from the total salt concentration in the liquid phase of ice, at a given temperature and salt composition, $[salt]_{ice}$ given in Fig. 1, times the fraction of Gdn in the salt mixture. The curves of Fig. 1 were obtained as follows. As $[salt]_{ice}$ is, by definition of T_f, the salt concentration of the solution having that particular freezing temperature, $[salt]_{ice}$ at various temperatures is normally read off an experimental plot describing the dependence of the freezing temperature on the salt concentration. As mentioned above, $[salt]_{ice}$ varies with T_f but also with the composition of the salt mixture. Ideally, T_f versus [salt] plots should therefore be constructed for every salt composition employed in the denaturation profile. Because there is only a moderate dependence of T_f on the % Gdn in the salt mixture, we have determined only seven T_f versus [salt] plots, covering salt compositions ranging from 30% to 90% in Gdn content, one for roughly every 10% increment, each of which over a 2–4 M concentration range (0.25 M increment in salt concentration) (in all 70 T_f data points). For a given freezing temperature the seven plots yielded seven distinct values of $[salt]_{ice}$, one for each salt composition. The curves of Fig. 1, describing the dependence of $[salt]_{ice}$ versus percent Gdn at selected freezing temperatures, represent polynomial fits of the seven salt concentrations read off the above experimental plots at each one of the selected temperatures.

For the determination of freezing temperatures 1-mL samples were placed in long round quartz cell (4-mm ID), sealed off from the atmosphere,

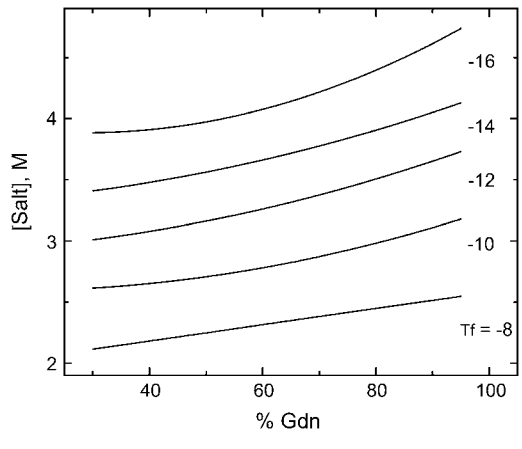

FIGURE 1 Total salt concentration in equilibrium with ice, $[salt]_{ice}$, as a function of the molar fraction of Gdn in the salt mixture, at selected freezing temperatures. The curves represent polynomial fits of [salt] versus percent Gdn data, interpolated at selected temperatures, from the seven experimental freezing temperature, T_f, versus [salt] plots, that cover salt compositions ranging from 30% to 90% in Gdn content.

and slowly frozen in a cold (−20°C) bath to avoid cracking of the cell. After 0.5 h equilibration, the bath temperature was raised by consecutive, 0.05°C, steps intercalated by 10-min equilibration time after each temperature change. Before ice was completely melted the solution was gently stirred (by means of a temperature equilibrated thin plastic rod) to eliminate any temperature and concentration gradient through the sample. The freezing temperature, measured with a precision (0.001°C) thermometer (Hart Scientific, model 1502, American Fork, Utah), was the temperature of the bath at which no further ice could be detected. The reproducibility of T_f measurements was generally better than 0.1°C.

Fluorescence measurements

Protein samples (150 μL) were placed in capped spectrosil quartz tubing, 4-mm ID, and frozen in a cold bath (−20°C). Subsequently, ice was melted leaving a small seed from which new ice is formed in a bath set at the same temperature of the experiment. It was observed that the reproducibility of denaturation data improved when freezing proceeded from an ice seed rather than from sudden cooling the solution to very low temperatures. Presumably, rapid freezing from undercooled solution gives rise to a more uneven distribution of solutes in frozen samples. After 0.5 h equilibration in the bath the samples were rapidly transferred to the sample holder of the fluorometer, set at the same temperature. Fluorescence spectra were taken after an additional 1 min thermal equilibration in the sample holder to balance for the small temperature increase occurring during sample transfer from the bath to the fluoremeter. Kinetic runs after temperature jumps show that both in ice and in liquid solution, after equilibration for 5 min at a given temperature no further changes on the degree of denaturation is observed up to a time as long as 1 week. At least three independent preparations were carried out for each V_L series and the fluorescence emission of every preparation was measured again after melting and refreezing of the samples. The results represent the average of these measurements.

Fluorescence spectra were measured on a homemade apparatus that implements pulsed UV laser excitation and emission detection by means of a CCD camera. The main advantage of laser over traditional lamp-monochromator sources is a considerable reduction of the spurious background signal, in the region of the fluorescence spectrum, which is observed in frozen samples. The merits of CCD detection over the traditional scanning monochromator-photomultiplier assembly are enhanced sensitivity to low light levels and simultaneous acquisition of the entire spectrum. The latter is crucial for avoiding spectral distortions caused by instability of excitation intensity, excitation/collection efficiency of the optical system and transmittance of the medium during spectral acquisition. Pulsed excitation (λ_{ex} = 290 nm), horizontally polarized with the respect to the optical plane, was provided by a frequency-doubled Nd/Yag-pumped dye laser (Quanta Systems, Milan, Italy) with pulse duration of 5 ns, pulse frequency up to 10 Hz and energy per pulse varying from 10 to 1000 μJ. The emission collected at 90° from the excitation direction was passed through a 290-nm long-pass filter (WG290, Lot-Oriel, Milan, Italy) and dispersed by a 0.3-m focal length triple grating imaging spectrograph (SpectraPro-2300i, Acton Research, Acton, MA) set to a band pass of 2.0 nm. The emission intensity in the spectral range 295–435 nm was monitored by a back-illuminated 1340 × 400 pixels CCD camera (Princeton Instruments Spec-10:400B (XTE), Roper Scientific, Trenton, NJ) cooled to −60°C. Fluorescence intensity variations among frozen samples were reduced by rotating the sample (3 Hz) during spectral acquisition and averaging 8–10 pulses during 1.5-s acquisition time. To avoid condense formation on optical components the whole apparatus was maintained under nitrogen atmosphere.

Data analysis

The fraction of native azurin, f_N, in the denaturation equilibrium was determined from the fluorescence intensity ratio $R = F_{308}/F_{357}$ according to the relationship

$$f_N = \left({}^{D}f_{308} - R\,{}^{D}f_{357}\right)/\left(R\Delta_{357} - \Delta_{308}\right), \quad (1)$$

where $\Delta_{\lambda i} = {}^{N}f_{\lambda 1} - {}^{D}f_{\lambda i}$ and, ${}^{N}f_{\lambda i} = {}^{N}F_{\lambda i}/F_{\lambda iso}$ and ${}^{D}f_{\lambda i} = {}^{D}F_{\lambda i}/F_{\lambda iso}$ are the fluorescence intensities at wavelength λi of native and fully denatured azurin, respectively, normalized by the fluorescence intensity at the isosbestic point, $F_{\lambda iso}$. Equation 1 is derived by assuming a two-state equilibrium as follows: at any wavelength λi the fluorescence intensity, $F_{\lambda i}$, is the sum of contributions from N and D species and is given by

$$F_{\lambda i}\,\alpha\, I_0\, c\left[f_N\,{}^{N}f_{\lambda}\mathrm{i} + (1 - f_N)\,{}^{D}f_{\lambda i}\right],$$

where I_0 and c are the excitation intensity and the protein concentration, respectively. Hence, the fluorescence intensity ratio R, expressed in terms of f_N, is given by

$$\begin{aligned} R &= F_{308}/F_{357} \\ &= \left\{f_N\left({}^{N}f_{308} - {}^{D}f_{308}\right) + {}^{D}f_{308}\right\}/\left\{\mathbf{f}_N\left({}^{N}f_{357} - {}^{D}f_{357}\right) + {}^{D}f_{357}\right\} \\ &= \left(f_N\Delta_{308} + {}^{D}f_{308}\right)/\left(f_N\Delta_{357} + {}^{D}f_{357}\right), \end{aligned}$$

which is a rearranged form of Eq. 1. It should be noted that above expression for f_N takes into account any variation of molar absorptivity and fluorescence yield between N- and D-states of the protein and, moreover, it is totally independent of variations in excitation intensity, extent of scattering and protein concentration within the exciting beam. Note also that because the fluorescence yield, of D in particular, varies substantially with temperature, the isosbestic point and each parameter of Eq. 1 was determined at every freezing temperature examined.

The thermodynamic parameters $\Delta G°$, m-value and $D_{1/2}$ (the midpoint denaturant concentration) were obtained from the linear extrapolation of ΔG_{Gdn} ($\Delta G_{Gdn} = -\,RT\ln\,(1/f_N - 1) = \Delta G° - m\,[Gdn]$) to zero denaturant concentration as described before (18,19).

RESULTS

Fig. 2 reports the fluorescence spectrum (λ_{ex} = 290 nm) of native, partially, and fully denatured C112S azurin in ice, at

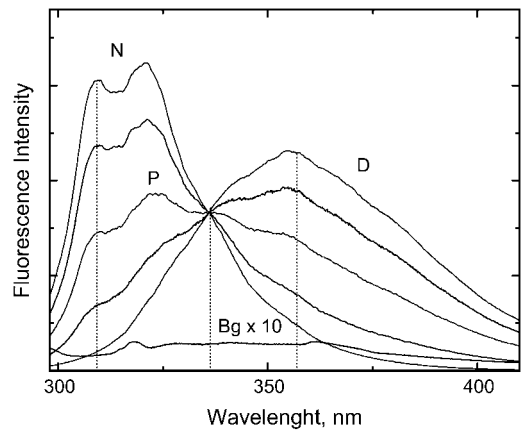

FIGURE 2 Fluorescence spectra of C112S azurin (6 μM) in ice, V_L = 0.57%, at −14°C and various extents of denaturation. Native (N) and fully denatured (D) spectra refer to 0 and 100% Gdn content in the 19 mM Gdn-NaCl + 1 mM Tris (pH 7.5) salt mixture, respectively. A typical background signal (Bg) from ice is also included. Vertical dashed lines indicate the wavelengths used to calculate the fluorescence intensity ratio R and the isosbestic point. The excitation wavelength is 290 nm. All spectra are corrected for the instrumental response.

−14°C showing a clearly identified isosbestic point. Both N and D spectra are practically indistinguishable from those in liquid solutions. A slight difference in the spectrum between liquid and frozen samples is owed to a larger background contribution in the solid state. In general background subtraction was found to be less practical because its contribution varies from sample to sample or even on changing sample orientation, up to twofold. We found that by choosing wavelengths where the fluorescence signal is large and the background signal is small (Fig. 1) the intensity ratio is less affected by background subtraction. The choice of wavelength is not critical, as the results are practically the same for $\lambda \pm 5$ nm. Experiments at larger protein concentration, where the background contribution is proportionally smaller, confirmed that under these conditions background subtraction made negligible differences on the estimate of f_N.

Caz denaturation in ice was demonstrated to be a completely reversible process, as reported for the protein in liquid solution at ambient temperature (11). This is indicated by the sudden and complete recovery of the native fluorescence upon thawing of fully denatured frozen samples. Further, in ice, where the denaturant concentration depends sharply on the freezing temperature (for example, at −14°C a change of ± 2°C is sufficient to shift the equilibrium from predominantly native to denatured), kinetic runs after temperature jumps show that a new equilibrium is established rapidly, within the time, 2–3 min, required to change the sample temperature. Most importantly they show that f_N remains constant at a given temperature even after several consecutive cooling-warming cycles. Such lack of hysteresis in the unfolding equilibrium is consistent with a highly reversible process which excludes the formation of protein aggregates.

Spatial uniformity of f_N in ice

Ice is a nonuniform medium in which the concentration of solutes can vary from one region to another, depending on the ice growth rate and the direction of the thermal gradient. Tests devised to check the spatial uniformity of the degree of azurin denaturation consisted in measuring the fluorescence spectrum in different regions of the sample after reducing the diameter of the excitation beam from 2.5 (normal) to 0.5 mm, to enhance spatial resolution. The results obtained with frozen samples of various V_L, with an f_N value close to 0.5 for maximum sensitivity, indicate that whereas the fluorescence intensity can vary considerably (up to 2.5-fold) across the sample, presumably reflecting distinct excitation/fluorescence collection efficiencies or spatially non uniform protein concentration, no significant differences could be detected in the degree of denaturation. Thus, it appears that at this spatial resolution the denaturant concentration in the liquidus and the stability of azurin in ice are constant throughout.

Effect of ice formation (V_L) on the unfolding equilibrium at constant temperature and solutes activity

The solution containing 1 mM Tris, pH 7.5 plus 1.58 M Gdn and 1.93 M NaCl (3.51 M total salt) has a freezing temperature of −14.0°C. Thus, when diluted samples prepared from this stock are brought to −14°C, water will freeze out until the concentration of the salt mixture in the liquidus reaches 3.51 M. This implies that, at −14°C, the solutes activity in the liquid water pool of frozen samples is the same as that of the original stock, independently of the initial dilution factor. The only difference between samples obtained from increased dilution of the salt mixture is that a proportionally grater fraction of water will form ice, i.e., the size of the liquid water pool (V_L) shrinks. Should ice not perturb the free energy of the protein in either N or D state, the unfolding equilibrium would not be affected by freezing and f_N be independent of V_L. Fig. 3 compares the value of f_N between the liquid stock solution and frozen samples obtained from progressive dilution of the same stock down to 10 mM total salt, corresponding to a decrease in V_L from 100 to 0.285% (for convenience we shall sometime indicate V_L in brackets by the salt concentration, mM, at 20°C). The results emphasize that, despite the constant activity of guanidinium, the unfolding equilibrium is a steep function of V_L. For this particular salt composition f_N decreases sharply from 0.9 at $V_L = 0.285\%$ to reach a plateau of 0.3 around $V_L = 1.4\%$ beyond which f_N attains a constant value, practically undistinguishable from that in liquid state. Hence, the f_N profile demonstrates unequivocally that when the amount of liquid water in equilibrium with ice is <1.5% the presence of the solid phase affects significantly the denaturation of azurin

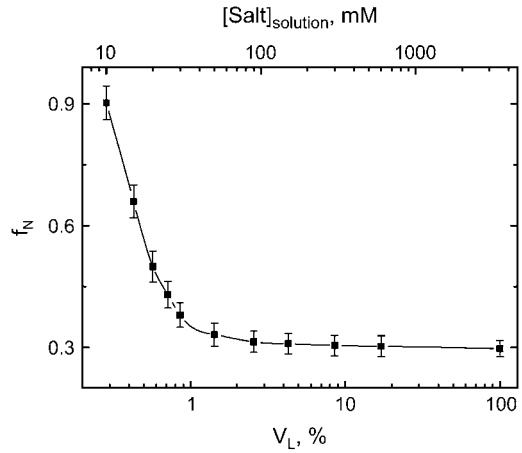

FIGURE 3 Fraction of native C112S azurin, f_N, in the denaturation equilibrium at −14°C, as a function of the fraction of the fraction of liquid water, V_L, in equilibrium with ice. The total concentration of the salt mixture, at 20°C, is indicated on the top axis. The Gdn content in the mixture is 45.05%. The salt concentration in the liquidus is 3.51 M. $V_L = 100\%$ refers to the liquid state. Other conditions are as in Fig. 2.

by Gdn. In view of the expected destabilizing effect of ice on the native protein fold, the increase of f_N is surprising because a shift of the equilibrium toward the folded state appears as a stabilizing influence of the ice. Above this V_L threshold the degree of unfolding is constant ($f_N = 0.3$) among frozen and liquid samples implying that there is no apparent influence of water freezing on the denaturation equilibrium. Indirectly, the above good agreement between the value of f_N of frozen and liquid samples is also a confirmation that the procedure to evaluate f_N in ice and the calculation of $[Gdn]_{ice}$ are quite accurate.

Denaturation profiles in ice at selected V_L

Equilibrium unfolding curves in liquid and in frozen samples, $V_L = 0.285$, 0.57, 1.425, and 5.7% (10, 20, 50 and 200 mM samples), at −14°C, are compared in Fig. 4 *A* (as stated under the Materials and Methods section the variation in denaturant concentration in ice is derived from the total salt concentration in the liquid pool at −14°C, given in Fig. 1, times the fraction of Gdn in the salt mixture). Error bars show that in ice the variability of f_N is somewhat larger relative to the fluid state, presumably because here small temperature variations, altering the salt concentration, have a larger impact on f_N.

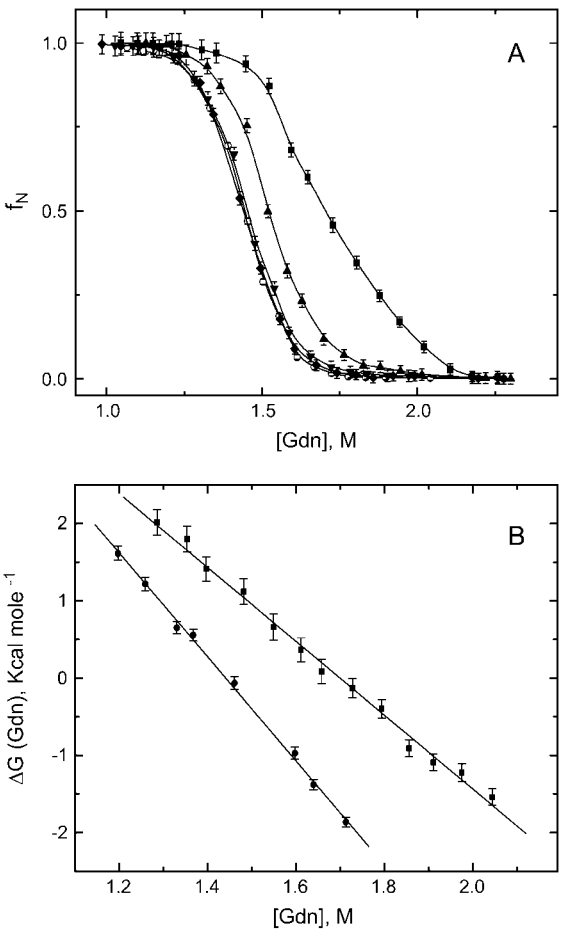

FIGURE 4 (*A*) Equilibrium denaturation curves of C112S azurin (6 μM), at −14°C, and at selected V_L: $V_L = 0.285\%$ (■), $V_L = 0.57\%$ (▲), $V_L = 1.425\%$ (▼), and $V_L = 5.7\%$ (♦). The denaturation profile in the liquid state (○), 3.51 M total salt concentration, is included for comparison. Other conditions are as in Fig. 2. (*B*) Linear free energy plots, $\Delta G = -RT\ln(1/f_N - 1) = \Delta G° - m[Gdn]$, for the denaturation of C112S azurin in ice $V_L = 0.285\%$ (■) and in solution (●), at −14°C (data from Fig. 4 *A*). ΔG^0 and *m*-values are calculated by a linear least-square fit.

Consistent with the f_N data of Fig. 3 we note that the denaturation profile at $V_L = 5.7\%$ is practically super imposable to that in the liquid state and that for $V_L = 1.425\%$ is only slightly shifted to higher Gdn concentration. For smaller V_L, however, the profile becomes progressively less steep and shifts to higher denaturant concentration. Fig. 4 *B* shows that the free energy plots ($\Delta G_{Gdn} = -RT\ln(1/f_N - 1) = \Delta G° - m[Gdn]$) are relatively linear on the denaturant concentration both in solution and in ice. The parameters $\Delta G°$, *m*-value, and $D_{1/2}$ (the transition midpoint) obtained by least-square fitting a two-state model to the data are collected in Table 1. As expected, at large V_L the magnitude of the thermodynamic parameters is practically identical to that in liquid solution, confirming that in the plateau region, $V_L > 1.5\%$, ice does not affect the stability of Caz. However, in frozen samples with <1% liquid water $\Delta G°$ decreases significantly (up to 31.5% for $V_L = .285\%$) relative to the liquid state, the data providing the first unequivocal proof that under certain conditions ice destabilizes the native fold of proteins. Most importantly, Table 1 shows that the lower protein stability in ''dry'' ice is correlated to the reduction in *m*-value. Because the gradient of the free energy plot decreases more steeply than $\Delta G°$ (for $V_L = .285\%$ the *m*-value decreases by 42%) the denaturation profile at small V_L actually shifts to higher denaturant concentrations (increase in $D_{1/2}$), relative to the liquid state, despite a smaller protein stability. The direct correlation established between the magnitude of the *m*-value and the change in solvent-accessible surface area of the macromolecule on unfolding (ΔASA) (20,21) suggests that the decreased stability in ice is associated to a remarkable reduction of ΔASA during guanidinium unfolding.

Effect of temperature on the denaturation profile in liquid and frozen samples

We have extended azurin denaturation studies in ice, formed from 10 mM salt mixtures ($V_L = 0.285$ at −14°C), and in the liquid state over the temperature interval of from −8 to −16°C, a range well above the eutectic temperatures of the salts in the mixture or the transition of liquid water to a glass state. The effect of temperature on the thermodynamic parameters is reported in Table 1.

In the liquid state, the main effect of cooling is to reduce $D_{1/2}$, from 1.58 M at −8°C to 1.41 M at −16°C, and raise by roughly an equivalent amount, from 6.6 to 7.5 kcal/mole, the *m*-value. The combined effect of the two opposing trends

TABLE 1 Thermodynamic parameters relative to the denaturation of Caz in liquid and frozen solutions at various V_L (%)

SAMPLE	T (°C)	ΔG^0 (kcal/mole)	m (kcal/mole)	$D_{1/2}$ (M)
Ice V_L= 0.285	−14	6.70 ± 0.56	3.97 ± 0.30	1.70
Ice V_L= 0.570	−14	8.04 ± 0.44	5.30 ± 0.27	1.52
Ice V_L= 1.425	−14	8.41 ± 0.34	5.81 ± 0.22	1.45
Ice V_L= 5.70	−14	10.00 ± 0.27	7.00 ± 0.18	1.44
Liquid	−14	9.78 ± 0.21	6.85 ± 0.14	1.44
Ice V_L= 0.285	−8	6.89 ± 0.31	3.63 ± 0.19	1.79
Ice V_L	−10	6.83 ± 0.38	3.77 ± 0.23	1.73
Ice V_L	−12	6.77 ± 0.38	3.85 ± 0.22	1.70
Ice V_L	−16	6.55 ± 0.77	3.99 ± 0.43	1.64
Liquid	−8	10.54 ± 0.18	6.66 ± 0.11	1.58
Liquid	−10	10.38 ± 0.23	6.71 ± 0.14	1.55
Liquid	−12	10.49 ± 0.20	6.98 ± 0.12	1.50
Liquid	−16	10.67 ± 0.19	7.54 ± 0.12	1.41
Liquid	+20	9.63 ± 0.40	5.53 ± 0.23	1.74

maintains relatively constant the value of $\Delta G°$. We should point out that the reduction of $D_{1/2}$ and the corresponding increase of *m*-value at lower temperature are partly apparent because f_N plots were constructed without taking into account the increase in denaturant concentration from the volume reduction on cooling (all concentrations refer to 20°C). Only appropriate density measurements will tell whether volume contraction accounts completely for the above trends. This trend is also confirmed by the denaturation profile at ambient temperature (20°C), which yielded $D_{1/2}$ =1.74 M, m = 5.5 kcal/mole and $\Delta G°$ = 9.63 kcal/mole, magnitudes similar to those reported by Sandberg et al (11)($\Delta G°$ = 9.0 kcal /mole, m = 5.74 kcal/mole, and $D_{1/2}$ = 1.57 M). Slight variations in the magnitude of the thermodynamic parameters from that study may arise from differences in experimental conditions (phosphate buffer versus Tris, pH 7.0 versus 7.5, and variable versus constant ionic strength across the transition).

The temperature dependence of the denaturation profile in ice shows a similar trend to that observed for the liquid state, except that the variation of $D_{1/2}$ and *m*-value is somewhat smaller and $\Delta G°$ decreases slightly on cooling. Considering that from −8 to −16°C the liquid water pool of 10 mM samples decreases from 0.44 to 0.25%, and that, as indicated above, these parameters are sensitive to V_L, the slightly different response to temperature between liquid and frozen samples could be largely attributed to V_L changes. Overall, between −8 and −16°C the stability of Caz is little affected by temperature both in liquid and frozen solutions. In ice, $\Delta G°$ remains 30–35% smaller that in solution and the decrease in protein stability is constantly associated to a 42–45% reduction in *m*-value. Such invariance to temperature attests to a predominant role of V_L on the ice perturbation.

DISCUSSION

This study reports a methodology that for the first time permits to assess the thermodynamic stability of proteins in frozen solutions. The results obtained with C112S azurin, a small, compact, β-barrel protein, as model system, demonstrate that the stability of the native fold may be significantly perturbed in ice depending mainly on the size of the liquid water pool in equilibrium with the solid phase. At −14°C, the data establish a threshold around V_L =1.5%, corresponding to ~50 mM concentration of a monovalent salt, below which $\Delta G°$ in ice decreases progressively relative to the liquid state, up to 3 kcal/mole for V_L = 0.285%. Naturally, the V_L threshold is expected to depend on the protein system and experimental conditions. Presumably, the threshold will be higher for less stable folds or for subunit dissociation of oligomeric proteins relative to global unfolding transitions. Above the threshold Gdn denaturation occurs apparently as in the liquid state, with no particular influence of ice on $\Delta G°$ and *m*-value. The implication here is that the protein free energy and the denaturant activity are not affected by the solidification of up 98.5% of the water in the sample. It is likely that solutes, other than the monovalent salts employed in this study, above this threshold have analogous effects on protein stability in both frozen and fluid solutions.

The above results predict that the effectiveness of stabilizing additives in preventing protein unfolding in the frozen state will be a combination of two influences: the ability to stabilize the N-state at low temperature and high solute concentration (a preferential hydration mechanism), plus a specific action of the additive to contrast perturbations deriving from protein-ice interactions. The f_N profile of Fig. 3 is particularly revealing as the presence of an ice threshold permits to distinguish the relative importance of these two factors on $\Delta G°$. In the perspective of protein stabilization studies, the utility of this discrimination is at least twofold: a), it serves to find limiting conditions of temperature, but particularly of solutes composition and concentration beyond which the ice perturbation is negligible (the plateau region) and need not be counteracted; and b), it can assess the specific effectiveness of an additive, at the high concentration generated in the liquidus, in attenuating the perturbation induced by solid phase (V_L below the threshold) as well in stabilizing the native protein fold in the plateau region (V_L above the threshold). Although, a constant stability of the protein in the plateau region of ice and in solution suggests that the latter can be determined by stability studies in the liquid state at cold temperature, for some compounds it is hard if not impossible to reach the high solute concentration attained in frozen samples. On the contrary, $\Delta G°$ obtained in the plateau region of ice may provide information on the thermodynamic effects of additives in liquid solutions at subfreezing temperatures simply by preparing dilute, mM solutions of the additive and exploit the up to 100-fold concentration factor induced by freezing.

Below the threshold there is a progressive decrease of $\Delta G°$ and *m*-value. Apart from increasing the protein concentration in the liquidus, the main effect of reducing V_L, the size of the liquid water pool, is to increase the surface area of ice in

contact with the liquid phase. Thus, the mechanism leading to increasing destabilization of N at small V_L is reminiscent of an adsorption process in which the macromolecule is partitioned between the surface of ice and the surrounding liquidus. As V_L decreases a greater fraction of the protein sample would adsorb to ice and change its stability. The decrease of $\Delta G°$ in ice implies that protein ice-interactions lower the free energy of the D-state to a greater extent than that of the N-state, suggesting that the interactions are stronger for the expanded protein fold having a larger solvent accessible surface area (ASA), which is reasonable for an adsorption process.

The decrease of $\Delta G°$ in dry ice is accompanied by an even steeper reduction in *m*-value. Several studies have demonstrated that the *m*-value is directly correlated to the change in solvent-accessible surface area (ΔASA) of the protein during unfolding (20,21). A smaller ΔASA in ice relative to the liquid state indicates that destabilization of the protein fold in ice is associated to either a larger ASA for the N-state or a smaller ASA for the D-state or both, namely that the structure of either N- or D-states, or both, is altered by the interaction with ice. Extreme scenarios contemplate that the main effect of adsorption of the protein to ice is partial unfolding of the N-state, and consequent increase in ASA^N, or alternatively, partial compaction of the expanded D-state, with consequent decrease of ASA^D. Practically no data is available on the structure of D in ice and the fluorescence spectrum, which is sensitive to the microenvironment of the chromophore, does not report alterations at small V_L that could be interpreted as less than full exposure of W48 to the aqueous phase. On the contrary, there is direct spectroscopic evidence of partial unfolding of N at small V_L. The phosphorescence lifetime of internal Trp residues in several proteins, azurin included, reports a drastic enhancement of the structural flexibility of the macromolecule in ice at small V_L that is consistent with substantial loosening of the compact protein core (22). More recent studies, based on ice induced binding of ANS to proteins, confirmed that in frozen solutions the globular structure is generally perturbed tending to evolve into a molten globule like state (23,24). Both Trp phosphorescence and ANS binding probes report an altered, expanded N-state that could largely account for decrease of $\Delta G°$ and *m*-value observed here. Assuming that the N-state is mostly affected by ice, the reduction of *m*-value predicts a maximum average increase of ASA^N of $\sim$30% in ice of $V_L = 0.285\%$.

In conclusion, this study reports the first determination of the thermodynamic stability of a protein in ice. The spectroscopic approach developed here, combined to chemical denaturation should find applicability to a large number of proteins. The results obtained with azurin as a model system show a sharp dependence of $\Delta G°$ on V_L that is consistent with adsorption of the protein to the ice surface as proposed before (5,22) for the perturbation of the native structure in the frozen state. The method opens now the possibility for examining in a more quantitative fashion the influence of various experimental conditions on the ice perturbation and in particular to test the effectiveness of numerous additives used in formulations to preserve labile pharmaco proteins.

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