Counteracting Effects of Thiocyanate and Sucrose on Chymotrypsinogen Secondary Structure and Aggregation during Freezing, Drying, and Rehydration*

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ABSTRACT Studies of numerous proteins with infrared spectroscopy have documented that unfolding is a general response of unprotected proteins to freeze-drying. Some proteins that are unfolded in the dried solid aggregate during rehydration, whereas others refold. It has been proposed for the latter case that aggregation is avoided because refolding kinetically outcompetes intermolecular interactions. In contrast, with proteins that normally aggregate after rehydration, minimizing unfolding during freeze-drying with a stabilizer has been shown to be needed to favor the recovery of native protein molecules after rehydration. The purpose of the current study was to examine first the opposite situation, in which a denaturant is used to foster additional unfolding in the protein population during freeze-drying. If the protein is not intrinsically resistant to aggregation under the study conditions (e.g., because of intermolecular charge repulsion) and the denaturant does not disrupt intermolecular interactions during rehydration, this treatment should favor aggregation upon rehydration. With infrared spectroscopy we found that at concentrations of the denaturant Na thiocyanate (NaSCN) that only slightly perturbed chymotrypsinogen secondary structure in solution before freeze-drying, there was a large increase in protein unfolding in the dried solid and in protein aggregation measured after rehydration. Bands assigned to intermolecular β sheet were present in the spectra of samples dried with NaSCN, indicating that aggregation could also arise in the dried solid. By examining the protein structure in the frozen state, we determined that in the absence of NaSCN the protein remains native. NaSCN caused structural perturbations during freezing, without the formation of intermolecular β sheet, that were intermediate to structural changes noted after freeze-drying. In contrast, samples treated in the presence of NaSCN and sucrose had native-like spectra in the frozen and dried states, and much reduced aggregation after rehydration. These results indicate that during freezing and drying the sugar can counteract and mostly reverse the structural perturbations induced by NaSCN before and during these treatments.

INTRODUCTION

Because of advances in biotechnology, there are now dozens of protein drugs approved for human use or under investigation for clinical safety and efficacy. The success of a protein as a therapeutic depends on the development of a formulation that maintains native structure and activity during shipping and long-term storage. An aqueous protein solution usually is most convenient and cost effective for the manufacturer and the end user. However, even under optimum conditions, proteins in aqueous liquid formulations are susceptible to degradation by several physical (e.g., agitation-induced aggregation) and chemical (e.g., hydrolysis or deamidation) mechanisms, all of which are mediated by water (cf. Manning et al., 1989, 1996).

Removal of water by freeze-drying (lyophilization) should minimize protein degradation by these pathways (Pikal, 1994; Carpenter and Chang, 1996). However, freezing and drying are severe stresses to proteins. To exploit the potential superior long-term stability afforded in a dried solid, it is essential that the protein survive the lyophilization process itself. Studies of numerous proteins with infrared spectroscopy have shown that unfolding is a general response of unprotected proteins to freeze-drying (Prestrelski et al., 1993a,b, 1994; Dong et al., 1995). Some proteins that are unfolded in the dried solid (e.g., interferon-γ and lactate dehydrogenase) aggregate during rehydration, whereas other proteins (e.g., α-lactalbumin and lysozyme) refold during rehydration and are not aggregated.

Prestrelski et al. (1993a) proposed that the results obtained after rehydration of an unfolded protein are dictated by kinetic competition between intramolecular refolding interactions and intermolecular aggregate-forming interactions. For unprotected proteins that are native after rehydration, aggregation is avoided because refolding outcompetes intermolecular interactions. In contrast, with proteins that normally aggregate after rehydration, minimizing unfolding during freeze-drying with a stabilizer is needed to favor the recovery of native protein molecules after rehydration. For example, sucrose inhibits unfolding during freeze-drying (e.g., of lactate dehydrogenase, phosphofructokinase, and interferon-γ), which results in increased recovery of activity and decreased aggregation after rehydration (Prestrelski et al., 1993a,b; Dong et al., 1995).

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*This paper is dedicated to Prof. Serge N. Timasheff on the occasion of his 70th birthday.

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One purpose of the current study was to test further the model proposed by Prestrelski et al. (1993a) by first examining the opposite situation, in which a denaturant is used to foster additional unfolding in the protein population during freeze-drying. If the protein is not intrinsically resistant to aggregation under the study conditions (e.g., because of intermolecular charge repulsion), this treatment should favor aggregation during rehydration. However, it is not known whether the presence of a denaturant would disrupt intermolecular interactions during rehydration, which would reduce the extent of aggregation. Another goal of the current study was to determine whether unfolding induced by a denaturant during freeze-drying could be counteracted by the presence of a protein stabilizer (e.g., sucrose) in the formulation. Such counteracting effects of cosolvents on protein stability are well established for aqueous solutions (e.g., Lin and Timasheff, 1994; Timasheff, 1995) but have not been examined during freezing and freeze-drying.

To address these issues, we chose α-chymotrypsinogen A as a model protein, because it aggregates after thermal and chemical unfolding, when treated at near neutral pH, indicating that under these conditions it is not intrinsically resistant to aggregation (Eisenberg and Schwert, 1951; Brandts and Lumry, 1963; Ismail et al., 1992). We tested sodium thiocyanate (NaSCN) as a denaturant and sucrose as a potentially counteracting protein stabilizer. Infrared spectroscopy was used to monitor the effects of these cosolvents on protein secondary structure in the initial aqueous solution, in the frozen and dried states, and after rehydration.

MATERIALS AND METHODS

Sample preparation

Bovine α-chymotrypsinogen A (C-4879) and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Before use, the protein was dissolved in, and dialyzed overnight against, 10 mM potassium phosphate buffer (pH 7.4 at 23°C) at 4°C. Insoluble material was removed by centrifugation at 10,000 × *g* for 20 min at 20°C. The protein in the resulting supernatant was brought to a final concentration of 50 mg/ml, with 10 mM potassium phosphate buffer alone or with buffer containing cosolvents, by mixing equal volumes of a 100 mg/ml protein solution with buffer containing 2 times the final cosolvent concentration(s). Protein concentration was determined spectrophotometrically at 282 nm (ϵ = 1.97 cm^2/mg; Lee and Timasheff, 1981). Samples (200 μl) were allowed to equilibrate with cosolvent(s) for 24 h at room temperature, before quantitation of the percentage of total protein forming insoluble protein aggregates in aqueous solution, and before freeze-drying or freezing. Preliminary experiments established that this duration of incubation of the protein in the presence of NaSCN was sufficient to achieve an equilibrium level of protein aggregation in aqueous solution (data not shown).

Quantitation of insoluble protein aggregates was achieved by centrifuging samples at 10,000 × *g* for 20 min (20°C). The protein concentration in the supernatant was determined spectrophotometrically as described above. The percentage of the total protein concentration that formed insoluble aggregates was calculated from the equation [(50 mg/ml − mg/ml protein in supernatant)/50 mg/ml] × 100.

For freeze-drying, samples (200 μl in 1.5-ml polypropylene Eppendorf test tubes) were frozen by immersion in liquid nitrogen and placed overnight on a Labconco 4.5 benchtop lyophilizer, at a chamber pressure of 10 mTorr. Sublimation cooling maintains the frozen samples at a temperature of about −15°C to −20°C during the initial stages of the lyophilization process. Unless otherwise indicated, samples were rehydrated with distilled water. A 200-μl aliquot was pipetted directly onto the dried powder, which was left in the 1.5-ml tube used for freeze-drying. The sample was equilibrated for 24 h at room temperature before further analysis.

Infrared measurement and amide I spectral analysis

Protein solutions were prepared for infrared measurement in a CaF_2 cell (Beckman FH-01) with a 6-μm spacer. To study the effects of freezing, the cell was mounted in a custom-made Peltier device, which allowed cooling to −15°C and precise control of sample temperature (±0.1°C). The temperature of the sample was monitored with a fine wire thermocouple, which was placed directly into a small depression in one of the CaF_2 windows, and a digital thermometer. The large, characteristic shifts in the infrared spectrum for water hydroxyls, which were noted upon formation of ice, were used to document that a sample was frozen and not just supercooled at subzero temperatures. Spectra shown for frozen protein solutions were obtained at a sample temperature of −15°C. Homogeneous protein suspensions containing insoluble aggregates were placed in a Perkin-Elmer variable-path-length cell set to 7 μm. Dried samples (~0.2-0.4 mg protein) were ground with 300 mg KBr and pressed into pellets, as described previously (Prestrelski et al., 1993b). This procedure for preparing KBr pellets does not alter the structure of proteins in the dried solid (Prestrelski et al., 1993b).

Infrared spectra were recorded at 25°C, unless otherwise noted, using a Nicolet Magna 550 spectrometer equipped with a dTGS detector. For each spectrum a 512-scan interferogram was collected in single-beam mode with a 4-cm^{-1} resolution. For aqueous and frozen samples, reference spectra were recorded under identical scan conditions with only the corresponding buffer (with or without cosolvents) in the cell. The spectra of liquid, solid, and gaseous water were subtracted from the observed protein spectra according to previously established criteria (Dong et al., 1990) with a double subtraction procedure (Dong and Caughey, 1994; Dong et al., 1995). Second-derivative spectra were obtained with the derivative func-

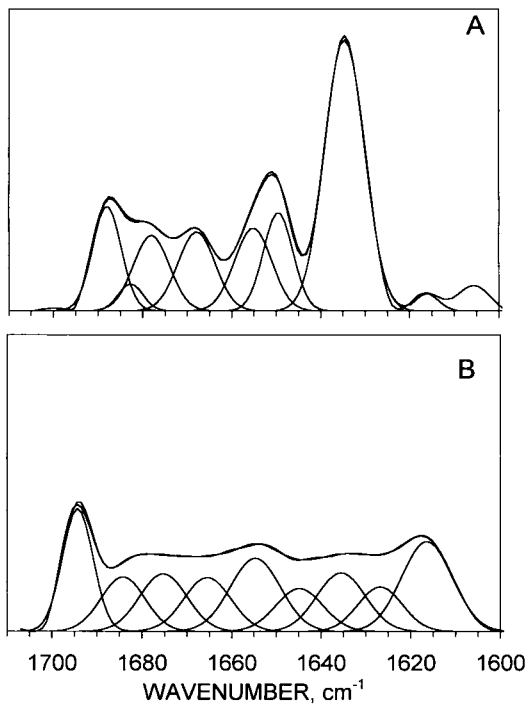

FIGURE 1 Representative examples of curve fitting to inverted second derivative amide I spectra. (*A*) Native, aqueous chymotrypsinogen. (*B*) Chymotrypsinogen freeze-dried in 250 mM NaSCN and rehydrated.

tion of Omnic software (Nicolet). The final protein spectrum was smoothed with a seven-point function to remove white noise. The inverted second-derivative spectra were obtained by factoring by -1 and then curve-fitted with Gaussian band profiles using SpectraCalc from Galactic Industries (Dong et al., 1992; Dong and Caughey, 1994; Dong et al., 1995). Examples of typical curve-fitted spectra are shown in Fig. 1. In cases where bands in the second-derivative spectrum were not clearly resolved, the fourth-derivative spectrum was calculated. With a high-quality original spectrum, the fourth-derivative spectrum can be used to aid in the determination of the number and positions of bands, to which curves are fitted in the second-derivative spectrum. For each sample, component bands determined by visual inspection and curve fitting were assigned to secondary structural elements based on wave number, according to the method of Dong and Caughey (1994).

The overall similarity between the spectrum of an experimental sample and that of native chymotrypsinogen was quantitated by the procedure of Kendrick et al. (1996). Briefly, second-derivative amide I spectra were normalized for area and overlaid. The area of overlap between the spectra was calculated using SpectraCalc. Spectral differences due to band shifting, and changes in relative band intensities and in bandwidths, which are the result of protein unfolding and aggregation, reduce the area of overlap (Kendrick et al., 1996). The value is expressed as the fractional area of the reference amide I region shared by an experimental sample's spectrum (i.e., the area of overlap). The lower the value, the less similar are the two spectra being compared. For all area of overlap values reported here, the reference spectrum was that for native chymotrypsinogen in aqueous solution.

Calorimetry

Thermal analysis of chymotrypsinogen solutions, which were identical to those used in the freeze-drying and freezing experiments, was performed using a Perkin-Elmer DSC-7 differential scanning calorimeter (Norwalk, CT). Protein solutions (50 μl) were sealed in aluminum pans and run with a pan in the reference oven containing buffer without protein. Samples were warmed from 10°C to 90°C at 5°C/min, and thermal data were obtained. The protein melting temperature (T_m) was defined as the point at which the maximum value of the endothermic peak was measured in this temperature range.

RESULTS AND DISCUSSION

Reversibility of lyophilization-induced unfolding during rehydration

The first goal of this study was to determine if using a denaturant to foster additional protein unfolding during lyophilization would favor the formation of nonnative protein aggregates during lyophilization and rehydration. To address this question rigorously, it is advantageous to select a model protein that, in the absence of a denaturant, unfolds during lyophilization but fully refolds during rehydration. Fig. 2 compares the second-derivative infrared spectra, in the conformationally sensitive amide I region (e.g., Susi and Byler, 1986; Dong et al., 1990; Surewicz et al., 1993), for chymotrypsinogen in aqueous solution (before lyophilization), in the dried solid, and after rehydration. Relative to that for the initial aqueous protein, the amide I spectrum of lyophilized chymotrypsinogen shows broadening and shifting of component bands, which are changes commonly noted when proteins are freeze-dried (Prestrelski et al., 1993b, 1994; Dong et al., 1995). The reduction in overall spectral similarity is reflected in an area of overlap between the spectra of 0.774. In contrast, the spectrum for the

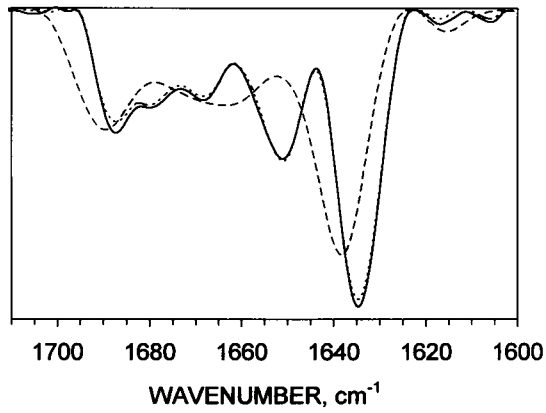

FIGURE 2 Area normalized second derivative amide I spectra of chymotrypsinogen. ——, In 50 mM phosphate buffer solution; – – –, lyophilized solid; ····, after rehydration back to 50 mg/ml with distilled water.

rehydrated sample is essentially identical to that of the native, control protein sample (area of overlap = 0.997). These results indicate that the rehydrated protein completely refolds. This conclusion is supported further by quantitation, with curve fitting, of relative band areas (Table 1) and overall secondary structural composition (Table 2); essentially identical values are obtained for samples before and after freeze-drying and rehydration. These results are also consistent with the observation that insoluble protein aggregates are not detected in the rehydrated sample (Fig. 3).

It has been demonstrated that the spectral changes noted for dried proteins are due to lyophilization-induced perturbation of protein secondary structure and are not simply due to the direct effects of removal of water on carbonyl stretching vibrations, which are the dominant absorbances in the amide I region (Prestrelski et al., 1993b; Gregory, 1995). However, the exact nature of the nonnative protein molecules in the dried solid is unknown. Investigations by other spectroscopic methods (i.e., NMR and Raman) have also shown that dried protein samples contain some fraction of nonnative molecules (reviewed in Prestrelski et al., 1994). Because the infrared spectral signal is an average of the contributions of all protein molecules in the sample, it is not possible to discern whether the alterations induced by freeze-drying are due to partial (or complete) unfolding of most (or all) molecules or to "complete" unfolding of some fraction of the population.

If the latter condition were met, then one might expect to see an increase in the absorbance of the infrared band due to disordered elements in the protein. However, the prominent band (at 1649 cm^{-1}) in the spectrum for aqueous chymotrypsinogen, which is assigned to these elements, is not apparent in the spectrum for the dried protein (Fig. 2) or detectable by quantitation of relative band areas (Table 1). Furthermore, based on quantitation of the overall secondary structural composition, the major structural alteration induced by freeze-drying is a loss of disordered components, which is compensated for by an increase in β sheet structure (Table 2).

TABLE 1 Curve fitting analysis of second derivative amide I spectra

Solution			Dried Solid			Rehydrated		
Band	%	Assignment	Band	%	Assignment	Band	%	Assignment
			Chymotrypsinogen in phosphate buffer					
1617	0.8	Side chain	1615	2.9*	Intermol. β#	1617	0.8	Side chain
1634	39.9	β sheet	1637	30.0	β sheet	1634	41.8	β sheet
1649	11.3	Unordered	1643	12.9	β sheet	1649	11.6	Unordered
1655	11.1	α-helix	1657	10.1	α-helix	1655	11.5	α-helix
1668	11.7	Turn	1666	12.6	Turn	1667	10.5	Turn
1678	8.6	Turn	1676	10.0	Turn	1678	10.1	Turn
1683	7.2	Turn	1685	3.8	Turn	1684	6.5	Turn
1688	9.3	β sheet	1691	17.6	β sheet	1688	7.1	β sheet
			Chymotrypsinogen in phosphate buffer + 100 mM NaSCN					
1617	0.2*	Intermol. β	1615	2.9*	Intermol. β	1619	11.3*	Intermol. β
1633	31.0	β sheet	1638	34.4	β sheet	1634	27.5	β sheet
1650	5.6	Unordered	1643	7.3	β sheet	1650	4.7	Unordered
1656	11.2	α-helix	1658	7.8	α-helix	1655	10.0	α-helix
1667	13.4	Turn	1666	12.5	Turn	1669	19.4	Turn
1676	14.0	Turn	1676	9.3	Turn	1678	4.3	Turn
1683	11.4	Turn	1687	4.4	β sheet	1684	9.5	Turn
1689	13.3	β sheet	1694	21.3	β sheet	1691	12.4	β sheet
						1696	1.0	Intermol. β
			Chymotrypsinogen in phosphate buffer + 250 mM NaSCN					
1616	1.3*	Intermol. β	1617	10.2*	Intermol. β	1617	18.2*	Intermol. β
1634	37.0	β sheet	1627	7.6	β sheet	1628	7.1	β sheet
1650	5.5	Unordered	1639	13.2	β sheet	1636	10.2	β sheet
1655	11.2	α-helix	1647	5.0	β sheet	1646	8.5	β sheet
1668	14.3	Turn	1657	12.3	α-helix	1654	10.9	α-helix
1678	11.7	Turn	1667	12.3	Turn	1665	13.0	Turn
1685	10.0	Turn	1677	8.7	Turn	1677	11.4	Turn
1689	9.1	β sheet	1687	8.3	β sheet	1686	8.1	Turn
			1697	22.4	Intermol. β	1695	12.6	Intermol. β

*Values indicated were corrected for the overlapping amino acid side-chain absorbance (0.8%) in this area.
#Intermolecular β sheet.

Actually, it appears that extended, random portions of the peptide backbone are not favored in the dried solid, because of the propensity of such regions to form intermolecular interactions in the absence of water. For example, with casein, which is essentially completely without secondary structure in aqueous solution, the amide I spectrum for the dried protein is dominated by bands arising from intermolecular β sheet, a major structural component in nonnative protein aggregates (Prestrelski et al., 1993b; Dong et al., 1995). Similarly, as will be described below, fostering ad ditional unfolding of chymotrypsinogen with NaSCN leads to the formation of intermolecular β sheet during freeze-drying, but not to an increase in disordered components in the dried protein.

Effect of thiocyanate on chymotrypsinogen aggregation

To test the effect of further perturbing protein structure during freeze-drying on the aggregation/refolding process during rehydration, we added the denaturing salt NaSCN to chymotrypsinogen solutions before freeze-drying. NaSCN was chosen for this purpose because this denaturant, unlike

TABLE 2 Secondary structural composition of chymotrypsinogen

Structure	Solution	Dried Solid	Rehydrated
	Chymotrypsinogen in phosphate buffer		
β sheet	49.6	60.5	48.9
α-helix	11.2	10.1	11.5
Unordered	11.4	0	11.6
Turn	27.5	26.4	27.2
Intermolecular β sheet	—	2.9	—
	Chymotrypsinogen in phosphate buffer + 100 mM NaSCN		
β sheet	44.4	67.4	39.9
α-helix	11.2	7.8	10.0
Unordered	5.7	0	4.7
Turn	38.7	21.8	33.2
Intermolecular β sheet	0.2	2.9	12.3
	Chymotrypsinogen in phosphate buffer + 250 mM NaSCN		
β sheet	46.1	34.1	25.8
α-helix	11.2	12.3	10.9
Unordered	5.5	0	0
Turn	36.0	21.0	32.5
Intermolecular β sheet	1.3	32.6	30.8

Values represent the sum of component band areas assigned to a specific secondary structural element.

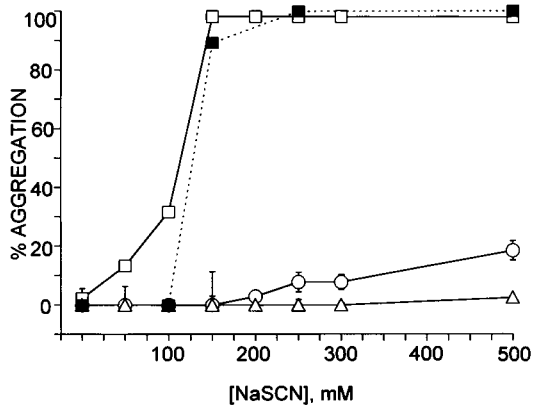

FIGURE 3 Effect of increasing NaSCN concentration on chymotrypsinogen aggregation. ○, Before lyophilization; □, after lyophilization in the presence of NaSCN and rehydration in distilled H_2O; △, after lyophilization in 50 mM phosphate buffer only and rehydration with distilled water containing NaSCN; ■, estimated aggregation in dried solid samples before rehydration based on percentage of amide I area occupied by intermolecular β sheet (see text for explanation of method).

urea or guanidinium salts, does not absorb in the infrared amide I region. At NaSCN concentrations up to 150 mM, protein aggregation is not measurable in solutions before lyophilization (Fig. 3). At higher NaSCN concentrations, protein aggregation is detected, with a maximum of 18% aggregation in the presence 500 mM NaSCN. Freeze-drying and rehydration greatly increase the effect of NaSCN on protein aggregation. Chymotrypsinogen samples lyophilized in ≥150 mM NaSCN and then rehydrated with distilled water are 100% aggregated (Fig. 3). The intermolecular interactions in aggregates formed by this treatment are noncovalent in nature, because the aggregated protein dissolves readily in 10% (w/v) sodium dodecyl sulfate or 8 M urea (data not shown).

To determine whether NaSCN is exerting its effects during rehydration, aggregation was measured in protein samples that were lyophilized from phosphate buffer alone and then rehydrated, as described in Materials and Methods, with distilled water containing NaSCN. This treatment leads to less aggregation than that noted when protein samples are incubated with identical concentrations of NaSCN before lyophilization (Fig. 3). This result may be due to the effects of sample history on the aggregation process. For samples exposed to NaSCN before lyophilization, chymotrypsinogen was already dissolved in phosphate buffer at 100 mg/ml and added to a solution containing 2 times the final NaSCN concentration. In contrast, NaSCN at its final concentration in distilled water was used to rehydrate chymotrypsinogen dried from phosphate buffer. The former treatment may transiently expose the protein to a higher NaSCN concentration, which could favor aggregation. More importantly, however, the results of this experiment document that aggregation of chymotrypsinogen lyophilized in the presence of NaSCN arises because of the effects of the denaturant during freeze-drying and not during rehydration.

Effect of thiocyanate on protein structure

Based on the above results, it seems most likely that NaSCN's effects are manifested as a perturbation of protein structure during freeze-drying. To test this suggestion, we used infrared spectroscopy to compare the effects of NaSCN on chymotrypsinogen secondary structure in solution, in the dried solid, and after rehydration. Representative spectra are presented in Fig. 4. For quantitation of changes, via analysis of component band areas and secondary structural composition (Tables 1 and 2), we chose to focus on sample conditions that are representative of three different protein behaviors during lyophilization and rehydration (Fig. 3). For these three systems the protein is prepared 1) without NaSCN and does not aggregate after rehydration; 2) with 100 mM NaSCN and exhibits an intermediate level of aggregation (32%) after rehydration; and 3) with 250 mM NaSCN and is 100% aggregated after rehydration.

Examination of amide I spectra indicates that before lyophilization NaSCN causes relatively small changes in secondary structure (Fig. 4 *A*, Tables 1 and 2). The primary changes are due to decreases in the absorbances, and shifts

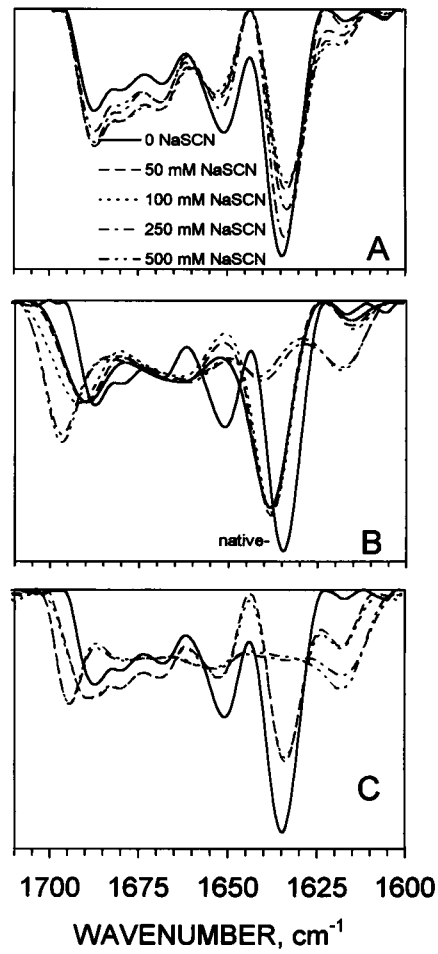

FIGURE 4 Area normalized second derivative amide I spectra of chymotrypsinogen showing effects of NaSCN (*A*) before lyophilization (*B*) in the dried solid, and (*C*) after rehydration.

in positions, of the most prominent bands at 1634 cm^{-1} and 1649 cm^{-1}, which are assigned to β sheet and unordered elements, respectively. The loss in these areas is compensated for mainly by increasing absorbances for bands in the region from about 1665 to 1690 cm^{-1}, which are due to β turn and β sheet.

These NaSCN-induced alterations in the protein's infrared spectrum are minor compared to those noted in the dried solid (Fig. 4 *B*, Tables 1 and 2). The samples prepared in the presence of 50 and 100 mM NaSCN have spectra that are slightly more altered than that for the protein lyophilized in the absence of NaSCN. The values for area of overlap with the spectrum for native aqueous protein for samples with 0, 50, and 100 mM NaSCN are 0.774, 0.733, and 0.712, respectively. The greater structural alteration induced in the presence of 50 and 100 mM NaSCN is sufficient to foster moderate levels of protein aggregation upon rehydration (Fig. 3). Much more drastic perturbation of protein structure is noted with samples lyophilized in the presence of 250 and 500 mM thiocyanate (Fig. 4 *B*). The values for area of overlap with the spectrum for native aqueous protein for these samples are, respectively, 0.628 and 0.624. This degree of protein structural alteration is sufficient to foster 100% aggregation after lyophilization and rehydration (Fig. 3).

Based on the infrared spectra of all of the dried samples and the aggregation levels measured after rehydration (Figs. 3 and 4), it appears that NaSCN-induced unfolding of chymotrypsinogen during lyophilization does favor protein aggregation during rehydration. An increase in the degree of protein structural perturbation noted in the dried solid (i.e., a decrease in spectral similarity to native, aqueous protein) correlates directly with an increase in aggregation noted after rehydration (Fig. 5). And once a threshold degree of perturbation is reached at 150 mM NaSCN, 100% aggregation is obtained at this and higher levels of NaSCN (Fig. 5).

However, these effects of NaSCN are not seen with all proteins. In similar studies with α-lactalbumin, we found that NaSCN fosters large increases in protein structural perturbation during freeze-drying. However, upon rehydration the protein remains soluble and unaggregated, but nonnative based on infrared spectroscopy (unpublished observations). In this case, it appears that the denaturant is disrupting the intermolecular contacts needed for aggregation and inhibiting refolding. The cause of the differences in the behavior of the two proteins is not known.

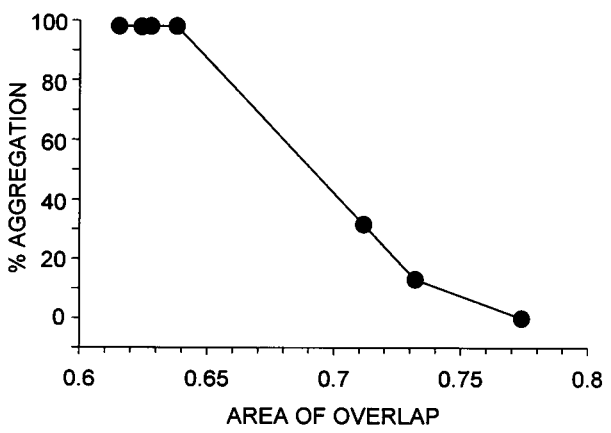

FIGURE 5 Relationship between percentage of chymotrypsinogen aggregated after rehydration and similarity of infrared spectrum of chymotrypsinogen in the dried solid versus that for native, aqueous protein.

By examining the spectra of chymotrypsinogen dried in the presence of 250 and 500 mM NaSCN, it is clear that sufficient alteration in protein structure has occurred to promote protein aggregation before rehydration (Fig. 4 *B*). The decreases in absorbances of the 1634-cm^{-1} β sheet band and the 1649-cm^{-1} band for disordered elements are compensated for almost completely by the appearance of prominent new bands at 1617 and 1697 cm^{-1} (Fig. 4, Table 1). These bands are characteristic of intermolecular β sheet (Clark et al., 1981; Ismail et al., 1992; Dong et al., 1995), which is due to nonnative protein aggregates in the dried solid. Thus, although high concentrations of NaSCN promote large-scale loss of native structure, any random structure that is induced during the denaturation must be immediately lost in the sample because of formation of intermolecular contacts between these regions of the dried protein molecules.

The great increase in NaSCN's protein denaturing capacity during freeze-drying, relative to that noted in aqueous solution (Fig. 4 *A* versus 4 *B*), can be ascribed to several factors. First, when ice is formed during freezing, the protein and NaSCN will partition into the non-ice phase and their concentrations will increase manyfold. This change in concert with the other stresses arising during freezing (e.g., subzero temperatures and formation of a large ice-water interface) should increase damage to the protein induced by a given initial NaSCN concentration. Experiments investigating the relative contribution of freezing-induced denaturation to the overall structural perturbations noted after freeze-drying will be discussed later. After freezing, the protein would then be subjected to the added insult of removal of its hydration shell during subsequent drying. Even if the protein survived freezing in its native state, the stress of drying alone would be sufficient to foster unfolding (cf. Prestrelski et al., 1993a; Carpenter and Chang, 1996). Finally, during drying there also would be extreme increases in NaSCN and protein concentrations, which should foster protein denaturation and aggregation.

Upon rehydration, the spectra for samples in 50 and 100 mM NaSCN partially regain some features noted before freeze-drying, such as the positions of the β-sheet band at 1634 cm^{-1}, the band for disordered elements at 1650 cm^{-1}, and β-turn bands at 1665–1685 cm^{-1} (Fig. 4). The area overlaps for these spectra with the spectrum for native chymotrypsinogen are 0.807 and 0.804, respectively. These results indicate that some portion of the protein molecular population has refolded. However, there are new spectral alterations noted after rehydration, which are due to moderate increases in the absorbance of bands associated with

intermolecular β sheet at 1619 and 1696 cm^{-1} (Fig. 4 and Tables 1 and 2). The magnitude of these bands is consistent with the intermediate levels of aggregation measured in these samples (Fig. 3).

There is neither intermolecular β sheet nor random structure when the samples with 50 and 100 mM NaSCN are in the dried solid (Fig. 4 *B*, Tables 1 and 2). Hence it appears that for these samples aggregation does not arise before rehydration, and that during this time nonnative structures other than random coil can participate in the formation of protein aggregates. In contrast, upon rehydration the spectra for the samples dried in the presence of 250 and 500 mM NaSCN change only slightly (Fig. 4 *C*). Therefore, for these two samples, the degree of protein structural change and aggregation is essentially already at a maximum in the dried solid.

To compare quantitatively the relative degrees of aggregation noted in the dried solid to those measured after rehydration, the relationship between percentage aggregated protein and intermolecular β sheet content was plotted, using data for various rehydrated samples (Fig. 6). With the equation for the fitted line, the percentage aggregation in the dried solid can be estimated from the intermolecular β sheet content, which is determined from the curve-fitted second-derivative spectra. Based on this analysis, at $\leq$100 mM NaSCN, aggregated protein is not detectable in the dried solid (Fig. 3). However, increasing the NaSCN concentration to 150 mM leads to almost 90% aggregation in the dried solid. And at 250 and 500 mM NaSCN, the protein is 100% aggregated in the dried solid.

Use of sucrose to counteract structural perturbations by thiocyanate

If sucrose can be used to counteract perturbation of chymotrypsinogen's structure by NaSCN during lyophilization, then upon rehydration aggregation should be reduced. Sucrose is known to stabilize proteins during both freeze-thawing and freeze-drying (Shikama and Yamazaki, 1961; Chilson et al., 1965; Brandts et al., 1970; Carpenter and Crowe, 1988; Prestrelski et al., 1993b; Dong et al., 1995; Carpenter and Chang, 1996). Numerous proteins, which unfold during freeze-drying, have been found to retain their native structure in the dried solid when freeze-dried with sucrose (Prestrelski et al., 1993b; Dong et al., 1995). In protein stabilization studies, usually a disaccharide concentration of about 300 mM has been found to be adequate to protect proteins during freeze-drying (e.g., Carpenter and Crowe, 1989; Prestrelski et al., 1993a,b; Carpenter and Chang, 1996).

Fig. 7 *A* shows the effect of 300 mM sucrose on chymotrypsinogen's infrared spectra in the initial aqueous solution for protein samples prepared in the presence of 0, 100, and 250 mM NaSCN. Sucrose has essentially no effect on the structure of native chymotrypsinogen (Fig. 7 *A*). Furthermore, the spectra of the protein in the presence of sucrose

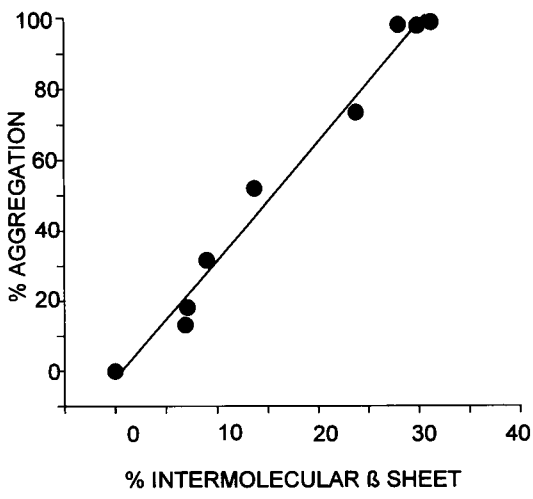

FIGURE 6 Correlation between percentage of chymotrypsinogen aggregated and intermolecular β sheet content. Intermolecular β sheet content is determined as percentage of total second derivative amide I area occupied by bands at 1617–1619 and 1695–1696 cm^{-1} in spectra for rehydrated protein samples. Percentage aggregation is measured after rehydration as described in Materials and Methods.

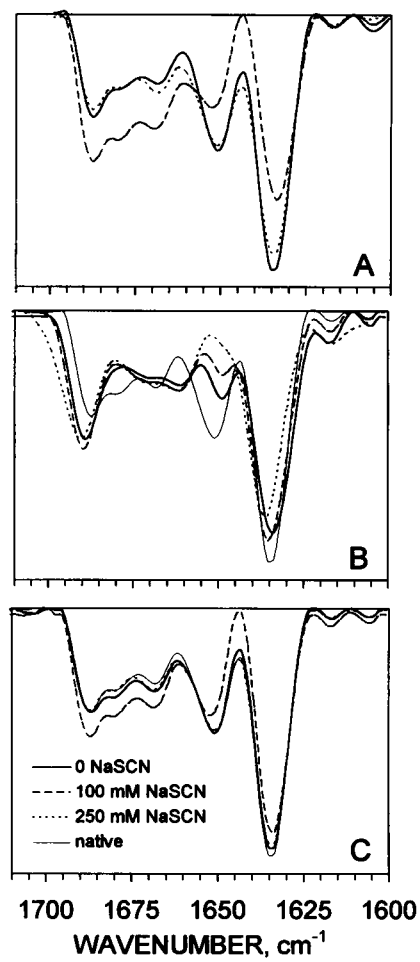

FIGURE 7 Area normalized second derivative amide I spectra of chymotrypsinogen-NaSCN mixtures containing 300 mM sucrose. (*A*) Before lyophilization; (*B*) in the dried solid; (*C*) after rehydration with distilled water.

and NaSCN are only slightly more native-like than those noted with NaSCN alone (Figs. 4 *A* and 7 *A*), indicating that structural perturbations induced by NaSCN are only minimally affected by 300 mM sucrose (Figs. 4 *A* and 7 *A*).

In contrast, after freeze-drying, samples prepared with sucrose were much more native-like than those freeze-dried without the sugar (Figs. 4 *B* and 7 *B*). The areas of overlap relative to native chymotrypsinogen are 0.896, 0.860, and 0.750, respectively, for samples dried in the presence of 300 mM sucrose, and 0, 100, and 250 mM NaSCN. Most interestingly, the band for disordered elements in the protein is retained in the spectra for samples freeze-dried with a combination of sucrose and 0 and 100 mM NaSCN (Fig. 7 *B*). As described earlier, this band, which is present in the spectrum of the native protein, is not apparent in the spectrum of the protein when it is lyophilized without sucrose (Fig. 4 *B*). The lyophilization-induced structural perturbation results in a loss of disordered elements, in favor of the formation of β sheet (Table 2). Thus during lyophilization of chymotrypsinogen, the stabilizing effect of sucrose is manifested, in part, in the retention of disordered elements in the protein, which reflects the general protection of the protein's native structure.

With 250 NaSCN the band for disordered elements is almost absent, indicating that a slightly greater degree of structural perturbation arose than that seen in the other two samples. However, the prominent bands associated with intermolecular β sheet are not seen in this spectrum. Taken together, the results indicate that not only does sucrose inhibit lyophilization-induced unfolding of chymotrypsinogen prepared without NaSCN, but that during freeze-drying the sugar can also counteract and mostly reverse the structural perturbations induced by NaSCN before and during freeze-drying.

This effect of sucrose might be due to its increasing concentration during freezing and drying. NaSCN might not have the same magnitude of increase in concentration in the amorphous phase with the protein, because the denaturant may crystallize during freeze-drying, as do many other salts (Chang and Randall, 1992). Sucrose, on the other hand, is known to remain amorphous when frozen and dried (Chang and Randall, 1992; Carpenter and Chang, 1996). Alternatively, it may simply be that the increase in protein stabilization as a function of sucrose concentration is greater than the concentration dependency for structural perturbation by NaSCN.

When the samples freeze-dried with sucrose are rehydrated, aggregation is greatly reduced relative to that noted in NaSCN alone. Aggregation in samples containing sucrose and 0, 100, or 250 mM thiocyanate are 0, 2.2, and 7.6%, respectively. In concert with these results, after rehydration the infrared spectra of all the samples freeze-dried with sucrose are similar to that for the native protein (Fig. 7 *C*). The areas of overlap of these spectra with that for native chymotrypsinogen are 0.888 (100 mM NaSCN + 300 mM sucrose) and 0.968 (250 mM NaSCN + 300 mM sucrose). Thus, when NaSCN-induced unfolding is inhibited by sucrose during freeze-drying, intermolecular associations are minimized during lyophilization and rehydration, making the reestablishment of native conformation more favorable.

Effect of freezing with and without cosolvents on chymotrypsinogen structure

Both the freezing and drying stresses of the lyophilization process can damage proteins (Carpenter et al., 1993; Prestrelski et al., 1993b; Carpenter and Chang, 1996). If freezing induces unfolding, then the protein will not be native in the dried solid, no matter how effective the protection against dehydration damage. Conversely, if a protein survives freezing, but is not adequately protected during dehydration, the native structure will not be retained in the dried solid. Therefore, it was important to determine the degree to which protein damage and the effects of cosolvents that are noted after freeze-drying are manifested during freezing.

To this end, infrared spectra were acquired for the frozen samples. The second-derivative infrared spectrum of chymotrypsinogen without cosolvents was not altered by freezing (area of overlap versus spectrum for native control = 0.966), indicating that at a 50 mg/ml concentration this stress does not denature the protein (Fig. 8 *A*). Therefore, in the absence of cosolvents, the structural perturbations noted after freezing and drying (Fig. 4 *B*) can be ascribed solely to damage induced during the dehydration step. The lack of unfolding in a frozen 50 mg/ml chymotrypsinogen solution

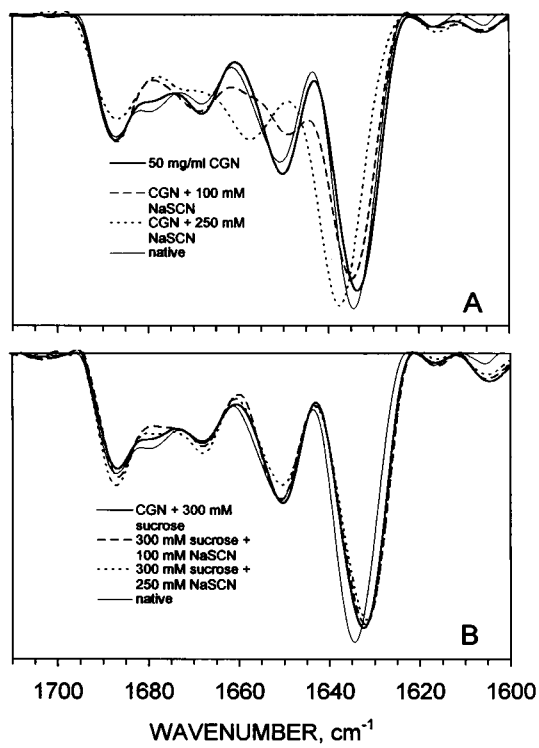

FIGURE 8 Area normalized, second derivative amide I spectra of chymotrypsinogen (CGN) in frozen solutions containing increasing amounts of NaSCN. (*A*) Samples prepared without sucrose; (*B*) samples prepared with 300 mM sucrose.

does not necessarily mean that the protein is inherently resistant to freezing. Rather, it is most likely due to the stabilizing effects of having the protein at a high initial concentration, which is needed to study the structure of proteins in H_2O with infrared spectroscopy. At much lower initial concentrations than studied here, chymotrypsinogen has been shown to undergo denaturation during freeze-thawing (Brandts et al., 1970). With many other labile enzymes the recovery of native active protein after freeze-thawing correlates directly with the initial protein concentration (Carpenter and Crowe, 1988; Carpenter and Chang, 1996). The mechanism for this "self-stabilization" during freezing is not known. It might be ascribed to the phenomenon termed by Minton (1981) as macromolecular crowding, which is due to excluded volume effects. Protein unfolding in highly concentrated solutions is inhibited by the steric repulsion between nearest-neighbor protein molecules. Alternatively, it can be argued that freeze-induced denaturation is associated with surface stress, due to the formation of an ice-water interface (cf., Strambini and Gabellieri, 1996). Assuming that a finite amount of protein could be unfolded at this interface, the relative amount of denatured protein would decrease as the initial protein concentration increased.

In contrast to the results for freezing chymotrypsinogen without cosolvents, when the protein is frozen in the presence of 100 and 250 mM NaSCN, its infrared spectra are altered (Fig. 8 *A*). Interestingly, during supercooling to −10°C, but before freezing, the spectra are not different from those noted at room temperature (data not shown). Thus, at least down to −10°C, low temperature alone is not sufficient to perturb the protein's secondary structure in the presence of 100 and 250 mM NaSCN. But immediately upon the formation of ice, the changes shown in Fig. 8 *A* are apparent, indicating that the freezing process is important for the structural changes noted. Areas of overlap for the spectra of frozen samples relative to that of native chymotrypsinogen are 0.914 and 0.815 for samples frozen in 100 and 250 mM NaSCN, respectively. Freezing in the presence of denaturant shifts the β sheet band from 1634 to 1636 cm^{-1} with 100 mM NaSCN and further to 1638 cm^{-1} with 250 mM NaSCN. The band for disordered elements, at approximately 1650 cm^{-1} in the native protein's spectrum, shifts to 1648 cm^{-1} with 100 mM NaSCN and is not apparent with 250 mM NaSCN. The appearance of a band at 1658 cm^{-1} with 250 mM NaSCN indicates an apparent structural rearrangement to form additional α-helix. Overall the magnitude of the spectral changes induced by freezing is less than that noted after freeze-drying, and intermolecular β sheet bands are not apparent in the spectra of the frozen samples. Thus, in the presence of 100 and 250 mM NaSCN, freezing-induced perturbations of chymotrypsinogen secondary structure contribute partially to the total denaturation noted after freeze-drying. And protein aggregation does not appear to occur during the freezing step.

When chymotrypsinogen is frozen with 300 mM sucrose, the spectral changes induced by NaSCN before and during freezing are almost completely reversed in the frozen state (Fig. 8 *B*). The spectrum for the frozen sample containing 100 mM NaSCN is almost identical to that noted for native chymotrypsinogen, and that for 250 mM NaSCN is only slightly altered. The areas of overlap versus spectrum for native chymotrypsinogen are 0.927 and 0.906, respectively, for samples with 100 and 250 mM NaSCN. This result is expected, because it has already been shown that in the presence of sucrose the protein freeze-dried with NaSCN has very native-like spectra (Fig. 7 *B*). Interestingly, in the spectra of all three samples frozen in the presence of 300 mM sucrose, the β sheet band, which normally is seen at 1634 cm^{-1} in the spectrum for native chymotrypsinogen in aqueous solution, is slightly shifted, to about 1631 cm^{-1} (Fig. 8 *B*). This shift is not seen before freezing (Fig. 7 *A*). Such a change most likely indicates that freezing in the presence of sucrose induces a minor alteration in the packing density of the β sheet elements giving rise to this band. More importantly, in the context of counteracting cosolvent effects, because this shift is noted in the absence or presence of NaSCN, it appears that this property of sucrose can also predominate over changes induced by NaSCN during freezing. As noted above for freeze-drying, all of these effects of sucrose could be due to a greater increase in its influence, due to increased concentration, relative to that of NaSCN during freezing.

Finally, it is important to consider that the freeze-dried protein samples were subjected to extremely low temperature (ca. −196°C) in liquid nitrogen (see Materials and Methods), whereas in our infrared spectroscopic studies of freezing-induced changes, the protein was exposed to only −15°C. Because lower temperature might foster further structural alterations (cf. Brandts et al., 1970) in the protein, especially in the presence of NaSCN, the degree of perturbation noted at −15°C might not reflect the actual changes arising during the freezing portion of our lyophilization cycle. It currently is not technically feasible for us to acquire protein infrared spectra at liquid nitrogen temperatures. Instead, to address these issues, chymotrypsinogen samples—containing no cosolvent, 100 mM NaSCN, 250 mM NaSCN, 300 mM sucrose, 300 mM sucrose and 100 NaSCN, and 300 mM sucrose and 250 mM NaSCN—were first frozen in a −15°C bath and then placed on the lyophilizer, in which sample temperature does not drop below about −20°C. The infrared spectra of the protein in the dried solids (data not shown) were almost identical to those shown for the respective samples in Figs. 4 *B* and 7 *B*. Thus, in the presence of NaSCN, freeze-drying leads to essentially equivalent perturbation of chymotrypsinogen structure with both freezing protocols. For the samples frozen at −15°C, the degree of protein damage noted in the frozen state (Fig. 8 *A*) accurately reflects protein unfolding that is intermediate to the degree noted in the dried solid. Moreover, it appears that the dehydration step, and not the final temperature achieved in the frozen state, is the limiting factor determining the effects of the denaturant on protein structure in our system. The same conclusion can be made for the protein prepared without cosolvent. Finally, the protective

action of sucrose during freeze-drying is manifested equally, whether the protein is exposed in the frozen state to liquid nitrogen temperature or to a temperature no lower than about −20°C.

Mechanisms for cosolvent effects during freezing and drying

As a solution is frozen, the protein will partition into the non-ice phase and should maintain interactions with any other components that also remain amorphous. Thus, as we have argued in the past, the interactions between the protein and any cosolvent molecules that do not crystallize, and the consequences of such interactions for protein stability, should be qualitatively similar to those arising in nonfrozen, aqueous solution (Carpenter and Crowe, 1988). For understanding what can appear to be complex, and sometimes counteracting, effects of cosolvents on protein stability in solution, we are indeed fortunate for Timasheff's decades of published research in this area. He has defined a single, universal thermodynamic mechanism that accounts for the effects of nonspecific cosolvents (i.e., those such as NaSCN and sucrose, which operate at high concentrations and have the same relative effect on any protein) on protein stability (reviewed in Timasheff, 1992, 1995). Briefly, Timasheff and colleagues have observed experimentally that all denaturing cosolvents examined to date (e.g., urea and guanidinium) are preferentially bound to the protein, which results in a reduction of protein chemical potential. These effects are much greater for the denatured state than for the native state, because unfolding leads to a much greater surface area to which denaturant can be bound preferentially. The net result is that the free energy of denaturation is lowered and the native state is rendered less stable.

Although preferential interaction measurements for NaSCN with proteins have not been made, it seems most likely Timasheff's mechanism is operative with this denaturant. We suggest that in the face of freezing and drying stresses and increased concentrations of NaSCN, because of preferential binding of NaSCN to the protein, the denatured state becomes more thermodynamically favorable than it was before freezing. The result is an increase in the apparent unfolding of protein samples in the frozen and dried states (Figs. 4 *B* and 8 *A*).

The opposite situation is seen with sucrose and numerous other stabilizers examined by Timasheff and colleagues (Arakawa and Timasheff, 1982a; Timasheff, 1992, 1995). Lee and Timasheff (1981) have shown that sucrose is excluded preferentially from the surface of chymotrypsinogen, which results in an increase in protein chemical potential. Because the surface area for preferential exclusion is greater in the denatured than the native state, the magnitude of the increase in chemical potential is greatest for the denatured state. Consequently, the thermodynamic barrier between the two states is increased and the native state is stabilized. This effect has recently been attributed to the increased unfavorability of exposing the peptide backbone to solvent in the presence of sucrose (Liu and Bolen, 1995).

Counteracting effects of cosolvents on protein stability also can be explained straightforwardly with the preferential interaction mechanism (Arakawa and Timasheff, 1982b; Lin and Timasheff, 1994). For example, with urea and trimethylamine *N*-oxide (TMAO), preferential binding of urea and preferential exclusion of TMAO are maintained at the same levels seen when only the individual cosolvents are present (Lin and Timasheff, 1994). Thus, the decrease in the free energy of denaturation due to urea is counteracted by an increase in this parameter due to TMAO. We propose that the same mechanism can account for the counteracting effects of sucrose and NaSCN on chymotrypsinogen stability during freezing.

To gain further insight into the effects of these cosolvents on chymotrypsinogen stability, we determined their influences on the protein's melting temperature (T_m). We took this approach because Lin and Timasheff (1994) have documented that the change in protein T_m due to the presence of a cosolvent can be used as an estimate of the relative effect of the cosolvent on the free energy of denaturation. Furthermore, they found that a change in T_m, and hence in the free energy of denaturation, due to the presence of cosolvent combinations is the algebraic sum of the separate effects of the different cosolvents. As expected, we found that NaSCN decreases the T_m for chymotrypsinogen, and the effect is greater at 250 than 100 mM denaturant concentration (Table 3). In contrast, 300 mM sucrose increases the T_m slightly. When 300 mM sucrose is combined with 100 mM NaSCN, the T_m of chymotrypsinogen is almost exactly the algebraic mean of the T_m's noted in the presence of individual cosolvents, and sucrose partially counteracts the effects of the denaturant. Sucrose reverses to a lesser degree the decrease in chymotrypsinogen's T_m induced by 250 mM NaSCN. These findings correlate well with the observation that sucrose has small effects on the perturbation of chymotrypsinogen secondary structure in aqueous solution before freezing (Fig. 7 *A*). In contrast, the greater relative stabilizing effects of sucrose seen during freezing and drying, as noted above, are most likely due to the increase in sucrose concentration during these treatments, which would not occur during heating.

Finally, several studies, including the current one, have documented that creating conditions (e.g., with high protein concentration and/or stabilizing cosolvents) that inhibit protein unfolding during freezing do not ensure retention of the

TABLE 3 Changes in chymotrypsinogen melting temperature in the presence of sucrose and/or Na thiocyanate

Sample	ΔT_m (°C)
CGN	—
CGN + 100 mM NaSCN	−3.9
CGN + 250 mM NaSCN	−6.6
CGN + 300 mM sucrose	+1.7
CGN + 300 mM sucrose + 100 mM NaSCN	−2.3
CGN + 300 mM sucrose + 250 mM NaSCN	−5.7

native protein after freeze-drying (Carpenter et al., 1993; Prestrelski et al., 1993a; Carpenter and Chang, 1996). It seems reasonable, however, to suggest that as water is initially being removed under vacuum during the drying step of lyophilization, the same relative effects of amorphous cosolvents will be maintained and Timasheff's thermodynamic mechanism can be used to explain cosolvent-induced alterations in protein stability. But near the end point of drying, the protein's hydration shell will be removed. At this point, the requisite thermodynamic arguments of the Timasheff mechanism are no longer applicable, because water is essential for cosolvent-protein interactions to be defined as preferential. The direct interaction of sucrose and other disaccharides with proteins in the dried solid can account for protein stabilization during drying. It has been found that stabilizing sugars can hydrogen bond to the dried protein in the place of water molecules that are lost as the protein's hydration shell is removed (Carpenter and Crowe, 1989; Prestrelski et al., 1993b; Allison et al., 1995). Such "water replacement" correlates directly with the inhibition of protein unfolding during dehydration. Under conditions where this interaction does not arise in protein-sugar mixtures during the terminal stages of dehydration (e.g., because of protein-sugar phase separation during freeze-drying), the protein is unfolded in the dried solid (Carpenter and Crowe, 1989; Allison et al., 1995).

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