

## Physical Factors Affecting the Storage Stability of Freeze-Dried Interleukin-1 Receptor Antagonist: Glass Transition and Protein Conformation

Byeong S. Chang,<sup>\*1</sup> Robert M. Beauvais,<sup>†</sup> Aichun Dong,<sup>†2</sup> and John F. Carpenter<sup>†</sup>

<sup>\*</sup>*Department of Pharmaceutics and Drug Delivery, Amgen, Inc., Amgen Center MS 8-1-A, Thousand Oaks, California 91320; and* <sup>†</sup>*Department of Pharmaceutical Sciences, School of Pharmacy, University of Colorado Health Center, Denver, Colorado 80262*

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The effects of glass transition of, and protein conformation in, the dried solid on the storage stability of freeze-dried recombinant human interleukin-1 receptor antagonist (rhIL-1ra) were examined. Glass transition is a temperature-dependent phenomenon. Amorphous materials become hard and brittle at temperatures below their characteristic glass transition temperatures (T<sub>g</sub>) such that diffusion of molecules along the matrix is not sufficient to cause large-scale structural changes. To ascertain the importance of the glass transition in protein storage stability, we compared 10 different lyophilized rhIL-1ra formulations, with T<sub>g</sub>s ranging from 20 to 56°C, during several weeks of storage at temperatures above and below the samples' T<sub>g</sub>s. Protein degradation, both deamidation and aggregation, was greatly accelerated at temperatures above T<sub>g</sub>, but for some formulations also arose below T<sub>g</sub>. Thus, storage of dried proteins below the T<sub>g</sub> is necessary but not sufficient to ensure long-term stability. To examine the effects of protein structure in the dried solid, we prepared formulations with various sucrose concentrations, all of which had a T<sub>g</sub> = 66 ± 2.5°C. With infrared spectroscopy, we determined that the protein lyophilized with ≤1% sucrose was unfolded in the initial dried solid. In contrast, in those formulations with ≥5% sucrose, conformational change was inhibited during lyophilization. When stored at 50°C, degradation of the freeze-dried protein varied inversely with sucrose concentration. These results indicate that structural changes arising during the lyophilization process led to damage during subsequent storage, even if the storage temperature was less than the T<sub>g</sub>.

<sup>1</sup>To whom correspondence should be addressed. Fax: (805) 498-8674.

<sup>2</sup>Current address: Department of Chemistry and Biochemistry, University of Northern Colorado, Greeley, CO 80639.

Together the results of these studies document that to obtain optimum stability of dried rhIL-1ra it was necessary to inhibit conformational change during lyophilization and to store at temperatures below the T<sub>g</sub> of the dried formulation. © 1996 Academic Press, Inc.

There are numerous unique, critical applications for proteins in human health care. However, even the most promising protein therapeutic will not be useful, if its stability cannot be maintained during shipping and long-term storage (1, 2). The inherent instability of proteins often precludes preparation of formulations as aqueous solutions (2). However, if the water is removed by freeze-drying (lyophilization), the dehydrated protein theoretically should be much more resistant to damage, even at ambient temperatures (3, 4). We have recently achieved such long-term stability (5) with freeze-dried recombinant human interleukin-1 receptor antagonist (rhIL-1ra).<sup>3</sup> An optimum formulation, containing 100 mg/ml rhIL-1ra, 2% (wt/vol) glycine, 10 mM sodium citrate (pH 6.5), and 10% (wt/vol) sucrose, could be stored for 56 weeks at 30°C with no detectable damage to the protein and at 50°C with only a 4% loss of native protein due to deamidation. The purpose of the current study was to investigate the physical bases for this stability and, in so doing, test rigorously the proposed mechanisms for storage stability of freeze-dried proteins.

Two physical criteria have been proposed to be important for long-term stability of dried proteins. First, in the dried powder, the protein is a component of a glassy phase that includes other additives which re-

<sup>3</sup>Abbreviations used: rhIL-1ra, recombinant human interleukin-1 receptor antagonist; T<sub>g</sub>, glass transition temperature.

main amorphous (e.g., sucrose). It has been proposed that proteins should be stable when stored below the characteristic glass transition temperature ( $T_g$ ) of this amorphous phase (3, 4). This is because the rate of diffusion-controlled reactions, putatively including protein unfolding, protein aggregation, and chemical degradative processes, should be greatly reduced relative to rates noted above  $T_g$  (3, 4). Published direct support for this mechanism is essentially limited to a study by Roy *et al.* (6), in which it was found that the temperature- and residual moisture-dependent changes in storage stability of a lyophilized monoclonal antibody-vinca conjugate could be explained by glass transitions. Indirect support comes from the observations that storage stabilities of ribonuclease (7) and galactosidase (8) were greatest when the proteins were freeze-dried in the presence of stabilizers that remained amorphous (e.g., sucrose). However, in these studies the glass transition temperatures of the samples were not determined and compared to the storage temperatures.

Secondly, Prestrelski and colleagues have suggested that in addition to storage below  $T_g$ , long-term stability is dependent on retaining the native protein structure in the dried solid (9, 10). Initially, their infrared spectroscopic studies documented that the acute stresses of freezing and drying induce unfolding of unprotected proteins (11, 12). Compounds, such as sucrose, which confer both acute stability (e.g., minimize aggregation of proteins rehydrated immediately after lyophilization) and storage stability to dried proteins, inhibit unfolding during freeze-drying. More recently, Prestrelski and colleagues (10) found that interleukin-2 lyophilized from a solution of pH 7 was unfolded in the dried solid and unstable during storage at 45°C. In contrast, lyophilization from a solution with pH 4 led to a native protein and storage stability. This storage temperature was most likely below  $T_g$ , because characteristically dried proteins have  $T_g$ s greater than 100°C (13, 14). Using protein in a pH 7 solution, they also compared denaturation during freeze-drying and storage stability conferred by carbohydrates of increasing molecular weight (10). As molecular weight increased, the inhibition of denaturation during freeze-drying decreased, but the  $T_g$  increased. The optimum stability during storage at 45°C was noted in samples stabilized with the tetrasaccharide, stachyose, in which the dried protein was native and  $T_g$  was greater than 45°C. The protein degraded in samples with lower molecular weight carbohydrates, which had native protein, but a  $T_g$  less than 45°C. Thus, even with a native protein, stability is dependent on maintaining the temperature below  $T_g$ . With the highest molecular weight dextrans, for which  $T_g > 45^\circ\text{C}$ , the protein was initially unfolded. However, no further damage was noted during storage, which supports the stabilizing role of the glassy state.

In the current study, we compared the degradation

of carefully selected lyophilized rhIL-1ra formulations, which have various  $T_g$ 's and differing degrees of conformational change during freeze-drying, in hopes of better understanding the critical physical factors affecting the storage stability of freeze-dried proteins.

## MATERIALS AND METHODS

**Materials.** Recombinant rhIL-1ra was produced and purified at Amgen, Inc. The protein was >99.5% homogenous based on a size-exclusion chromatography and approximately 98% based on a cation-exchange chromatography. Other chemicals used for this study were analytical grade or better.

**Methods.** Each formulation was made by exchanging the buffer with a testing formulation by gel filtration on Sephadex 25. To prepare freeze-dried samples, 1.0 ml of each formulation was pipetted into a 3-ml glass vial. The vials were placed into an Edwards Supermoldulyo lyophilizer (Edwards High Vacuum, Inc.) and freeze-dried as described previously (5). The storage stability experiments on the dried protein samples and the analyses of protein degradation after storage and rehydration were performed as described previously (5). Briefly, degradation was assessed by rehydrating samples after storage and quantitating deamidation by cation exchange high-performance liquid chromatography and aggregation by turbidity determinations (5).

The  $T_g$  of a freeze-dried powder was determined using a Perkin Elmer DSC-7 as previously described by Chang and Randall (15). Approximately 10 mg of powder was sealed in a 50- $\mu\text{l}$  volatile aluminum pan and loaded to a sample compartment. The sample was equilibrated at  $-20^\circ\text{C}$ . Then, data were collected during warming at a rate of  $5^\circ\text{C}/\text{min}$  between  $-20$  and  $150^\circ\text{C}$ . A second order transition, which was reversible upon rescanning within 1 h, was considered as a signal for  $T_g$ . The glass transition signal occurred over a temperature range of approximately  $20^\circ\text{C}$ . The midpoint of the signal was used to represent the  $T_g$ .

Infrared spectra of aqueous protein solutions and dried powders were recorded at  $25^\circ\text{C}$  with a Nicolet Magna 550 Fourier transform infrared spectrometer, equipped with a dTGS detector. Protein solutions were prepared for infrared measurement in a sample cell (Beckman FH-01) that employed  $\text{CaF}_2$  windows separated by a 6- $\mu\text{m}$  spacer. For dried solids, approximately 0.5–1.0 mg of dried protein was combined with 300 mg of potassium bromide (KBr) and ground into a fine powder. The powder was annealed into a disc using a hydraulic press. We have previously established that this procedure does not alter the conformation of proteins in dried powders (11). Dried samples for spectroscopic analyses were lyophilized and stored for 24 weeks at  $50^\circ\text{C}$  and  $-70^\circ\text{C}$ . The latter samples were equivalent to samples examined immediately after lyophilization.

For each spectrum, a 512-scan interferogram was collected in single beam mode, with a  $4\text{-cm}^{-1}$  resolution. For aqueous samples, reference spectra were recorded under identical conditions with only buffer in the cell. The spectra for liquid and gaseous water were subtracted from the protein spectra, as appropriate, according to previously established criteria (16, 17). The final protein spectrum was smoothed with a 7-point function to remove white noise. Second derivative spectra were calculated with the derivative function of Nicolet Omnic software. To quantitate the secondary structural content represented by the second derivative spectra, the spectra were inverted by multiplication by  $-1$  and curve-fitted (SpectraCalc Software from Galactic Industries) with Gaussian band profiles (18).

## RESULTS

### *Glass Transition and Storage Stability*

If the glass transition mechanism (3, 4) for long-term stability is correct, then rhIL-1ra should be stable in

TABLE I  
Formulations Selected for Tg Study

Stabilizer	Bulking agent	Buffer	Tg
1% Sorbitol	5% Glycine	Sodium citrate	20
1% Sucrose	4% Mannitol + 2% glycine	Sodium phosphate	26
	4% Mannitol + 2% glycine	Sodium citrate	46
1% Alanine	5% Glycine	Sodium citrate	47
1% Sucrose	4% Mannitol	Sodium citrate	47
1% Trehalose	5% Glycine	Sodium citrate	50
0.5% Sucrose	5% Glycine	Sodium citrate	50
1% Sucrose	4% Mannitol + 2% glycine	Sodium citrate	51
1% Sucrose	5% Glycine	Sodium citrate	54
1% Sucrose	2% Glycine	Sodium citrate	56

the dried state during storage at temperatures below Tg and should have greatly reduced stability at temperatures above Tg. Also, such a relationship should hold for any freeze-dried formulation, as long as the none of the components are obviously protein destabilizers (e.g., urea). To test this mechanism rigorously, we prepared 10 different formulations of freeze-dried rhIL-1ra, which had Tgs ranging from 20 to 56°C (Table I). These formulations were selected for examining protein storage stability because they met the following criteria: (1) There was full recovery of native protein, with no chemical degradation, when the samples were rehydrated immediately after freeze-drying, indicating that irreversible acute damage during drying was not sustained. These results also support the contention that none of the additives were protein destabilizers. (2) There was the presence of amorphous additives. (3) The Tg could be measured by DSC. (4) Residual moisture determined by Karl Fisher method was less than 0.01 g H<sub>2</sub>O/g dried powder. (5) The dried cakes maintained structural integrity and did not collapse during freeze-drying. The latter condition is important because collapse means a loss of structure of the amorphous phase during the freeze-drying process, which could lead to excessively high residual moisture and/or heterogeneous distribution of water in the dried cake. In turn, such retention of water could result in water-induced instability during storage (cf. 19).

When the selected formulations were stored at various temperatures for several weeks, rehydrated, and analyzed for degradation products, there was generally an increase in the rates of deamidation and aggregation during storage above Tg (Fig. 1). Also, in most instances the rates of degradation were greatly reduced at temperatures below Tg.

However, there were several instances where storage below Tg did not ensure stability (Fig. 1). For example, even at temperatures almost 40°C below Tg, the formulation containing 1% sucrose, 4% mannitol, 2% glycine and sodium phosphate had deamidation rates substan-

tially higher than those for other formulations in the same range of storage temperatures below Tg (Fig. 1A). Also, in many of the formulations, damage, which was manifested as aggregation upon rehydration, was noted after storage at temperatures as much as 45°C below Tg (Fig. 1B). Therefore, storage of dried rhIL-1ra below the formulation Tg was not always sufficient to ensure long-term stability.

#### *Lyophilization-Induced Structural Change and Storage Stability*

As noted in the introduction, the second factor to which storage stability has been ascribed is the retention of the native structure in the dried solid, i.e., inhibition of lyophilization-induced unfolding (9, 10). Thus, the hypothesis is that both storage at a temperature below the Tg and a native protein structure are necessary to confer stability. To test this hypothesis we prepared freeze-dried formulations containing from 0 to 10% (wt/vol) sucrose, in combination with 2% glycine, 10 mM sodium citrate (pH 6.5), and 100 mg/ml rhIL-1ra. This range of formulations was chosen because we had previously documented that increasing the sucrose-to-protein ratio, while keeping other formulation components constant, led to increased stability (based on final levels of degradation after 56 weeks at 50°C) during storage in the dried solid (5). Indirect support for the contention that the increased stability may be due, at least in part, to the effect of sucrose on protein structure, comes from our earlier work with several other proteins that showed that sucrose could prevent acute protein unfolding during freeze-drying (11, 20).

The formulations with varying amounts of sucrose were also selected for this study because their Tgs were almost identical at  $66 \pm 2.5^\circ\text{C}$  (Table II). Thus, during storage at 50°C, all formulations would be about 16°C below Tg, and any difference in stability should be attributable factors other than a glass transition. The results shown in Fig. 2 indicate that there were dra-

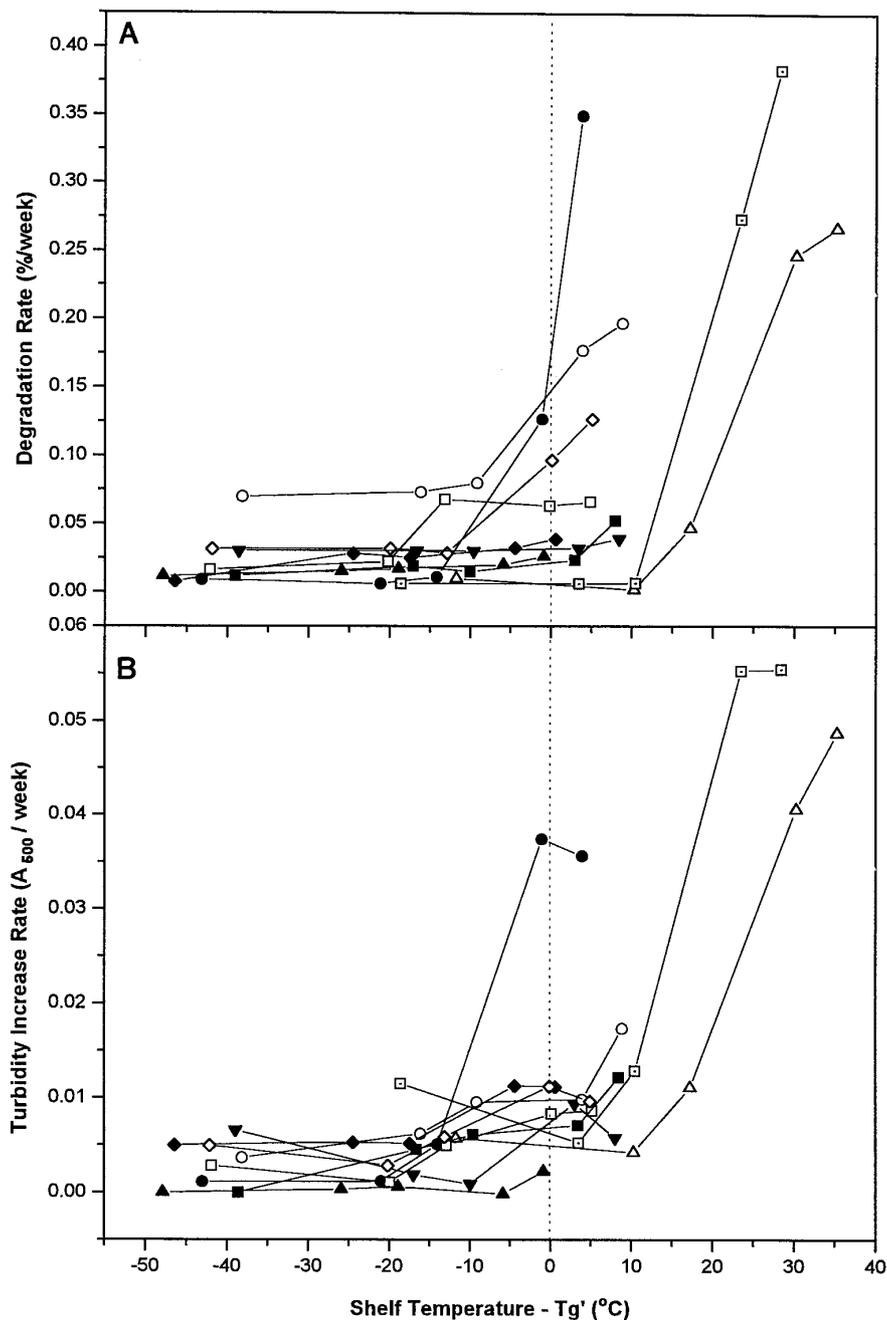


FIG. 1. Rates of deamidation (A) and precipitation (B) for rhIL-1ra in freeze-dried formulations, as a function of the difference between storage temperature and formulation Tg. Precipitation and deamidation were measured after storage and rehydration. Figure symbols: 1% sorbitol/5% glycine/citrate ( $\Delta$ ), 1% sucrose/4% mannitol/2% glycine/phosphate ( $\square$ ), 4% mannitol/2% glycine/citrate ( $\circ$ ), 1% alanine/5% glycine/citrate ( $\blacksquare$ ), 1% sucrose/4% mannitol/citrate ( $\blacktriangledown$ ), 1% trehalose/5% glycine/citrate ( $\diamond$ ), 0.5% sucrose/5% glycine/citrate ( $\square$ ), 1% sucrose/4% mannitol/2% glycine/citrate ( $\bullet$ ), 1% sucrose/5% glycine/citrate ( $\blacklozenge$ ), 1% sucrose/2% glycine/citrate ( $\blacktriangle$ ).

matic differences in protein stability between the formulations and that increasing the sucrose content led to less degradation. In all samples, degradation reached an apparent maximum level within about 24 weeks. In the absence of sucrose, significant protein

degradation due to both aggregation and deamidation was detectable within 5 weeks of storage. Aggregation, as documented by turbidity after rehydration, reached such high values after only 16 weeks of storage that measurements after further storage were off scale for

TABLE II

The Glass Transition Temperatures (T<sub>g</sub>s) of Selected Formulations with Varying Concentrations of Sucrose

Sucrose concentration (w/v %)	T <sub>g</sub> (°C)
0	66.5
1	68.5
3	67.8
5	66.2
10	64.6

Note. All formulations contain 2% (w/v) glycine, 10 mM sodium citrate buffer at pH 6.5. See Materials and Methods for the procedure of DSC analysis.

the spectrophotometer (Fig. 2A). The addition of 1% sucrose attenuated both the rate and extent of damage. In the presence of 5 and 10% sucrose degradation due to aggregation was not detectable, and only about 4–5% of the protein was lost due to deamidation, even after 56 weeks of storage.

To determine the acute effects of lyophilization and the presence of the varying amounts of sucrose on pro-

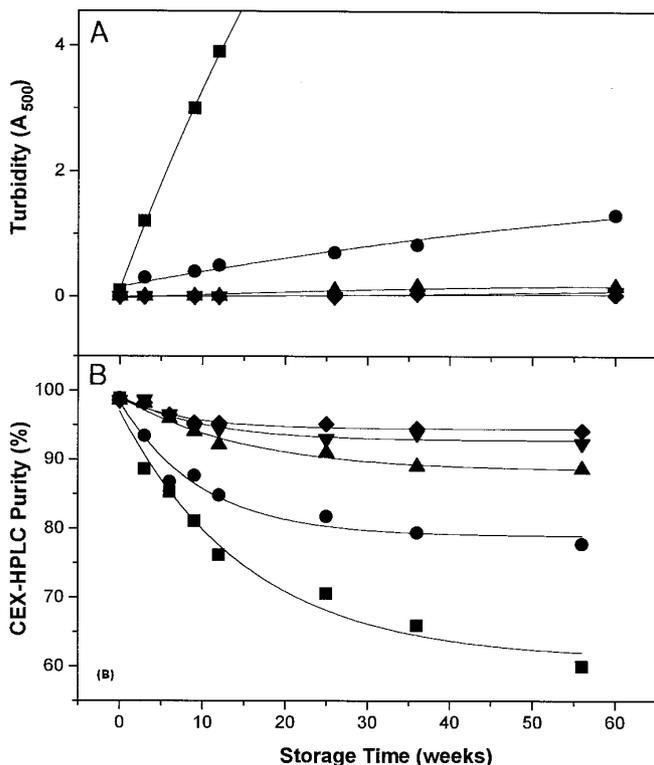


FIG. 2. Effect of initial sucrose content on precipitation and deamidation of rhIL-1ra during storage of dried solid at 50°C. Initial sucrose contents: 0 (■), 1% (●), 3% (▲), 5% (▼), and 10% wt/vol (◆). Precipitation and deamidation were measured after storage and rehydration.

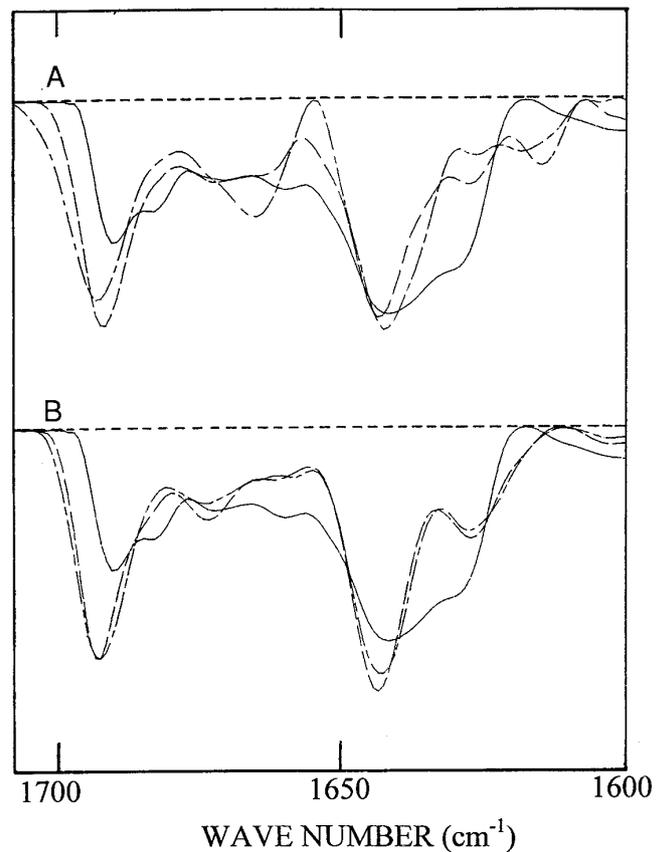


FIG. 3. Effects of sucrose on lyophilization-induced alterations in the second derivative infrared spectra of rhIL-1ra. (A) Aqueous protein (—) and protein dried with no sucrose (---) and with 1% wt/vol sucrose (- · -). (B) Aqueous protein (—) and protein dried with 5% (- · ·) and 10% wt/vol sucrose (· · ·).

tein structure, we compared the second derivative infrared spectra in the conformationally sensitive amide I region for the aqueous protein to that for the dried formulations. This comparison was made first by simply overlaying the second derivative spectra, which had been normalized for total area (Figs. 3–5). In our experience this approach is often most valuable for ascertaining overall alterations in protein structure (19). However, it is also of interest to quantitate the relative contribution of different component bands, and hence secondary structural elements, to the total area of the amide I contour. This is accomplished (cf. 16, 18) by fitting curves to the inverted second derivative spectrum (Fig. 6), calculating relative areas for the fitted curves, and assigning the bands to secondary structure based on frequency (Table III).

The spectrum for the aqueous protein prepared in the absence of sucrose had relatively broad bands, especially in the region between 1625 to 1645 cm<sup>-1</sup>, in which bands are assigned (cf. 17) to  $\beta$ -sheet structure (Fig. 3A). This spectrum for the aqueous protein solution

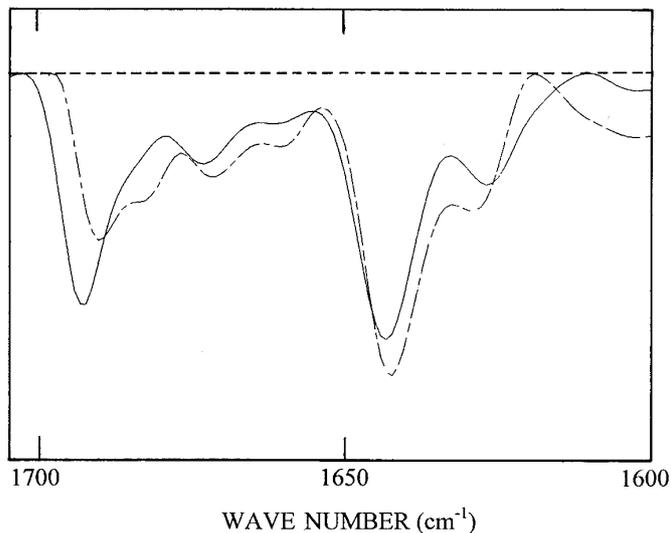


FIG. 4. Comparison of the second derivative infrared spectrum for rhIL-1ra in solution with 60% wt/vol sucrose (---) to that for the protein dried with 10% wt/vol sucrose (—).

might be due to a relatively mobile conformation, in which the peptide chain is packed into a volume larger than the minimum. With other proteins that have more regular, compact structure in solution, drying usually leads to band shifts and increases in band width, which are ascribed to changes in conformation and the presence of multiple "static" conformations (9, 11). When rhIL-1ra was freeze-dried in the absence of sucrose, there were several alterations in the infrared spectrum (Fig. 3A). First, bands in the 1625- to 1645- $\text{cm}^{-1}$  region were more clearly resolved, which could indicate that lyophilization has simply led to a "compaction" of the native conformation. However, other spectral changes indicate that the overall protein structure was altered (i.e., the protein was unfolded) by freeze-drying (Fig. 3A and Table 3). There was the appearance of a new large band centered at 1663  $\text{cm}^{-1}$  and the loss of the band at 1661  $\text{cm}^{-1}$ , which are usually assigned to  $3_{10}$  helix structure (17). Also there was the loss of other bands in the region (ca. 1670–1690  $\text{cm}^{-1}$ ) assigned to  $\beta$ -turn (17). There was a decrease in the absorbance at 1652  $\text{cm}^{-1}$ , indicating a reduction in disordered content (17), which was compensated by an overall increase in  $\beta$ -sheet and  $\beta$ -turn content (Fig. 3A and Table 3).

Also, there was a shift of the band normally found at 1690 to 1693  $\text{cm}^{-1}$  and a large increase in absorbance in this region (Fig. 3A and Table 3). The appearance of a new band at 1618  $\text{cm}^{-1}$  could be due to formation of intermolecular beta sheet in the dried solid (20). The broadening and shift to a higher frequency of the band at 1690  $\text{cm}^{-1}$  is consistent with this interpretation, because often intermolecular beta sheet leads to a weaker band at wave numbers above 1690  $\text{cm}^{-1}$ . These spectral

changes are often associated with the formation of protein aggregates (20). However, aggregates formed during lyophilization must dissociate in samples rehydrated immediately, because aggregation was not detectable prior to storage.

The addition of 1% sucrose led to further narrowing of the bands in the 1625- to 1645- $\text{cm}^{-1}$  region (Fig. 3A). There was also a greater increase in the absorbance at 1692  $\text{cm}^{-1}$ , relative to that for the aqueous protein. The other alterations noted for the sample dried without sucrose were attenuated (Fig. 3A). The spectra for samples dried with 5 and 10% sucrose were very similar to each other, but were much different than that for aqueous control protein (Fig. 3B and Table 3). The bands in the 1625- to 1645- $\text{cm}^{-1}$  region were narrower than for other samples, indicating the most compact, organized structure of all samples tested. The band at 1663  $\text{cm}^{-1}$ , noted with 0 or 1% sucrose, was not present

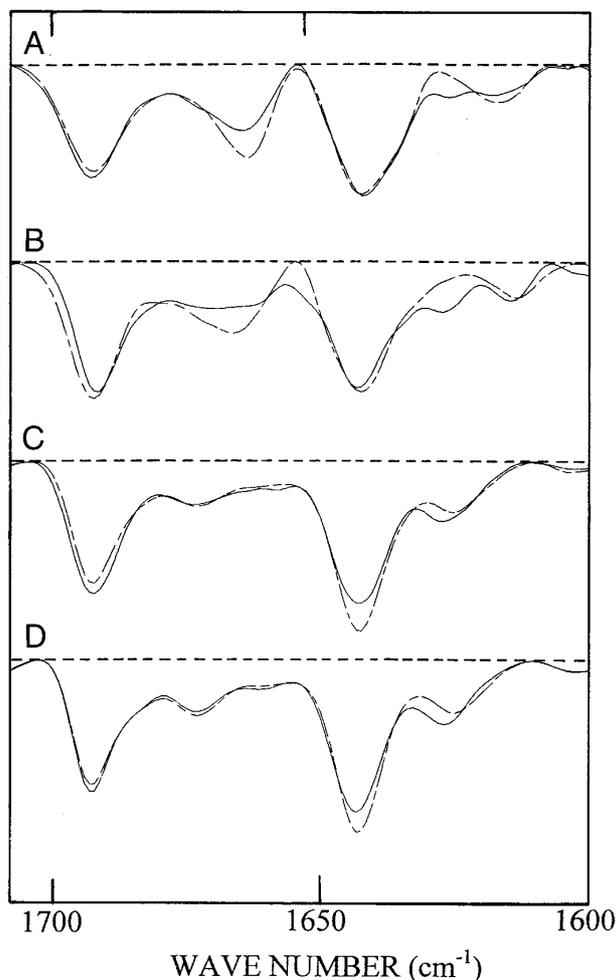


FIG. 5. Effect of 24 weeks of storage at 50°C on the second derivative infrared spectra of dried rhIL-1ra. Samples were lyophilized with 0 (A), 1% (B), 5% (C), and 10% wt/vol sucrose (D). Samples held at  $-70^{\circ}\text{C}$  (—) and samples stored at  $50^{\circ}\text{C}$  (---).

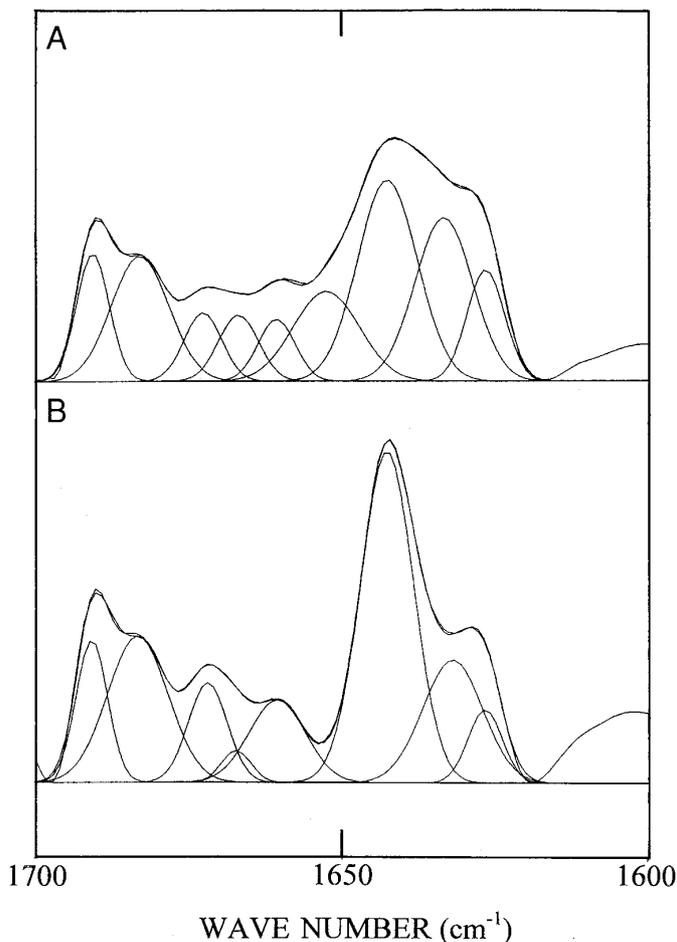


FIG. 6. Representative examples of curve fitting to inverted second derivative infrared spectra for rhIL-1ra. (A) Aqueous protein in the absence of sucrose. (B) Aqueous protein in the presence of 60% wt/vol sucrose.

with 5 and 10% sucrose. Also, the beta turn band noted in the aqueous protein's spectrum at about  $1672\text{ cm}^{-1}$  was retained, as was a somewhat smaller  $3_{10}$  helix band at  $1661\text{ cm}^{-1}$ . The band at  $1618\text{ cm}^{-1}$  was not present in the samples dried with 5 and 10% sucrose, nor was the band for disordered components at the region from  $1648\text{--}1652\text{ cm}^{-1}$  (Fig. 3B and Table 3). Taken together these results indicate that freeze-drying the protein in the presence of 5 and 10% sucrose not only inhibited unfolding, but actually induced an apparently more highly structured "native state" in the dried solid.

In a separate study on storage stability of rhIL-1ra in aqueous solution, we found that addition of large amounts of sucrose (i.e.,  $>40\%$  wt/vol) could also cause similar alterations in the protein's infrared spectrum (Chang, Beauvais, Dong, and Carpenter, unpublished observations). These alterations were indicative of a structure that was more compact and ordered than that noted in the absence of sucrose and that correlated

with increased long-term stability. We compared the spectrum for the aqueous protein solution in the presence of 60% sucrose to that for the protein freeze-dried from a solution containing 10% sucrose (Fig. 4 and Table 3). All other formulation components noted above were held constant. These spectra were more similar to each other than either was to that for the protein in aqueous solution without sucrose (Figs. 3 and 4 and Table 3). In both spectra, there was similar band narrowing in the  $1625\text{--}1645\text{ cm}^{-1}$  region. Bands ascribed to  $3_{10}$  helix at  $1661\text{ cm}^{-1}$  and turn structure at ca.  $1671\text{ cm}^{-1}$  were present in both spectra. In both cases, a band assigned to disordered components was not retained. The total secondary structure contents of the two protein samples were more similar to each other than to that for the aqueous protein without sucrose (Table 3). The major differences were a greater absorbance and higher wave number for the high frequency beta turn band (ca.  $1690\text{ cm}^{-1}$ ) and a reduction in absorbance of the ca.  $1683\text{ cm}^{-1}$  band for the dried sample, relative to sample in solution with 60% sucrose. Finally, it should be noted that sucrose at concentrations up to 10% (wt/vol) had essentially no effect on the protein's infrared spectrum in solution (data not shown).

To ascertain if structural alterations were arising in the dried solid during high temperature storage, we acquired infrared spectra of the dried formulations after 24 weeks of storage at  $50^\circ\text{C}$ . By this point in the storage study, degradation had essentially reached a maximum (Fig. 2). As can be seen in Fig. 5, even though all formulations were held about  $16^\circ\text{C}$  below their  $T_g$ , those that had 0 and 1% sucrose had relatively large changes in their infrared spectra during storage at  $50^\circ\text{C}$  in the dried solid. In the sample without sucrose, there was a large increase in the absorbance of the  $1663\text{ cm}^{-1}$  band, which was initially induced by freeze-drying. Also, there was an increase in the absorbance at  $1618\text{ cm}^{-1}$ , indicating that there was an increase in intermolecular beta sheet structure in the dried solid. These absorbance increases were compensated by a decrease in absorbance at  $1627\text{ cm}^{-1}$ . Similar changes were noted in the sample with 1% sucrose, except that there was not an increase in the absorbance in the intermolecular beta sheet band at  $1618\text{ cm}^{-1}$ .

In contrast, the formulations prepared with 5 and 10% sucrose had relatively minor changes in their infrared spectra after 24 weeks at  $50^\circ\text{C}$ . In both cases, the spectra for the dried proteins showed a small increase in absorbance at  $1643\text{ cm}^{-1}$  and a small decrease in absorbance at  $1693\text{ cm}^{-1}$ . The band at  $1626\text{ cm}^{-1}$  decreased slightly in absorbance and shifted to  $1625\text{ cm}^{-1}$ . This apparent resistance to structural alterations in the dried solid correlated well with the resistance to deamidation and aggregation noted upon rehydration after long-term storage at  $50^\circ\text{C}$  (Fig. 2).

TABLE III  
Relative Areas and Assignments of Infrared Second-Derivative Amide I Band Components of rhIL-1ra

Aqueous				Dried solid				Assignment
0% sucrose		60% sucrose		0% sucrose		10% sucrose		
$\nu$ ( $\text{cm}^{-1}$ )	Area (%)							
—	—	—	—	1618	4.4	—	—	$\beta$ -Aggregate
1627	8.4	1627	5.6	1626	4.0	1627	16.4	$\beta$ -Sheet
1633	18.5	1632	13.5	1636	15.8	—	—	$\beta$ -Sheet
1642	23.9	1643	35.0	1643	18.9	1643	35.6	$\beta$ -Sheet
1652	12.2	—	—	1649	3.2	—	—	Unordered
1661	4.5	1661	9.4	1663	8.6	1661	6.7	$^3$ 10-helix
1667	5.1	1667	2.0	—	—	—	—	Turn
1673	5.3	1672	7.7	1671	12.7	1673	11.5	Turn
1683	14.3	1683	18.0	1681	2.5	1686	12.4	Turn
1690	7.9	1691	8.8	1693	29.9	1693	17.4	$\beta$ -Sheet/ $\beta$ -aggregate

## DISCUSSION

The two degradation pathways that we monitored to assess the storage stability of lyophilized rhIL-1ra, precipitation and deamidation, are the most common for proteins (1, 21). Therefore, rhIL-1ra is a good model system for evaluating the physical factors affecting the long-term stability of dried proteins. We found that storage of lyophilized formulations at temperatures above the Tg greatly accelerated degradation (Fig. 1), which is consistent with the glass transition mechanism for stabilization (3, 4). However, in many of the formulations tested, comparable rates of degradation occurred at temperatures below the Tg (Fig. 1). Actually, if all that were needed for stability was a Tg greater than the storage temperature, then proteins freeze-dried in the absence of protective additives should be extremely stable. Proteins freeze-dried alone have relatively high Tgs (13, 14). For example, dry legumin has a Tg of about 140°C, and even in the presence 10% residual water (by weight), the Tg is still ca. 50°C (13). However, proteins lyophilized without protective additives have very poor storage stability, even if they are held at temperatures much below the range associated with the Tg for dried proteins (8, 22).

The glass transition mechanism for storage stability can be qualified by stating that another amorphous compound must be present with the protein. For example, amorphous additives conferred storage stability to  $\beta$ -galactosidase, whereas compounds that crystallized during lyophilization did not (8, 22). However, all of the formulations we tested contained amorphous additives, yet in many instances the protein degraded during storage at temperatures below Tg (Figs. 1 and 2). Thus, maintaining the protein below the Tg, even with an additional amorphous compound, is not sufficient for storage stability.

All of these observations actually are consistent with the proposal that in addition to keeping the temperature below the formulation Tg, storage stability is dependent on inhibiting acute protein unfolding during freeze-drying (9, 10). As noted in the introduction, Prestrelski *et al.* (10) found that storage stability of interleukin-2 was greatest when both criteria were met. To obtain this requisite acute protection, additives must remain amorphous during lyophilization. For example, compounds that crystallized failed to inhibit lyophilization-induced unfolding of lactate dehydrogenase and phosphofructokinase, and, hence, to foster recovery of enzyme activity after rehydration (23). In contrast, sufficient initial concentrations of amorphous agents (e.g., sucrose) acutely protected these and many other proteins during freeze-drying (11, 12, 20); native proteins were present in the dried solids and function was recovered after rehydration.

The importance of protein protection during freeze-drying in long-term storage stability also can be inferred from earlier studies that did not determine protein structure in the dried solid. For example, Izutsu *et al.* (8) found that storage stability of  $\beta$ -galactosidase at 70°C for 7 days was greatest in the presence of amorphous compounds (e.g., trehalose and sucrose), which also led to full enzyme activity recovery in samples rehydrated immediately after lyophilization. Structural changes during freeze-drying, irreversible loss of activity, and further instability during storage were noted in samples lyophilized without stabilizers or with agents that crystallized. Since minimizing acute loss of enzyme activity correlates with inhibition of unfolding during lyophilization (11, 12), Izutsu's data support the contention that increased storage stability conferred by amorphous excipients is due to the maintenance of the native protein structure during lyophilization. The Tgs

of the final dried solids were not measured. It is most likely that the Tgs of the stable formulations were higher than the storage temperature of 70°C and/or the duration of storage was not sufficient for degradative processes to be manifested in samples with Tgs lower than 70°C.

Our infrared spectroscopic results with rhIL-1ra formulations containing 0–10% sucrose provide direct support for the role of inhibition of acute lyophilization-induced protein unfolding in storage stability. As is the case with most proteins (9–12, 20), the infrared spectrum of rhIL-1ra dried without sugar was greatly altered relative to the initial aqueous protein's spectrum, indicating that lyophilization induced unfolding (Fig. 3A). Even though it was stored below its Tg, this sample had extensive degradation due to deamidation and aggregation, which were noted after rehydration (Fig. 2). However, it appears that the stabilization of rhIL-1ra by sucrose involves more than a simple retention of the original aqueous protein structure in the dried solid. In the infrared spectra for rhIL-1ra dried with 5 or 10% sucrose some bands were more narrow than those for the native, aqueous control protein, and the other bands matched closely those for the native protein (Fig. 3B and Table 3). Also, there was the loss of the band due to disordered components. These results suggest that the structured had become more ordered in the dried sample.

A similar alteration of the protein's infrared spectrum was noted in aqueous solution, when the protein was exposed to 60% (wt/vol) sucrose (Fig. 4 and Table 3). In a recent study of the storage stability of aqueous solutions of rhIL-1ra at 30°C, we found that degradation was also reduced when in samples containing  $\geq 40\%$  sucrose. Thus, it appears that the most stable rhIL-1ra structure in both liquid and dried solids is the more organized state induced by sucrose.

We propose that the transition to an apparently more ordered native structure by high concentrations of sucrose can be explained by Timasheff's stabilization mechanism for proteins in aqueous solution (reviewed in 2, 24). Accordingly, sucrose is excluded preferentially from the surface of proteins, which increases protein chemical potential. The free energy of the system is minimized by reducing the surface area of the protein from which solute is excluded and, hence, minimizing the effect of excluded solute on protein chemical potential. Speculatively, if a protein's native state in aqueous buffer is not as compact as possible, then preferentially excluded solutes could cause a more ordered native structure, which has a decreased surface area, to be favored.

Much lower initial sucrose concentrations were needed to cause this structural transition during freeze-drying than in aqueous solution. This is probably because the concentrations of all solutes, including sucrose and protein, are greatly increased as a sample

is frozen and dried. Then why was a 1% initial sucrose concentration not also effective at inducing a more ordered and stable IL-1ra conformation during lyophilization? Proteins must be protected during both freezing and drying to inhibit lyophilization-induced unfolding (12, 23). The degree of protection during freezing is directly related to the initial concentration of solute and can be explained by Timasheff's preferential solute exclusion mechanism (25, 26). A 1% sucrose concentration may not be sufficient to protect rhIL-1ra from freezing damage. Alternatively, there might also be insufficient sucrose to protect the protein during the dehydration step, by hydrogen bonding in the place of water molecules (11, 27). Such perturbation of protein structure would offset the effect of sugar on favoring a more compact conformation.

The next question that arises is why are nonnative proteins not stable in the dried solid, even when stored at a temperature below the sample's Tg? First, as recently reviewed by Pikal (28), this might be due, at least in part, to the fact that even below Tg there can be significant molecular mobility, which is permissive to degradative reactions. And a recent study by Hancock and colleagues found that glasses of sucrose and Polyvinylpyrrolidone must be cooled to more than 50°C below Tg for molecular motions to be negligible over the normal lifetime (e.g., months) of a typical pharmaceutical product (29). Second, in aqueous protein solutions, perturbing the native structure (e.g., with denaturants) can greatly accelerate degradation by deamidation, even if deamidation is negligible in the native protein (30–33). Kossiakoff (32) has argued that minor changes in the backbone structure can cause significant rearrangement of side chains, so that a microenvironment favorable to deamidation is created. It appears that the structural alterations in rhIL-1ra induced by freezing–drying are also sufficient to provide an environment conducive to deamidation, and that there is sufficient mobility, even 16°C below the Tg, to allow such degradation.

Finally, in addition to an environment permissive to chemical degradation, the glassy state also allowed sufficient mobility for further structural alterations in, and intermolecular interactions between, unfolded rhIL-1ra molecules. In samples prepared with  $\leq 1\%$  sucrose, the infrared spectra after 24 weeks of storage at 50°C were altered relative to those for rhIL-1ra immediately after lyophilization (Figure 5). The increased intensity of the bands at 1618  $\text{cm}^{-1}$ , which is indicative intermolecular beta sheet, suggests that protein aggregation was arising during storage in the dried solid. These spectral changes were not seen in the preparations containing 5 and 10% sucrose, in which the initial lyophilization-induced protein unfolding was inhibited (Fig. 5). Thus, it appears that the native structure is more resistant to structural alterations in the dried solid.

However, the current data do not allow us to rule out the possibility that one aspect of the glassy transition mechanism played at least some role in the structural stability during storage. Namely, it has been proposed that glass forming additives such as sucrose serve to "dilute" protein molecules in the amorphous phase of the dried solid (3, 4). This effect, combined with a restriction in mobility noted at temperatures below  $T_g$ , could make it more difficult for dried protein molecules to form intermolecular contacts. Further work with a protein system in which an added glass forming compound does not confer structural stabilization during freeze-drying or in which the protein is inherently resistant to structural perturbation during freeze-drying is needed to differentiate between the relative roles of native structure and glassy additives in preventing structural alterations during storage in the dried solid.

## CONCLUSIONS

The results of the current study, together with those published previously by Prestrelski *et al.* (10), document that maintaining a dried protein formulation below its glass transition temperature is necessary for long-term storage stability. Even if the protein is native in the dried solid, there can be degradation above the sample  $T_g$  (10). Therefore, for optimal storage stability a protein must both be native in the dried solid and stored below the formulation  $T_g$ . The major effect of amorphous, stabilizing additives (e.g., sucrose) appears to be inhibiting the unfolding arising during lyophilization. Finally, since a high  $T_g$  and a native protein structure are essential for stability of dried proteins, we recommend that differential scanning calorimetry and infrared spectroscopy, respectively, be used to measure these physical parameters. Such determinations should help make design of optimally stable lyophilized protein formulations a more rational, straightforward process.

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