Optimization of Lyophilization Conditions for Recombinant Human Interleukin-2 by Dried-State Conformational Analysis Using Fourier-Transform Infrared Spectroscopy

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Purpose. Examination of the dried-state conformation of interleukin-2 (IL-2) was used to determine the pH conditions and stabilizers that provide optimal storage stability for the lyophilized product. ***Methods.*** Fourier-transform infrared spectroscopy and accelerated stability studies which examined solubility, aggregate formation, and covalent cross-linking were used. ***Results.*** Varying the pH in the absence of excipients resulted in dramatic differences in the dried state conformation of IL-2. At pH 7, IL-2 unfolds extensively upon lyophilization while at pH below 5 it remains essentially native. Additional unfolding was observed upon incubation at elevated temperatures. A strong direct correlation between the retention of the native (aqueous) structure during freeze-drying and enhanced stability is demonstrated. IL-2 prepared at pH 5 is approximately an order of magnitude more stable than at pH 7 with regard to formation of soluble and insoluble aggregates. A similar pH profile was observed in the presence of excipients, although the excipients alter the overall stability profile. Additional accelerated stability studies examined the stabilizers necessary for optimal stability. ***Conclusions.*** Excipients with the capacity to substitute for water upon dehydration better preserve the native structure resulting in enhanced stability. Those that have high glass transition temperatures provide the highest level of stability during storage, although they do not prevent dehydration induced unfolding.

KEY WORDS: lyophilization; interleukin-2; protein conformation; infrared spectroscopy; stability.

INTRODUCTION

Freeze-drying (or lyophilization) is often used to enhance the long-term stability of protein pharmaceuticals when the stability of aqueous formulations is insufficient. Due to the limited conformational stability of many proteins, the lyophilization process can also result in protein unfolding and consequent destabilization. To maximize stability, both the lyophilization cycle (1) and the product formulation (2) must be optimized for product development. The stability of proteins during drying and storage is a combination of several interrelated factors. Thus, a thorough understanding of all factors that can affect protein stability is necessary for achieving truly optimal stability.

Lyophilization has long been used to preserve the functionality of and enhance the storage properties of foods (3) and traditional (small-molecule) pharmaceuticals (4,5). Thus, it is not surprising that the first attempts to rationalize the stability of lyophilized proteins would draw on the accumulated knowledge of the food and pharmaceutical industries. However, there is one crucial property of proteins that distinguishes them from foods and small molecule drugs; the absolute dependence on a unique three-dimensional conformation for biological activity (6). Proteins are chemical entities and are susceptible to chemical degradation processes, as are foods and drugs (7). However, the necessity of maintaining a three-dimensional conformation for biological activity, and therefore pharmaceutical functionality, is unique to proteins.

Protein conformation is a complex function of numerous, often opposing, weak forces and is typically the least stable feature (6). Proteins have variable sensitivities to the different stresses encountered during freeze-drying (*e.g.*, low temperatures, dehydration, concentration changes, etc.). Thus, the present state of the art for optimizing lyophilization parameters for proteins is often reduced to a semi-empirical screening of formulations and cycles to arrive at a stable product. Such an empirical technique can be time consuming as well as fail to arrive at the optimal level of stability. More rational approaches based upon fundamental physical principles are necessary to determine the optimal formulation and lyophilization cycle for a given protein product.

Recent studies have used Fourier-transform infrared (FT-IR) spectroscopy to examine protein conformation before and after freeze-drying, and have demonstrated that lyophilization-induced spectral changes are due to unfolding of proteins during lyophilization (8). Further, stabilizers were found to function through preventing lyophilization-induced unfolding (8–10). These results suggest that FT-IR spectroscopy may be useful in rationally optimizing lyophilization conditions for proteins. It is hypothesized that lyophilization of proteins in a manner that best preserves the native, active conformation of the protein will result in the highest long-term stability. In this report, the optimization of the pH and excipients for interleukin-2 (IL-2) during lyophilization is described. Previous studies have indicated that IL-2 is particularly susceptible to degradation during lyophilization and storage and thus provides a suitable model to examine the relationship between conformation and stability (11). It is demonstrated that the conformation of the dehydrated protein is a strong factor in determining stability.

MATERIALS AND METHODS

Materials

IL-2 (Ala-125) was produced recombinantly at Amgen. Prior to lyophilization any aggregates were removed from the stock IL-2 solutions using a preparative Superose-12 column (5 × 60 cm, Pharmacia) and a Pharmacia fast protein liquid chromatography system at a flow rate of 2.5 ml/min.

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The eluent used was 20 mM sodium citrate, pH 4. At this pH non-specific binding of the IL-2 to the column is minimal. Filtering the solutions through a 0.2 m filter was found to be equally useful and was used for some of the later preparations. Solutions of purified monomeric IL-2 were dialyzed to exchange the column buffer for the lyophilization buffer and the concentration was adjusted to 0.8 mg/ml. One ml aliquots were placed into 3 cc glass vials for lyophilization. Solutions were buffered in the pH range 5–7 using MES buffer; pH 4–5 with homopipes buffer; pH 2.5–4, citrate buffer; pH 1.5–2.5, phosphate buffer. Buffers were chosen for buffering capacity in the region of interest, infrared transparency in the Amide I region, and lack of stabilizing capacity.

Lyophilization

Samples were frozen by placing them on the lyophilizer (The Virtis Company, Gardiner, NY or FTS Systems, Stone Ridge, NY) shelf, which was pre-cooled to −50°C, for 1 hour. Primary drying was achieved at a shelf temperature of −45°C for 24–36 hours. Secondary drying was carried out at −10°C and then +20°C each for 24 hours. The product temperature was monitored using a thermocouple placed in a sample vial and was maintained at or below the shelf temperature throughout the run. All samples were dried without evidence of collapse. After the completion of secondary drying, the vials were capped under vacuum.

Accelerated Stability Studies

After lyophilization, vials containing the dried samples were placed in an incubator at either 45°C or 29°C. At various intervals samples were removed for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel permeation chromatography (GPC), ultraviolet (UV) spectroscopy and FT-IR spectroscopy. SDS-PAGE was performed using Novex (San Diego, CA) precast gels, 14%, followed by silver staining. UV spectra were collected on the reconstituted samples using a Beckman DU-650 ultraviolet spectrophotometer. The baseline intensity at 330 nm, resulting from the light scattering from high molecular weight aggregates, was used as a measure of turbidity. GPC was performed using a Superose-12 column (1 × 30 cm, Pharmacia) and a FPLC system (Pharmacia) at 0.5 ml/min. The mobile phase was 20 mM sodium citrate, pH 4. For each sample, 300 l of 0.8 mg/ml reconstituted solution was centrifuged to remove precipitates and the supernatant was injected onto the column. The eluent was monitored at 280 nm.

Infrared Spectroscopy

Samples for infrared spectroscopy were prepared as described in Prestrelski *et al.* (8). Briefly, a sufficient amount of sample powder containing 0.2–0.4 mg protein was mixed with ~300 mg KBr, mixed and pressed into a pellet. Spectra of unlyophilized aqueous proteins were collected using infrared cells with CaF_2 windows and 6 m spacers. For IR spectra, the protein solutions were concentrated to 30 mg/ml. All spectra were collected using a Mattson Research Series (Mattson Instruments, Madison, WI) spectrometer using liquid nitrogen cooled MCT detector. 4000 scans (15 min collection time) were coadded for each spectrum. The spectral resolution was set at 4 cm^{-1}. Second derivative spectra were calculated according to the method of Susi and Byler (12) and 9-point smoothed. Spectral correlation coefficients (r values) were calculated as described in Prestrelski *et al.* (8).

Differential Scanning Calorimetry (DSC)

DSC measurements were performed using a Perkin-Elmer DSC-7 differential scanning calorimeter (Perkin-Elmer, Norwalk, CT). The glass transition temperature, Tg, was determined for dried mixtures according to the method described by Wolanszyk (13). Approximately 10-20 mg of material was place into sealed pans and the scan rate was 10°C/min. Reported Tg values refer to the mid-point of the observed transition determined using the Perkin-Elmer software. A glass transition is observed as a reversible increase in heat capacity that leads to a step change in the baseline. Thus, each sample was scanned twice to ensure that the observed transitions were indeed glass transitions.

Moisture Analysis

Dried samples were analyzed for residual moisture content by the Karl Fisher technique using an Aquastar C2000 titrator equipped with an EV-6 solid evaporator (EM Science, Gibbstown, NJ). Samples were transferred directly from the vial to the purged solid evaporator to prevent uptake of moisture.

Biological Activity Assay

The biological activity of IL-2 was measured using a method based on radiolabeled thymidine uptake by a cultured murine tumor specific cytotoxic T-cell line as described by Gillis *et al.* (14).

RESULTS

Optimization of pH

The first series of experiments in this study examined the effects of lyophilizing IL-2 in the pH range 5–7 using a 10 mM MES buffer, pK ~6. The pI of IL-2 is approximately 8. The control spectra of the aqueous protein at various pH values prior to lyophilization showed no observable differences. All spectra of aqueous samples in the pH range from 7 to 1.5 had spectral correlation coefficients (r values) greater than 0.99 when compared against one another. Further, the infrared spectra of IL-2 obtained here are in good agreement with the secondary structure observed in the reported crystallographic structure (15).

Figure 1 shows the second derivative infrared spectra in the amide I region (C = O stretch, *ca.* 1720–1610 cm^{-1}) of IL-2 in the aqueous state and immediately after lyophilization from pH 7, 6, and 5 solutions. These spectra indicate that lyophilizing from the different pH solutions has a marked effect on the conformation of dried IL-2. The spectrum of IL-2 lyophilized from pH 7 is drastically altered compared to that of the aqueous spectrum. In particular, a new, strong band has become apparent at 1617 cm^{-1} indicating formation of a non-native structure during dehydration. This band and the enhanced absorbance near 1690

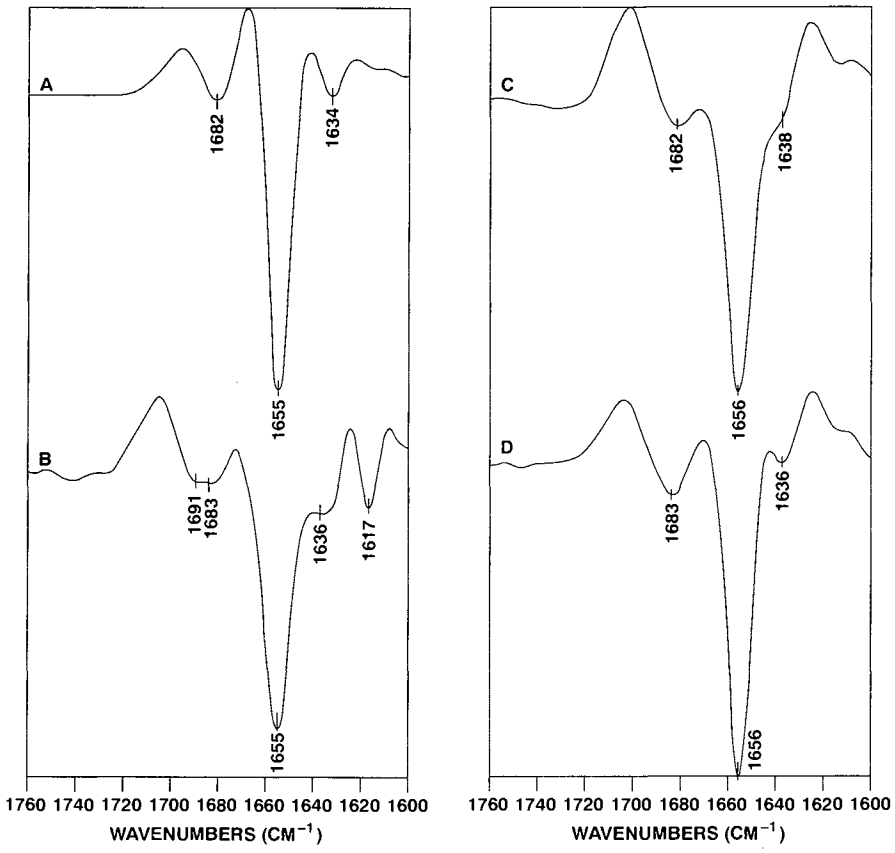

Fig. 1. Second derivative infrared spectra of IL-2 in the amide I region. A) aqueous, pH 5; B) lyophilized, pH 7, C) lyophilized, pH 6, D) lyophilized, pH 5.

cm^{-1} are most likely indicative of a β-sheet structure formed by unfolding and aggregation of the protein during lyophilization. Additional spectral changes are observed near 1636 cm^{-1} where a well-resolved band observed in the aqueous spectrum has become a shoulder on the predominant peak at 1656 cm^{-1}. In summary, lyophilization of IL-2 from pH 7 solution results in unfolding of the native polypeptide structure and possibly aggregate formation.

The spectrum of the dehydrated protein lyophilized from the pH 6 solution (Figure 1C) is indicative of lyophilization-induced unfolding, although not as severe as that observed for pH 7. The spectrum shows a general broadening of the peaks likely indicative of static peptide chain disordering (8). Further, the peak at 1633 cm^{-1} in the aqueous spectrum is observed only as a shoulder in the spectrum of the lyophilized protein. In contrast to the spectra of dried IL-2 lyophilized from pH 6 and 7 solutions, the spectrum of the protein lyophilized from the pH 5 solution resembles closely the spectrum of the aqueous protein, showing only a slight deviation from the native protein conformation. Table I lists the correlation coefficients for the spectra of the freeze-dried proteins when compared to the spectrum of the unlyophilized aqueous protein. The correlation coefficients provide a measure of the overall spectral differences between two spectra. These results indicate that in the pH range examined here, lyophilization from a pH 5 solution results in the highest degree of retention of the native structure during the freeze-drying process; lyophilization from pH 7, the lowest.

Table I also lists the correlation coefficients for lyophilized IL-2 after two and four weeks of incubation at 45°C. Incubation has resulted in further unfolding, indicating that the dried proteins possess some degree of conformational flexibility. The spectra of the pH 5 and 6 preparations begin to resemble the pH 7 spectrum taken at zero time (Figure 1B), showing enhanced intensity near 1615 and 1690 cm^{-1}. However, the pH 7 samples appear little changed over the course of the incubation.

Table II shows the ultraviolet spectroscopy results from the accelerated stability studies of the freeze-dried proteins. Based on results of the UV spectroscopy (turbidity), the pH 5 protein shows the highest level of stability for IL-2 lyophilized from a MES buffer. At higher pH values the turbidity increases rapidly upon incubation. After four weeks, the protein lyophilized from pH 6 and 7 solutions were essentially insoluble. SDS-PAGE analysis (Figure 2), indicates

Table I. Spectral Correlation Coefficients for IL-2 Lyophilized from MES Buffers at Various Times of Incubation at 45°C

	r value		
Time (wk)	0	2	4
pH 7.0	0.75	0.74	0.74
pH 6.0	0.83	0.80	0.79
pH 5.0	0.92	0.86	0.83

Table II. Turbidity of Reconstituted IL-2 After Various Incubation Times as Measured by the Baseline Intensity in UV Spectra at 330 nm

Buffer pH		Incubation time (wk) 0	2	4	8
MES					
7.0	29°C	0.232	0.246	0.708	—
	45°C		ins[a]	ins	—
6.0	29°C	0.118	0.265	0.412	—
	45°C		ins	ins	
5.0	29°C	0.130	0.176	0.343	—
	45°C		0.442	0.373	—
Homopipes					
5.0	29°C	0.076	0.095	0.102	0.219
	45°C		0.107	0.258	
4.0	29°C	0.065	0.068	0.062	0.074
	45°C		0.068	0.057	0.076

[a] Determination of the intensity at 330 nm was not possible for these samples due to the intense scattering from the large fraction of insoluble aggregates.

that at the higher pH values, formation of covalently linked oligomers (dimers, trimers, tetramers) is also accelerated.

GPC was used to further analyze the lyophilized samples after reconstitution. Analysis of pre-lyophilized control samples showed monomeric peaks of equivalent intensity, indicating that the effect of pH alone on aqueous IL-2 is negligible. GPC profiles of the lyophilized and rehydrated samples, after storing for 2 weeks at 29 or 45°C are shown in Figure 3. For the pH 5 sample, the peak eluting at 14 min corresponding to the monomeric form is slightly smaller at 45°C than 29°C, while the monomeric peak is much smaller for the pH 7 sample of 29°C and negligible at 45°C. Intermediate intensities are observed for the pH 6 sample. The decreased protein recovery during GPC is due to aggregation which caused the insoluble aggregate fraction of the protein to be removed by centrifugation prior to GPC analysis. In addition to the observed decrease in monomeric peak, the pH 7 sample incubated at 45°C shows the presence of soluble aggregates eluting with the void volume at 10 min. The disappearance of the monomeric protein during incubation is more clearly demonstrated by plotting the monomer peak intensity versus storage time at 45°C in Figure 4, showing that the pH 5 sample exhibits only a small decay of the monomer even at 45°C, while the pH 6 and 7 samples display sharp decreases with time. After 4 weeks, very little monomeric form is present for the latter two samples. Figure 5 shows the apparent first order rate constants for loss of monomeric protein plotted against the spectral correlation coefficient. It is noted that decreasing the pH of the solution from 7 to 5 enhances stability by approximately an order of magnitude.

Thus, it is apparent that unfolding of the proteins during dehydration and subsequent storage leads to instability. However, unfolding alone does not necessarily result in instability. The samples lyophilized from pH 7 are extensively unfolded at zero time but are almost completely recoverable as monomeric protein after reconstitution. With incubation the protein does not unfold significantly further but recovery of soluble is dramatically reduced. More likely, unfolding predisposes the protein to chemical and physical changes resulting in loss of solubility.

A second experiment was designed to examine the effects of lyophilizing IL-2 at pH values lower than 5. A homopipes (pK 4.5) buffer was used to prepare solutions of IL-2 at pH 5 and 4. The pH 5 sample was prepared in homopipes buffer to compare with the pH 5 MES buffer. Table III lists the correlation coefficients for spectra of IL-2 lyophilized from 10 mM homopipes at pH 5 and 4 (spectra not shown). These values indicate that little or no difference results from lyophilizing IL-2 at pH 4 relative to pH 5. The results of the ultraviolet analysis of the turbidity in Table II shows that lyophilizing at pH 4 relative to pH 5 results in a

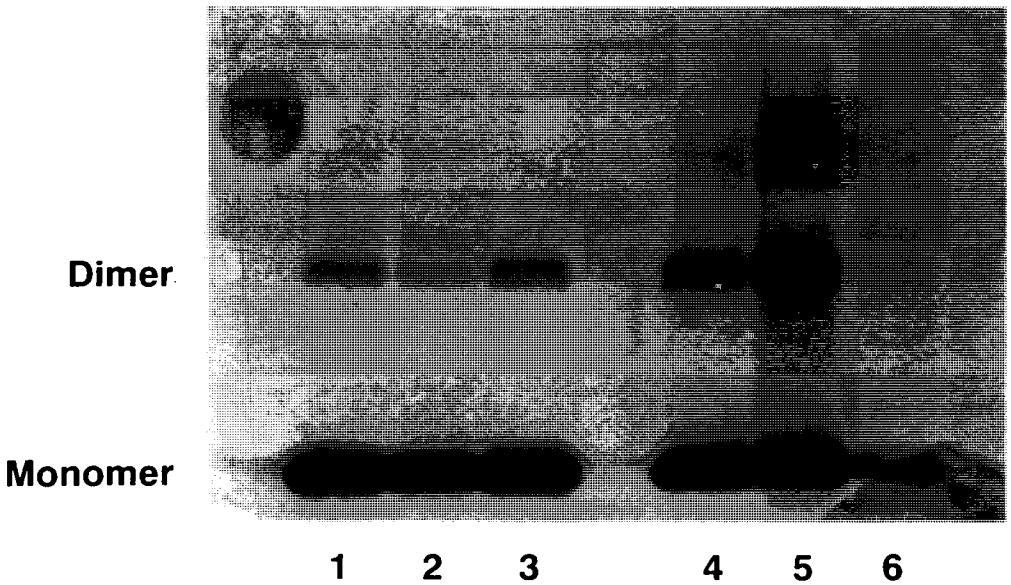

Fig. 2. Non-reduced SDS-polyacrylamide gel of lyophilized IL-2 reconstituted after two weeks of incubation. Lanes 1-3 are for pH 5, 6, and 7 stored at 29°C and lanes 4-6 are for pH 5, 6, and 7 stored at 45°C.

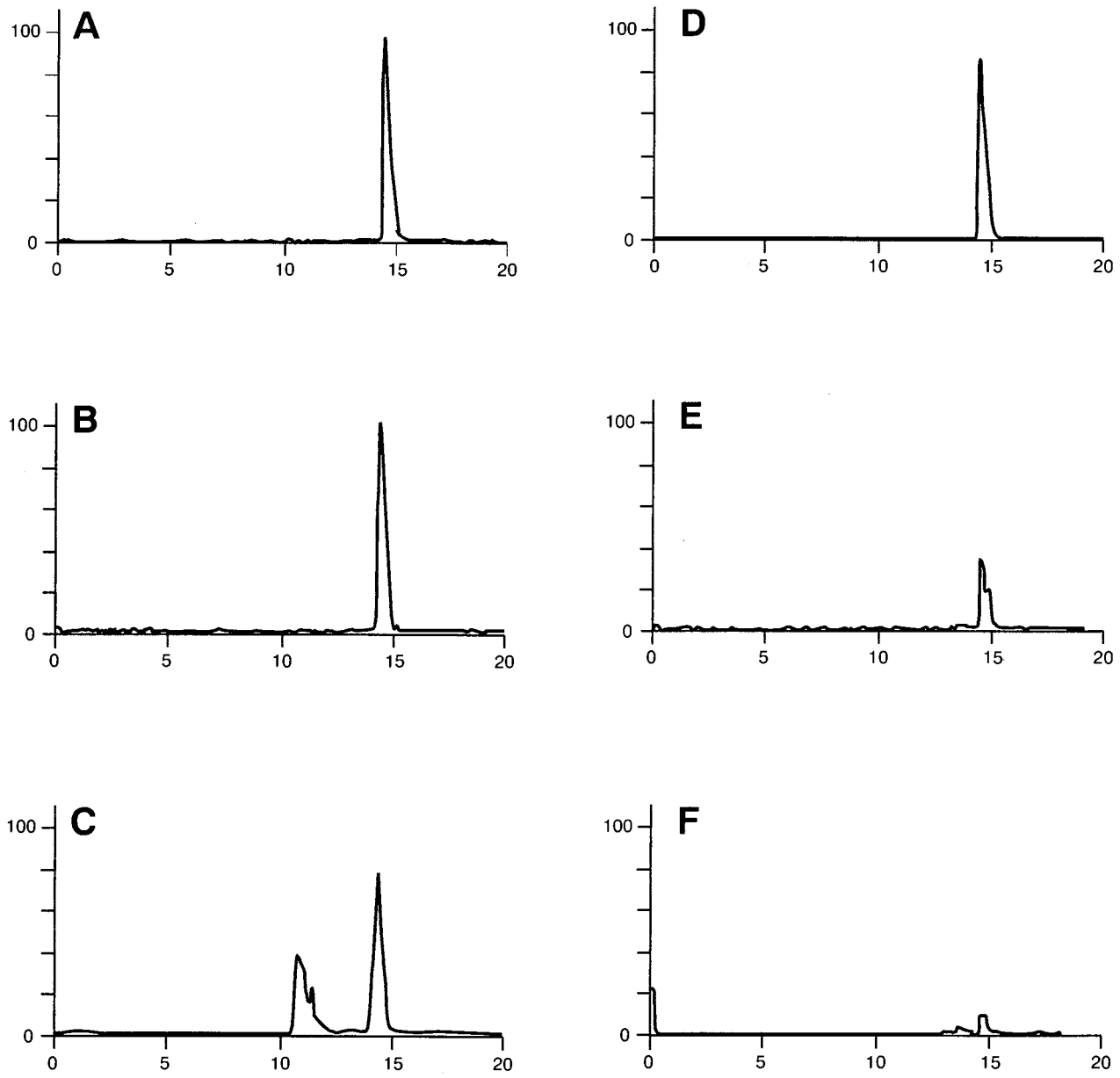

Fig. 3. GPC elution curves for lyophilized IL-2 incubated for 2 weeks at 29°C and 45°C. A and D) pH 7, B and E) pH 6, C and F) pH 5.

small but significant enhancement in stability. GPC results (not shown) indicate that the protein remains essentially monomeric with no aggregate formation at both pH values even after 8 weeks of incubation at 45°C. Additionally, SDS-PAGE analysis (Figure 6) indicates that a slight increase in covalent cross-linking occurs at pH 5 relative to the pH 4 samples. However, even after 8 weeks of incubation the difference is minimal. Further, the similar stability observed in the pH 5 samples in the MES and homopipes buffers indicates that the change in buffer has an insignificant effect on the stability.

A third study was designed to examine the pH dependence of the stability of lyophilized IL-2 at pH values from 4.5 to 1.5. An overlapping series of pH buffers was used: pH 4.5 to 3.5, 10 mM homopipes; pH 3.5 to 1.9, 20 mM sodium citrate; pH 2.5 to 1.5, 20 mM sodium phosphate. Samples were lyophilized as described above and incubated at 45°C for various periods. Table III shows the correlation coefficients for the samples lyophilized from the various buffers at low pH. All samples show r values in the 0.91–0.93 range although there is a slight decrease at the lowest pH values. The results of turbidity measurements are shown in Table IV. Across the pH range examined in this experiment, no significant increases in turbidity were observed. Similar results were observed with GPC (data not shown) which showed no measurable loss in soluble protein due to aggregation. However, a slight increase in formation of covalently linked aggregates is observed at the lowest pH samples (2.5–1.5) using SDS-PAGE (Figure 7).

Effect of Excipients on pH Dependence

An additional experiment was designed to examine the

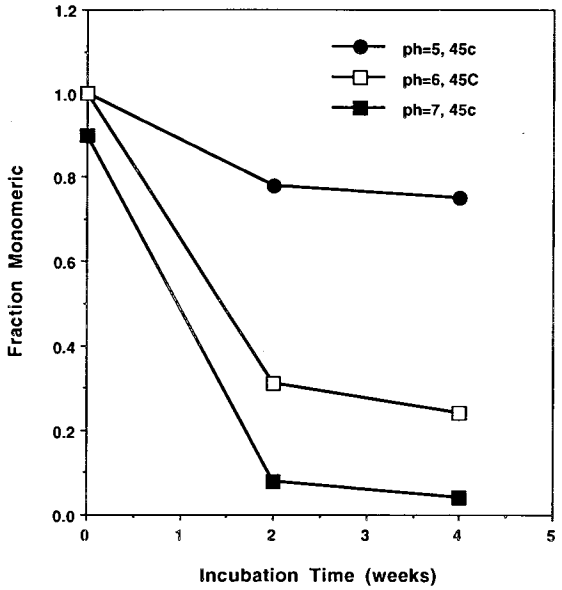

Fig. 4. Fraction of monomeric IL-2 remaining after various incubation times at 45°C as estimated from the GPC elution curves.

pH profile of IL-2 stability in the presence of excipients. It is possible that in the presence of a stabilizer the observed pH stability profile will become degenerate. That is, a stabilizer may effectively eliminate the observed stability enhancement at lower pH. Thus, IL-2 was lyophilized at the observed extremes of stability, pH 7 (MES buffer) and pH 4 (homopipes buffer), in the presence of 5.0% mannitol or 0.5% sucrose, two commonly employed excipients in lyophilized protein formulations. The weight ration of sucrose to protein in this formulation is approximately 6:1. The higher mannitol concentration was used to ensure its crystallization. Lyophilization in the presence of mannitol results in a dried state conformation similar to that observed in the absence of excipients. In contrast, lyophilizing with sucrose results in spectra (r values of ~0.90) more similar to the aqueous spectra for both the high and low pH solutions.

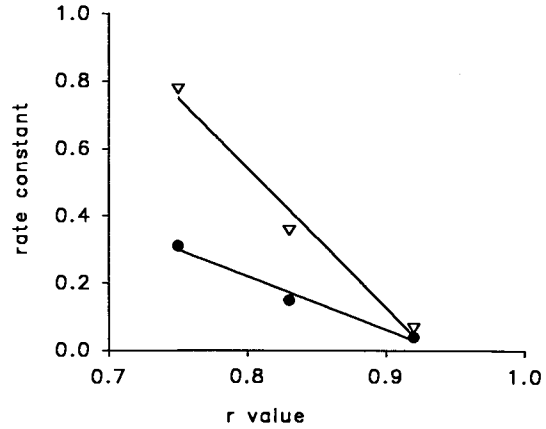

Fig. 5. Apparent first order rate constants for loss of monomeric proteins plotted against the spectral correlation coefficients in the pH range 5 to 7 and incubated at 29°C (●) and 45°C (▽). Lines are best fits from linear regression analysis (r = 0.98 for both temperatures).

Table III. Spectral Correlation Coefficients for Lyophilized IL-2 for the Various Low pH Solutions

Solution	r value
10 mM Homopipes	
pH 5.0	0.91
pH 4.5	0.93
pH 4.0	0.92
pH 3.5	0.91
20 mM Citrate	
pH 3.5	0.92
pH 2.5	0.92
pH 1.9	0.92
20 mM Phosphate	
pH 2.5	0.92
pH 1.5	0.90

Thus, while the pH 4 spectrum is improved slightly, the pH 7 spectrum is improved dramatically by the sucrose. In the presence of sucrose the pH 4 and 7 spectra are essentially identical, indicating that a stabilizer eliminates the sensitivity to unfolding at pH 7.

Figure 8 shows the fraction of monomeric IL-2 after various periods of incubation at 45°C. At a given pH value, addition of sucrose to the formulation resulted in enhanced stability and mannitol results in decreased stability. In addition, the pH stability profile is similar in the absence and the presence of the two excipients. Similar results were observed using SDS-PAGE analysis (data not shown). The formation of covalently linked aggregates was higher for all of

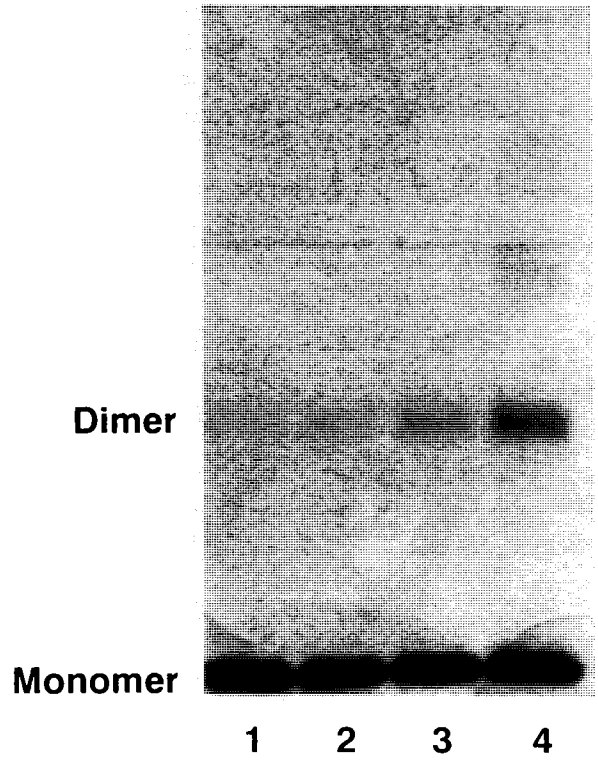

Fig. 6. Non-reduced SDS-gel of lyophilized IL-2 reconstituted after eight weeks of incubation. Lanes 1 and 2 are pH 4 and 5 stored at 29°C and lanes 3 and 4 are at 45°C.

Table IV. Turbidity of Reconstituted IL-2 After Various Incubation Times at 45°C as Measured by the Baseline Intensity in UV Spectra at 330 nm

Buffer pH	Incubation time (wk) 0	2	6
Homopipes			
4.5	0.035	0.031	0.043
4.0	0.013	0.027	0.029
3.5	0.025	0.022	0.029
Citrate			
3.5	0.016	0.045	0.039
2.5	0.014	0.025	0.036
1.9	0.020	0.029	0.029
Phosphate			
2.5	0.020	0.022	0.036
1.5	0.020	0.024	0.041

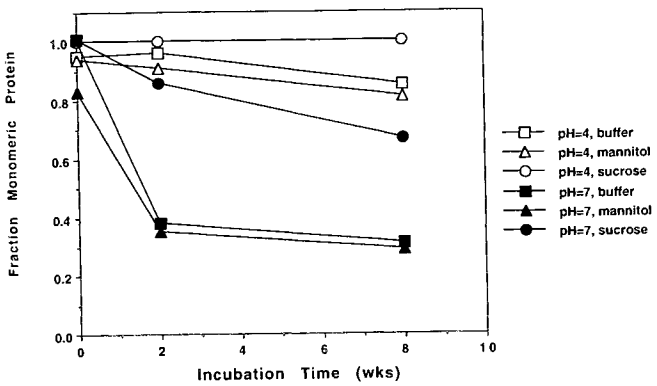

Fig. 8. Fraction of monomeric IL-2 remaining after various incubation times at 45°C.

the pH 7 samples relative to the pH 4 samples. In addition, for this experiment, a biological activity assay was performed for the 4-week samples. These data are shown in Table V. The bioassay results mimic the fraction of soluble protein indicating that the soluble protein retains its bioactivity.

Molecular Weight Dependence of Stabilizers

The next experiment examined the effect of the molecular weight of stabilizers using non-reducing carbohydrates of various sizes. IL-2 samples with the various stabilizers (at 5 mg/ml) were prepared at a concentration of 0.8 mg/ml in 10 mM MES, pH 7. The higher pH was chosen to accelerate degradation upon incubation. Samples were incubated for accelerated stability studies at 45°C, reconstituted and analyzed using UV spectroscopy, SDS-PAGE, and GPC.

The stabilizers used and the glass transition temperatures, Tg, of the dried protein/stabilizer mixtures and the correlation coefficient of the dried protein are listed in Table VI. All samples had similar residual moisture contents in the range of 2.5–3.5%. All samples showed a substantial increase in the correlation coefficient compared to those ly-

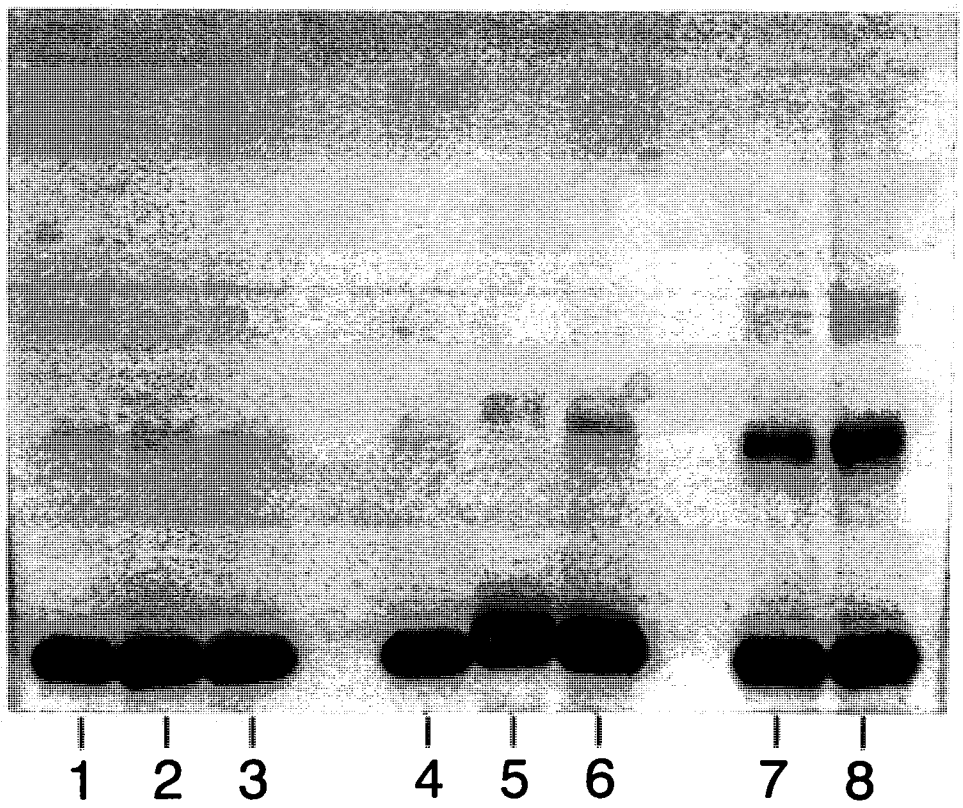

Fig. 7. Non-reduced SDS-polyacrylamide gel of lyophilized IL-2 reconstituted after storage for 6 weeks at 45°C. Lanes 1-3, homopipes pH 4.5, 4, and 3.5, respectively; lanes 4-6, citrate, pH 3.5, 2.5, and 1.9; and lanes 7, 8, phosphate, pH 2.5 and 1.5.

Table V. Biological Activity of Lyophilized IL-2 Samples After 4 Weeks or Incubation at 45°C Expressed as a Percent of Control (Unlyophilized) Sample Activity

	Mean	S.E.*
pH 4, buffer	106	12
pH 4, mannitol	—	—
pH 4, sucrose	118	13
pH 7, buffer	47	4
pH 7, mannitol	28	4
pH 7, sucrose	86	8

* Standard error, n = 6.

ophilized from pH 7 buffer alone (see Table I) indicating that all of the stabilizers tested have some capacity to prevent the dehydration-induced unfolding observed for IL-2 lyophilized from a pH 7 solution. However, significant differences are observed among the various stabilizers. The lower MW stabilizers, up to and including stachyose, all result in the dried protein having a correlation coefficient of about 0.90. The higher MW stabilizers are less effective at inhibiting unfolding and show decreasing r values down to 0.85 for the higher molecular weight dextran (MW = 40,000). Figure 9 shows the second derivative spectra of the extremes, sucrose and dextran 40. Again, while both are clearly improved relative to the unstabilized protein (Figure 1), the sucrose results in a more native-like conformation. As expected, the data in

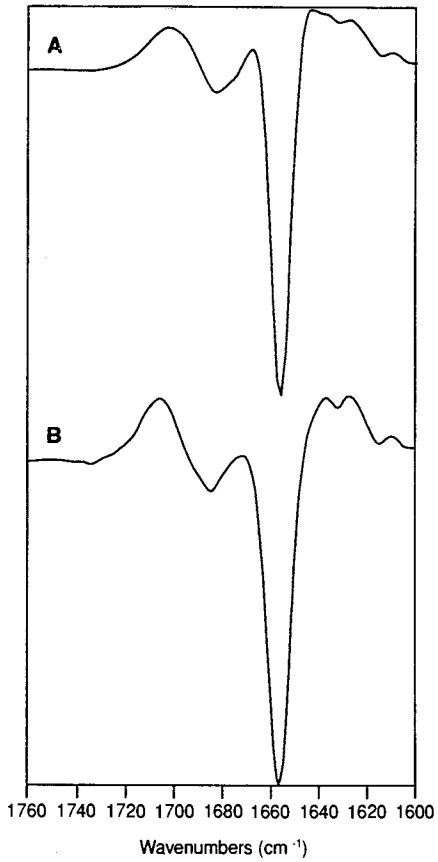

Fig. 9. Second derivative infrared spectra in the amide I region of lyophilized IL-2 (10 mM MES, pH 7) in the presence of A) 0.5% sucrose and B) 0.5% dextran 40,000.

Table VI. Molecular Weights and Glass Transition Temperatures for Various Stabilizers and Correlation Coefficients for IL-2 Lyophilized with Them

	MW	Tg (°C)	r
Glucose	180	4	0.90
Sucrose	342	31	0.90
Trehalose	342	46	0.90
Raffinose	505	47	0.89
Stachyose	667	65	0.90
β-Cyclodextrin	1135	108	0.87
Dextran 10	10,000	91	0.87
Dextran 40	39,100	101	0.85

Table VI show a direct correlation between the Tg and the molecular weight of the stabilizers. The Tg values which range from 4°C for the glucose sample to ~100°C for the higher MW dextran and β-cyclodextrin. Storage at 45°C, thus, will result in some samples being stored below their Tg, some near their Tg, and some above.

Figure 9 illustrates the effect of the stabilizer on the aggregation of IL-2 as measured by turbidity in samples that were incubated for up to 36 weeks at 45°C. For samples reconstituted at zero time, the observed turbidity increases with increasing stabilizer molecular weight. Samples containing dextran 40 show the highest increase in turbidity relative to the pre-lyophilized controls. (All control samples prior to lyophilization gave $A_{330\ nm}$ readings of 0.055 ± 0.01, indicating that preparation of aqueous solutions of IL-2 in the presence of different stabilizers had no effect.) However, if one examines the samples over the time course of incubation it is observed that the increase in turbidity during storage is inversely proportional to the molecular weight and hence the glass transition temperature.

DISCUSSION

The conformation of lyophilized proteins has been examined in detail by Prestrelski *et al.* (8). It was reported that proteins fall into three categories: a) proteins that unfold upon lyophilization and upon reconstitution form irreversibly denatured forms, b) proteins that unfold during lyophilization but refold to form the native conformation upon reconstitution and c) proteins that are resistant to unfolding during lyophilization and thereby remain after reconstitution. This behavior was apparently protein dependent. The pH dependence of the conformation of IL-2 upon lyophilization indicates that this behavior is also dependent on the protein's environment. At pH 5 (and below) IL-2 is resistant to unfolding; at pH 6 unfolding is observed but it is reversible; and, at pH 7, IL-2 unfolds upon lyophilization and aggregates irreversibly.

A comparison of the results of the accelerated stability studies for IL-2 lyophilized from different pH solutions and infrared spectroscopic analyses of the dried proteins indicates that for IL-2 a direct correlation exists between the retention of the native structure upon freeze-drying and increased stability. All other factors being equal, retention of the native (aqueous-like) structure during the freeze-drying process results in a higher level of storage stability. The stability of dried IL-2 lyophilized at pH 6 and 7 (where the

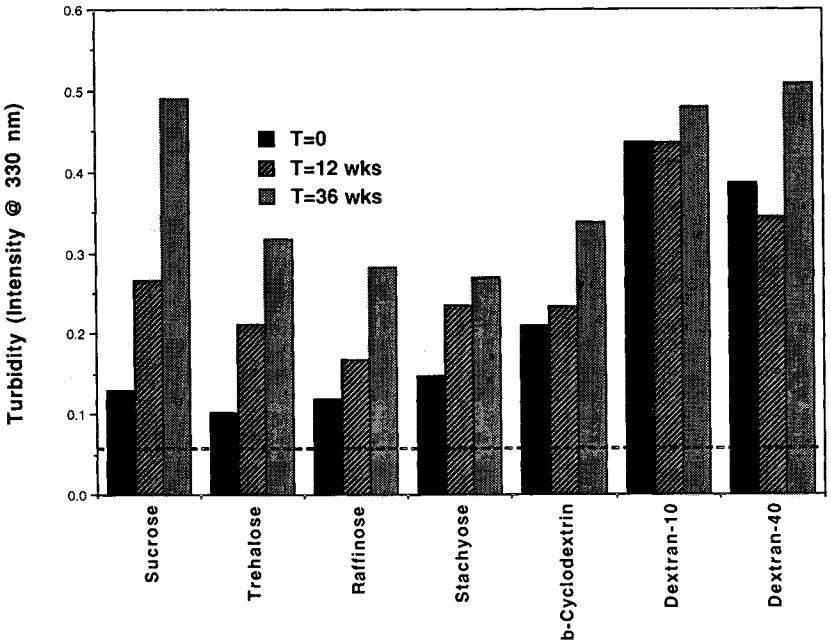

Fig. 10. Bar graph representing the turbidity (absorbance at 330 nm) of the reconstituted IL-2 samples after various periods of incubation at 45°C. (----) control turbidity value.

infrared spectra indicate extensive unfolding upon lyophilization), as examined by aggregate formation and covalent cross-linking, is significantly reduced. At pH values of 5 and below, where the protein shows minimal unfolding upon dehydration, stability is enhanced.

Several mechanisms are plausible to explain the enhanced covalent and non-covalent aggregation observed for the unfolded forms of IL-2. A partially unfolded protein is likely to have smaller barrier to further unfolding. Furthermore, an unfolded protein will have a greater exposure of its side chains exposed leading to greater reactivity for reactive chemical groups. This may in part be the mechanism by which the covalent cross-linking is enhanced in the more unfolded forms of IL-2 observed here. Additionally, unfolded proteins are more likely to interact intermolecularly with other proteins through H-bonding and hydrophobic interaction, leading to aggregate formation. It is difficult to speculate on the nature of hydrophobic interactions in the dehydrated state; however, the observed aggregation may also occur upon rehydration if unfolding takes place upon dehydration and storage.

Stabilizers, including carbohydrates, polyols, amino acids, polymers, and other compounds, are often added to protein formulations for lyophilization to enhance the stability of the dried product. Two schools of thought exist to explain the rationale for choice of different stabilizers. One school claims that effective stabilizers function by raising the glass transition temperature of a given formulation. Effective stabilization is a result of the protein being trapped in a glassy matrix where the viscosity is so high that physical and chemical degradation processes are essentially halted (3,5,16). A second school relates to the "water replacement" hypothesis, which focuses upon the capacity of a stabilizer to replace water bound to the protein upon dehydration (17,18). Stabilizers that can replace bound water prevent lyophilization-induced unfolding, thereby stabilizing the protein.

The experiments that examined the molecular weight dependence of stabilizers provide an insight into the relative degrees of importance of water substitution and a high glass transition temperature in achieving optimal stability. The data suggest that stabilizers that act as water substitutes are necessary during the lyophilization process but that a high glass transition temperature is likely most important for long-term storage stability. That is, compounds that act as water substitutes are necessary to prevent lyophilization-induced unfolding. This is evident from the data for samples reconstituted immediately after lyophilization. The stabilizers with the lowest molecular weight showed the greatest stabilization toward aggregate formation. However, the data for samples incubated at elevated temperature for extended periods suggest that storage stability (independent of stability during lyophilization) is optimal with higher molecular weight and higher glass transition temperature compounds. An optimal stabilizer, or possibly a mixture of stabilizers, that takes into account both the lyophilization process and storage stability, then, should have capacity to prevent lyophilization-induced unfolding as well as foster a high glass transition temperature. At 12 and 36 weeks, raffinose and stachyose, respectively, provide the highest level of stability among the compounds examined here.

While raffinose and/or stachyose appear to have the greatest stabilizing capacity for IL-2 lyophilized at pH 7 and stored at 45°C, it is insightful to ask what would be predicted for other conditions. For example, at pH 4, it is observed that IL-2 has a lesser tendency to unfold. This may shift the optimum toward those stabilizers with high Tg values since water substitution capacity, necessary to prevent unfolding, is less important at low pH. Conversely, storing the lyophilized preparation at lower temperatures would shift the optimum toward the lower MW compounds because they would then be below their Tg temperatures but have greater capacity as water substitutes.

The data reported in this paper represent the first examination of the relationship between dried-state protein structure and long-term stability. Thus, the mechanisms proposed above must be regarded as speculative. It has been demonstrated, at least for IL-2, that retention of the native conformation during lyophilization is an important determinant of stability. It is expected that this will hold true for proteins whose predominant degradation pathways are conformational in nature. Decomposition of proteins during storage can occur through chemical reactions as well. Thus, in these cases, the conformation of the dehydrated protein may not be an effective indicator of stability in proteins whose predominant degradation pathways are chemically related. However, several reports have shown that chemical instability may be related to the conformation in cases where in a folded protein a reactive group is buried or conformationally constrained but becomes exposed or more flexible upon unfolding (19,20). Thus, the conformation of the dehydrated protein may indirectly correlate with stability against chemical degradation. Clearly, more research is necessary to fully understand the complex pathways which lead to protein degradation in the dried state. The results presented here demonstrate that Fourier-transform infrared spectroscopic analysis of the conformation of the dried proteins can be an effective tool for increasing our understanding of the stability of dried proteins and further, for optimizing the lyophilization conditions for maximal stability.

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