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Replacing succinate with glycolate buffer improves the stability of lyophilized interferon-γ

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Abstract

Lyophilization is commonly used to dry protein pharmaceuticals to enhance their shelf life. During the freezing step of this process, significant events (e.g. pH shifting) can occur in the uncrystallized, liquid portion which influence the stability of the product. Herein, we present evidence of such an effect and the impact on the quality of recombinant human interferon-γ (IFN-γ) lyophilized from mannitol-containing succinate buffer at pH 5. In the frozen matrix, we hypothesize that the monosodium form of succinic acid crystallized, as evidenced by electrical resistance data, affecting the buffer system's ability to maintain pH, as probed by Fourier-transform infrared (FT-IR) spectroscopy. The latter indicated that the succinate buffer lyophilized from aqueous solution at pH 5 exhibited an ionization state corresponding to that of some 1–2 pH units lower. In exploring the implications for stability, we found that IFN-γ exhibited a marked bioactivity loss during aqueous incubation at pH 3 compared with pH 5. This loss correlated with (reversible) unfolding of the IFN-γ molecule at low pH, as determined by both FT-IR spectroscopy and circular dichroism. We also examined the stability of IFN-γ following lyophilization from pH 5 in two different buffer systems, succinate and glycolate. The latter, which appeared to minimize the freeze-induced pH shifting, exhibited superior solid-state stability upon 4-week incubation at 25°C. Both samples had a similar cake structure (based on X-ray diffraction and differential scanning calorimetry) and had the same residual moisture content. The data suggest that the difference in stability was a consequence of the freeze-induced pH shifting in the succinate buffer system, resulting in a more perturbed (solid-state) structure for IFN-γ. This is consistent with our FT-IR spectroscopic analysis of the lyophilized protein.

Keywords: Circular dichroism; Fourier-transform infrared spectroscopy; Interferon-γ; Lyophilization; pH; Protein stability

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1. Introduction

The stability of many protein pharmaceuticals is strongly dependent upon the pH. Many deleterious pathways are pH-dependent; an unfavorable pH can cause protein to degrade (Cleland et al., 1993). Thus, in formulating therapeutic proteins, it is important to select a buffer in which the formulation can be steadily maintained at optimal pH.

The solubility and ionization constant of a buffering agent are often affected by temperature. The effects leading to a change in solution pH have been documented for various buffers under nonfreezing conditions (Fasman, 1989). In contrast, literature on the pH change occurring in a buffer system during freezing is relatively limited (Keilin and Hartree, 1949; Van den Berg and Rose, 1959; Larsen, 1973; Orri and Morita, 1977).

Freezing is the first operational step in lyophilization, a process commonly used to prepare dry protein pharmaceuticals. When freezing a protein solution, supercooling, ice nuclei formation, ice crystal growth and glass formation generally occur. During the phase transition of water into ice, protein and buffer components become progressively concentrated in the uncrystallized, liquid portion of the system. It has been suggested that such concentration could influence the pH of the liquid phase and result in significant protein deterioration (Pikal, 1990). This scenario has been invoked to interpret the degradation of proteins and enzymes caused by the freeze–thaw operation (Finn, 1932; Williams-Smith, 1977). In this report, we demonstrate this effect and its influence on the quality of lyophilized recombinant human interferon-γ (IFN-γ). We found that minimizing the pH change occurring in the frozen state during lyophilization improves IFN-γ stability.

2. Materials and methods

2.1. Materials

Recombinant DNA-derived IFN-γ (MW = 16 440, pI = 9.9) was purified from *Escherichia coli* extracts using a series of chromatographic methods (Rinderknecht and Burton, 1985). All chemicals were reagent grade and obtained from commercial sources.

2.2. Protein lyophilization

For solid-state stability studies, IFN-γ was lyophilized from an aqueous solution consisting of 0.2 mg/ml protein, 5 mM succinate (or glycolate) buffer at pH 5.0, 220 mM mannitol and 0.01% (w/v) polysorbate 20. This is the same formulation as that of the marketed liquid product. For the solid-state Fourier-transform infrared (FT-IR) spectroscopic studies, the protein was lyophilized from 0.78 mg/ml in water (excipient-free) at various pH values (see below), achieved with addition of 0.1 N HCl or NaOH. For all experiments, samples were titrated to an accuracy of 0.1 pH unit.

Formulations (1.0 ml) were sterile-filtered (0.22 μm pore size, Millipore, USA) and aseptically filled into 3-cc glass vials. Filled vials were loaded into a 20-ft^2 lyophilizer (Leybold model GT20, Germany) prechilled to a temperature of -55°C. All vials were held in the dryer for 5 h before proceeding with lyophilization. Lyophilization was conducted at a pressure of 250 mTorr and a primary shelf temperature of -10°C for 18 h, followed by secondary drying at 18°C for 7 h. After lyophilization, all vials were stoppered at this lyophilizer pressure. Residual moisture contents were measured by Karl Fischer titration, and lyophilized cake structures were investigated by X-ray diffraction (XRD) (to determine the degree of crystallinity) and differential scanning calorimetry (DSC) (to determine the glass transition temperature), as described previously (Hsu et al., 1995).

2.3. Electrical thermal analysis

Electrical thermal analysis was performed to observe any events (e.g. crystallization) occurring in the frozen solution leading to changes in resistance (Her et al., 1994). Each solution tested (0.5 ml) was subjected to controlled temperature in cooling, warming and hold steps in a programmable rate freezer (Planer model Kryo 10/16

series III, UK) controlled digitally (Planer model K10–22 series III, UK). Sample temperature and electrical resistance were monitored by a probe connected to a digital resistance meter (model LMS 10, Hieronetics, USA). The signals were transmitted to a PC-compatible computer running Hieronetics LPS software for data analysis.

Samples were initially held at 5°C for 15 min to ensure temperature equilibration. The sample was then cooled at 5°C/min to −70°C. After a 15-min hold, warming was carried out at 0.5°C/min to 10°C. Some samples were heated from −70 to −20°C, cooled a second time to −70°C, and then warmed a second time, to 10°C. Again, there was a 15-min hold between each step to ensure temperature equilibration.

2.4. Circular dichroism (CD) measurements

Circular dichroism (Aviv 60DS spectropolarimeter, USA) was performed at room temperature to determine the effect of pH on the secondary and tertiary structure of IFN-γ in the succinate solution. IFN-γ was concentrated by ultrafiltration from 0.2 to 1.0 mg/ml (the succinate and mannitol concentrations were not affected). Spectra were obtained in triplicate in the far-UV (200–250 nm) and near-UV (250–325 nm) regions with a bandwidth of 1.0 nm and a sampling interval of 0.2 nm. The CD data were expressed as mean residue ellipticity using 117.6 g/mol as the mean residue weight of IFN-γ.

2.5. FT-IR spectroscopy

All measurements were performed on an ATI-Mattson galaxy 5022 IR spectrophotometer (Mattson Instruments, USA) as previously described (Costantino et al., 1996a). For measuring protein spectra, mannitol and buffer were removed from the IFN-γ by dialysis (6000–8000 molecular weight cutoff; Spectra-Por, USA) in order to simplify the vibrational spectrum of the formulation. This solution (0.78 mg/ml protein) was then concentrated to 20 mg/ml using a microconcentrator (Amicon, USA) and aqueous spectra were obtained using a 15-μm spacer in a liquid cell equipped with CaF_2 windows. The pH was varied from 3 to 6, as indicated in the text. The water signal was subtracted to obtain the pure protein spectrum (Griebenow and Klibanov, 1995). In order to obtain solid-state spectra, 4-ml aliquots of the dialyzed IFN-γ solution were filled in 10-cc glass vials, pH adjusted to various levels from 3 to 5, and lyophilized as described above (all pH-adjustments were accomplished by addition of HCl or NaOH). The spectra of these lyophilized powders were obtained by transmis-

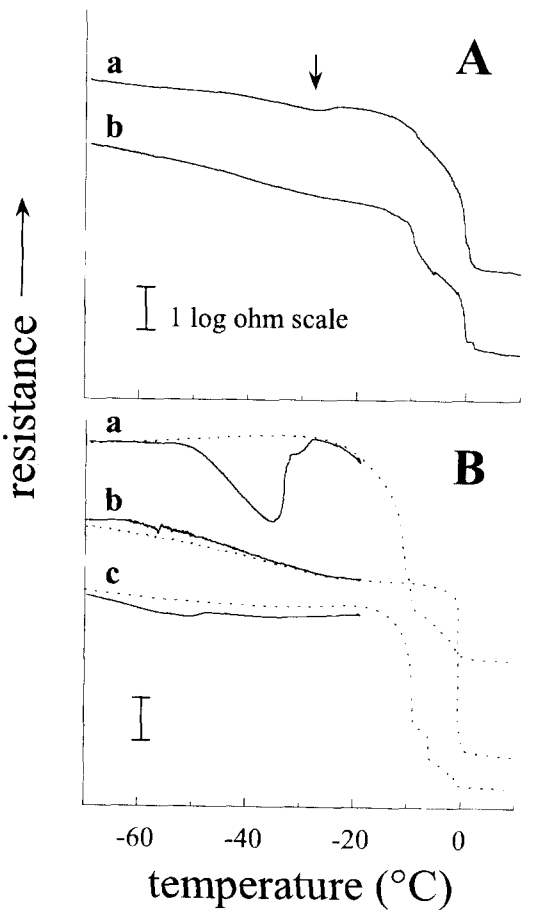

Fig. 1. Electrical thermal analysis of various aqueous solutions. (A) Thermograms of (a) 5 mM succinate buffer (pH 5) and (b) 5 mM glycolate buffer (pH 5). The data are shown for the warming of the frozen solution from −70 to +10°C (data for cooling not shown). (B) Thermograms of (a) 100 mM succinate buffer (pH 5), (b) 100 mM succinic acid and (c) 100 mM disodium succinate. The data are shown for the warming of the frozen solution from −70 to −20°C (——), and for a second warming to 10°C (- - -) (data for cooling not shown).

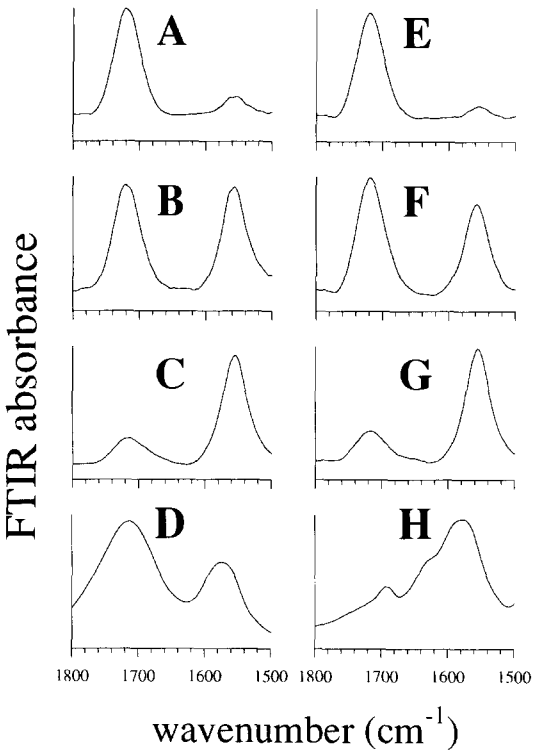

Fig. 2. IR absorption of succinate and glycolate buffers. Spectra are shown for succinate buffer in aqueous solution (100 mM) at (A) pH 3, (B) pH 4 and (C) pH 5, and for (D) solid lyophilized from an aqueous solution (5 mM, pH 5). Also shown are spectra for glycolate buffer in aqueous solution (100 mM) at (E) pH 3, (F) pH 4 and (G) pH 5, and for (H) solid lyophilized from an aqueous solution (5 mM, pH 5).

sion IR spectroscopy using an ATI-Mattson Quantum infrared microscope (Costantino et al., 1996a).

FT-IR was employed to study the succinate and glycolate buffers in a similar fashion. Aqueous (100 mM; pH 3, 4 or 5) spectra were collected using a 15-μm spacer in a liquid cell equipped with CaF_2 windows. The pure buffer's aqueous spectrum was then obtained following subtraction of water. Spectra were also obtained for powders freeze-dried from 5 mM succinate or glycolate (pH 5), using the FT-IR microscope (described above).

2.6. Secondary structure determination from FT-IR spectra

To estimate the secondary structure of IFN-γ, we performed Gaussian curve-fitting of the amide

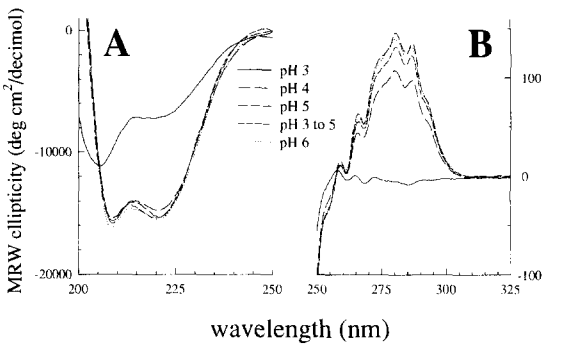

Fig. 3. CD spectra of aqueous IFN-γ in the (A) far- and (B) near-UV range. The aqueous solution contained 1 mg/ml protein, 220 mM mannitol, 0.01% (w/v) polysorbate 20 and 5 mM sodium succinate, pH as indicated.

III region, which is sensitive to changes in protein structure (Griebenow and Klibanov, 1995; Costantino et al., 1995, 1996a). Spectral peaks were located by second derivatization (smoothed with a 11-point function) using OMNIC 1.2 software (Nicolet, USA). For aqueous and solid samples, the number of peaks and their locations were found to be very similar. Gaussian curve-fitting (of original, non-smoothed spectra) was accomplished with GRAMS/386 software (Galactic Industries, USA) using band locations

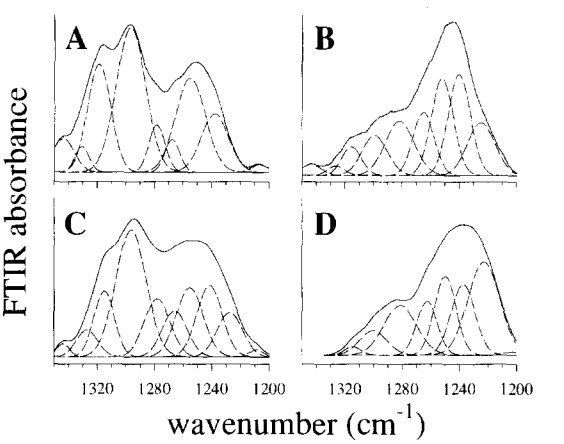

Fig. 4. IR spectra of IFN-γ in the amide III region and their Gaussian curve-fitting: (A) aqueous solution at pH 6; (B) aqueous solution at pH 3; (C) solid lyophilized from pH 6; (D) solid lyophilized from pH 3. The solid and dotted curves represent the superimposed original spectra and curve-fit, and the dashed curves are individual Gaussian bands.

obtained from second derivatization as a starting point (Costantino et al., 1996a). The percentages of secondary structure were based on areas of individual bands as a fraction of the total area in the amide III region. Discussion of the assignment of bands in the amide III region to elements of secondary structure can be found elsewhere (Fu et al., 1994; Griebenow and Klibanov, 1995; Costantino et al., 1996a).

2.7. Product stability

For the solution stability studies, IFN-γ was formulated at 1 mg/ml protein in 5 mM succinate buffer, 220 mM mannitol, 0.01% (w/v) polysorbate 20, at various pH values, as indicated. For the solid-state stability studies, we used two formulations of IFN-γ, one in succinate and the other glycolate, both lyophilized from pH 5 (as measured in aqueous solution at room temperature prior to freezing) as described above. The incubation time was 4 weeks at 2–8°C and 25°C for the aqueous and solid protein, respectively. The latter sample was reconstituted with Water for Injection (USP), for testing.

The total protein concentration was measured by UV absorption at 280 nm using an extinction coefficient of 0.75×10^{-4} M^{-1} cm^{-1}. The biological activity of IFN-γ was measured by two methods: (i) enzyme-linked immunosorbent assay (ELISA) to yield immunoreactive protein and (ii) bioassay, i.e. the ability to protect A549 cells against infection by an encephalomyocarditis virus (Rinderknecht and Burton, 1985). Both techniques gave similar results in terms of active protein present.

The level of soluble, non-sodium dodecyl sulfate (SDS)-dissociable protein aggregates was measured by size exclusion chromatography (SEC) using a Spherogel TSK3000SW column (Tosohaas, Japan), with UV detection at 214 nm. The protein was eluted isocratically with 0.2 M sodium phosphate and 0.1% SDS (pH 6.8) at a flow rate of 0.5 ml/min. Silver-stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions was performed to detect protein fragmentation and to confirm the presence of non-SDS-dissociable protein aggregates. Deamidation of IFN-γ was detected by cation exchange chromatography using a TSK SP-5PW column (Tosohaas), with protein detected by UV at 214 nm. The protein was eluted with 0.3 M NaCl and 50 mM sodium phosphate (pH 7.0) at a flow rate of 1 ml/min.

3. Results and discussion

IFN-γ, an important therapeutic protein used to treat chronic granulomatous disease, is marketed as an aqueous solution containing 5 mM succinate as buffer (consisting of succinic acid, mono- and disodium succinate) (pH 5), mannitol and polysorbate 20. We tested the feasibility of preparing a freeze-dried solid from this formulation. First, we tested the electrical thermal behavior of the succinate buffer system upon freezing and warming. This technique monitors the electrical resistance of the frozen matrix as a function of temperature, allowing for identification of changes in physical states leading to changes in resistance, as described by Her et al. (1994).

Our data show that at approximately −30°C, 5 mM succinate buffer exhibits a transition, which we hypothesize to be crystallization occurring in the frozen solid (Fig. 1A, curve a). In order to examine this phenomenon more closely, we also tested a 100-mM solution of succinate buffer (pH 5). During the first warming, a transition was clearly seen (Fig. 1B, solid line of curve a). Subsequent cooling and rewarming (dotted line) showed the absence of transition, consistent with our view that an (irreversible) transition from the amorphous to crystalline state occurred during the first warming. In addition, we examined solutions of the pure acid and base (disodium salt) forms (Fig. 1B, curves b and c, respectively). Since these two solutions did not exhibit behavior indicating a significant change in their physical state, we conclude that it is the monosodium form of succinate which is prone to crystallize in the solution frozen from pH 5.

Table 1
Results of Gaussian curve-fitting of IR spectra in the amide III region for aqueous and lyophilized IFN-γ [a]

Sample	Band position (cm^{-1})		Area (%)	Assignment
	2nd derivative	Gaussian curve-fitting		
Aqueous solution (pH 6)	1328 ± 1	1328 ± 4	3 ± 1	α-helix
	1318 ± 1	1319 ± 1	18 ± 2	α-helix
	1296 ± 1	1297 ± 1	34 ± 1	α-helix
	1280 ± 1	1279 ± 1	5 ± 1	Unordered
	1267 ± 1	1268 ± 1	4 ± 1	Unordered
	1251 ± 2	1255 ± 1	24 ± 1	Extended chain
	1238 ± 1	1238 ± 1	12 ± 2	Extended chain
	1225 ± 3	1224 ± 1	<1	β-sheet
Aqueous solution (pH 3.0)	1326 ± 1	1328 ± 2	2 ± 1	α-helix
	1317 ± 1	1315 ± 1	7 ± 2	α-helix
	1297 ± 1	1298 ± 1	10 ± 1	α-helix
	1285 ± 2	1281 ± 1	15 ± 1	Unordered
	1266 ± 1	1265 ± 1	11 ± 2	Unordered
	1249 ± 1	1251 ± 2	24 ± 5	Extended chain
	1240 ± 1	1239 ± 2	20 ± 2	Extended chain
	1225 ± 6	1223 ± 2	11 ± 2	β-sheet
Lyophilized from pH 6	1326 ± 1	1324 ± 4	3 ± 1	α-helix
	1317 ± 1	1314 ± 2	12 ± 1	α-helix
	1294 ± 1	1294 ± 1	26 ± 3	α-helix
	1279 ± 1	1278 ± 1	10 ± 2	Unordered
	1266 ± 2	1267 ± 1	9 ± 2	Unordered
	1254 ± 1	1255 ± 1	16 ± 2	Extended chain
	1240 ± 2	1240 ± 1	15 ± 1	Extended chain
	1224 ± 3	1226 ± 1	9 ± 1	β-sheet
Lyophilized from pH 3	1314 ± 3	1315 ± 1	2 ± 1	α-helix
	1298 ± 2	1300 ± 1	9 ± 4	α-helix
	1283 ± 3	1283 ± 2	16 ± 2	Unordered
	1263 ± 1	1264 ± 3	12 ± 1	Unordered
	1251 ± 3	1251 ± 1	17 ± 2	Extended chain
	1243 ± 3	1239 ± 2	17 ± 5	Extended chain
	1225 ± 5	1225 ± 3	27 ± 2	β-sheet

[a]All data are the average and S.D. of at least four independent determinations.

Since such crystallization will probably affect the succinic buffer system's capacity to maintain pH, it was desirable to examine another buffer system. To this end, we studied the electrical thermal behavior of 5 mM glycolate buffer (pH 5) and found no evidence of such a transition in the frozen sample (Fig. 1A, curve b). This finding was further confirmed with 100 mM glycolate buffer, pH 5 (data not shown).

Based on these results, we hypothesize that glycolate buffer has a greater ability to maintain pH in the frozen state than succinate. It follows then that the pH in the lyophilized solid (i.e. the ionization state) should be affected accordingly (assuming that drying does not preferentially volatilize acidic or basic species). One approach that can be used to investigate this is FT-IR spectroscopy, which can be applied to both the aqueous and lyophilized states (Costantino et al., 1996b). Both succinate and glycolate species contain the carboxyl group, which undergoes an ionization-sensitive stretching vibration.

We obtained FT-IR spectra of succinate and glycolate in aqueous solution over the pH range

Table 2
Effect of pH on the secondary structure of IFN-γ in the aqueous and lyophilized states as determined by Gaussian curve-fitting their IR spectra in the amide III region[a]

Sample	pH	α-Helix (%)	β-Sheet (%)	Unordered (%)
Aqueous solution[b]	3	19 ± 2	11 ± 2	70 ± 3
	4	47 ± 2	5 ± 1	48 ± 1
	5	55 ± 1	1 ± 1	44 ± 2
	6	55 ± 2	0	45 ± 1
Lyophilized powder	3	11 ± 4	27 ± 2	62 ± 4
	4	37 ± 3	11 ± 1	52 ± 3
	5	38 ± 3	10 ± 1	52 ± 3
	6	41 ± 3	9 ± 1	50 ± 4

[a]All data are the average and S.D. of at least four independent determinations.
[b]Data are shown for protein in buffer-free media; at a given pH, the protein spectra were essentially identical in the presence of 5 mM succinate or glycolate.

3–5, and compared these data with those obtained for powders lyophilized from solutions buffered at pH 5 (Fig. 2). We focused on peaks occurring at approximately 1720 and 1560 cm^{-1} wavenumbers, arising from stretching of C=O (COOH) and C=O (COO^-), respectively, as assigned from the literature (Venyaminov and Kalnin, 1990). The pH can be followed by inspecting the relative intensity of these two peaks. For example, as either buffer system was varied from pH 3 to 5 in aqueous solution, the band arising from ionized species exhibited a relative decrease, as expected (Fig. 2A–C for succinate and Fig. 2E–G for glycolate). However, the spectrum of the succinate powder lyophilized from an aqueous solution of pH 5 (Fig. 2D) exhibited an ionization state corresponding to the aqueous solution between pH 3 and 4 (Fig. 2A and B). In contrast, the spectrum of glycolate lyophilized from pH 5 (Fig. 2H), although evidencing some band shifts and band-splitting, was much closer to that of the (starting) aqueous solution (pH 5) (Fig. 2G), compared with the other pH values, suggesting that ionization state was maintained.

Both the electrical thermal and spectral data are consistent with a pH shift occurring in the frozen succinate buffer formulation. Furthermore, this effect is not apparent in these data for glycolate. Accordingly, replacing succinate with glycolate in the IFN-γ formulation would help to avoid any pH shifting.

What are the implications of a pH shift for IFN-γ stability? To address this question, we first examined how the pH affects the protein structure in aqueous solution. Thus, we measured the protein's CD spectra in the far- and near-UV range (Fig. 3). The data indicate that the structure of IFN-γ is slightly perturbed at pH 4 and highly perturbed at pH 3, both compared to either pH 5 or 6 (all aqueous solutions contained succinate buffer). For example, at pH 3 the far- and near-UV CD spectra are relatively flat, indicating a loss of ordered secondary and tertiary structure, respectively. This may be related to the dissociation of the IFN-γ dimer, which occurs below pH 3.5 (Hsu and Arakawa, 1985).

IFN-γ that had been in the pH 3 succinate buffer at room temperature for 5 h was titrated with a 0.1-N NaOH solution to pH 5 and its CD spectrum was measured. Both near- and far-UV spectra for the titrated IFN-γ protein were found to be essentially identical to the spectra for the protein initially formulated at pH 5 (Fig. 3). These results suggest that the pH-induced unfolding was reversible. Our observations agree with those reported by Arakawa et al. (1987) for IFN-γ formulated in ammonium acetate and NaCl.

We wished to quantitate this unfolding in terms of IFN-γ secondary structure. The far-UV CD spectra can be used to obtain such information (Johnson, 1990), although the data-fitting to obtain secondary structure may be problematic

(Shire et al., 1991). Therefore, we employed another, more versatile approach, namely FT-IR spectroscopy and Gaussian curve-fitting of the amide III region to estimate the secondary structure (Griebenow and Klibanov, 1995; Costantino et al., 1995). This approach can be applied to both liquids and solids (facilitating the study of lyophilized IFN-γ as discussed below).

FT-IR spectra of IFN-γ in aqueous solution were obtained spanning the range of pH from 3 to 6 (no excipients or buffers were present so that the protein's behavior could be observed solely as a function of the pH). Some significant differences were observed in the absorption in the amide III region (compare Fig. 4A and B). In order to quantitate this difference in terms of secondary structure, we identified the peaks in the spectra by second derivatization, used this information to perform Gaussian curve-fitting, and assigned individual peaks to elements of secondary structure.

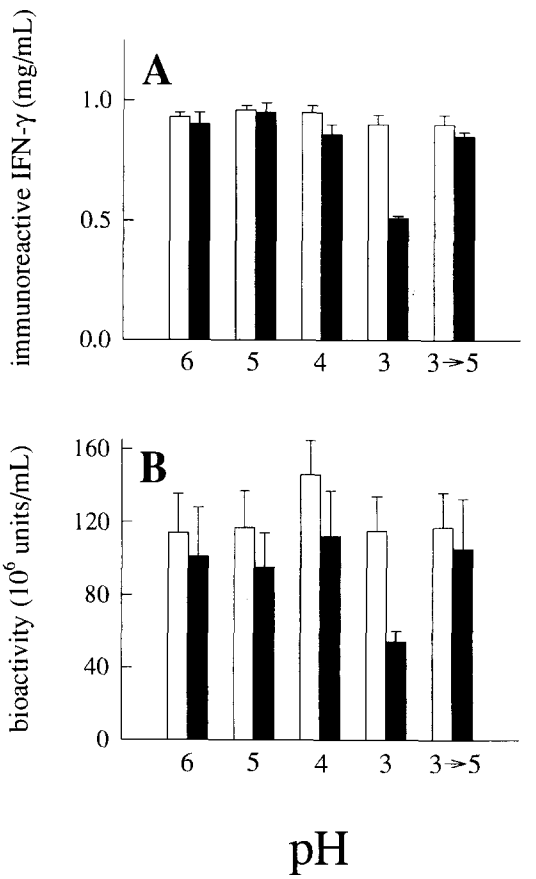

Fig. 5. The stability of IFN-γ in an aqueous solution at various pH values as determined by (A) ELISA and (B) bioassay. The open bars represent the initial value (no incubation) and the solid bar represents data from a 4-week incubation at 5°C. The aqueous solution contained 1.0 mg/ml protein, 220 mM mannitol, 0.01% (w/v) polysorbate 20 and 5 mM sodium succinate, pH as indicated. The last bar on the right represents the sample adjusted from pH 5 to 3, and then re-adjusted back to pH 5 again. All data are given as average and S.D.

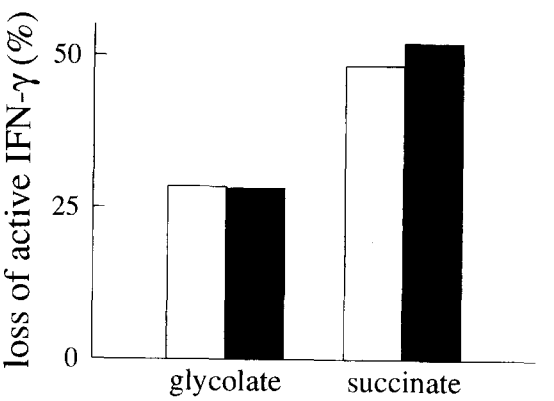

Fig. 6. The solid-state stability of IFN-γ lyophilized from either 5 mM sodium succinate or glycolate (pH 5). Both formulations contained 0.2 mg/ml protein, 220 mM mannitol and 0.01% (w/v) polysorbate 20. The data represent the loss of active protein determined by ELISA (open bars) and bioassay (solid bars) following a 4-week incubation at 25°C.

The results of the curve-fit for IFN-γ in aqueous solution at pH 6 and pH 3 are given in Table 1. Although the two spectra have their peaks at similar wavenumber locations, the relative areas of the peaks show an alteration of secondary structure. Based on secondary structural assignments, we found that IFN-γ in aqueous solution at pH 6 was composed of approximately 55% α-helix with no β-sheets (the data are summarized in Table 2). The remaining structure was unordered. This is consistent with the native conformation of IFN-γ which is about 62% α-helix, no β-sheets, as elucidated from the X-ray crystal structure (Ealick et al., 1991). As the pH was dropped, no change in secondary structure was detected at pH 5, and only a slight change was detected at pH 4 (Table 2). However, at pH 3 there was a major structural alteration, largely from α-helix to unordered structure.

Next, we examined the effect of the pH on IFN-γ stability during aqueous incubation. We followed the bioactivity of IFN-γ during incubation in aqueous sodium succinate at 2–8°C from pH 3 to 5 by ELISA and bioassay (Fig. 5A and B). The initial performance of the protein was independent of the pH, i.e. the samples did not immediately lose potency upon the pH adjustment. However, over 4 weeks, it was seen that the stability of IFN-γ decreased significantly at pH 3 (as evidenced by some 40% loss of IFN-γ activity) compared with the higher pH values. In addition, we found that if the pH was re-adjusted from pH 3 up to pH 5 prior to incubation, no activity loss was observed.

These data indicate that IFN-γ loses its native structure in aqueous solution at pH 3 where it exhibits decreased stability. It is likely that the stability of the lyophilized protein will similarly be influenced by the pH of the solution from which it was frozen and subsequently dried. For example, the solid-state aggregation of various pharmaceutical proteins depends on the pH in solution prior to lyophilization (Liu et al., 1991; Costantino et al., 1994, 1995). To test this, we lyophilized from pH 5 in two different buffer systems, that of glycolate and succinate. Both lyophilized formulations exhibited essentially identical XRD patterns and DSC thermograms (data not shown). Furthermore, their residual water contents were identical, 2% (w/w). However, the two samples differed in their ability to maintain pH during the freezing stage of lyophilization. Therefore, it was interesting to compare their solid-state stability.

To this end, the stability of the two formulations was examined following 4 weeks at 25°C (Fig. 6). The biological activity of IFN-γ decreased much faster in the lyophilized succinate formulation than in the lyophilized glycolate formulation as measured by ELISA and the bioassay. To investigate whether this was the result of aggregation, we examined both samples by SEC. Neither sample exhibited the formation of insoluble aggregates (as evidenced by total recovery of OD at 280 nm). Both samples exhibited formation of soluble, non-SDS-dissociable aggregates (about 8%), as evidenced by SEC. Since the level of soluble aggregate formation was similar for IFN-γ formulated in both glycolate and succinate, it is not likely that this particular process is related to the observed difference in bioactivity loss. A similar conclusion regarding the formation of soluble aggregates was drawn from SDS-PAGE (data not shown). We also checked for deamidation, a common intramolecular degradation, to determine if it was related to the bioactivity loss. We found that the solid-state incubation resulted in substantial deamidation, but the extent was comparable for both samples (34% and 38% for IFN-γ in the glycolate and succinate formulations, respectively) making it unlikely that this pathway was directly responsible for the significant difference in the loss of bioactivity.

Since the solution stability of IFN-γ correlated with a loss in its native conformation, it was logical to also study the conformation of the protein in the lyophilized state. The freezing step of lyophilization may induce a drop in pH in the succinate buffer system but not in the glycolate buffer system, as discussed above. As a result of this pH shift, the IFN-γ in the succinate buffer system most likely unfolds in the freeze-concentrated phase. In contrast, there is minimal pH-induced unfolding in IFN-γ formulated in the glycolate buffer system. Furthermore, it has been shown that lyophilization itself results in structural reorganization of proteins (Prestrelski et al., 1993a,b; Griebenow and Klibanov, 1995; Costantino et al., 1995, 1996a,c).

In order to probe whether IFN-γ loses its native structure upon lyophilization, and if the extent depended on the pH, we lyophilized the protein from aqueous solutions from pH 6 to pH 3. In this case, we did not include mannitol or buffer since we wanted to observe protein behavior solely as a function of pH. (In this case, no pH shifting was observed, data not shown). The FT-IR spectra were measured for these samples, and the amide III region was analyzed to estimate the secondary structure in the lyophilizate (Fig. 4C and D). The lyophilized protein exhibited peaks in the amide III at similar wavenumbers as observed for the aqueous solution (Table 1). Again, we observed that the relative areas were different, reflecting differences in secondary structure (Table 2).

The data reveal that the extent of lyophilization-induced secondary structural reorganization of IFN-γ is approximately the same from pH 6 to pH 4. Over this range, the lyophilized protein's total α-helix content decreased by 10–17%, with a concomitant increase in β-sheet, as compared with the corresponding solution structure. Recently, the lyophilization-induced α-helix-to-β-sheet transition has been reported for various proteins (Prestrelski et al., 1993a,b; Dong et al., 1995; Griebenow and Klibanov, 1995; Costantino et al., 1995, 1996a,c). When lyophilized from pH 3 (which results in a significant unfolding of the protein in aqueous solution as discussed above), the aberration from the native structure was much greater: both α-helical and unordered structures decreased and the β-sheet content increased significantly.

In summary, IFN-γ exhibits superior solid-state stability when lyophilized using glycolate as buffer instead of succinate. In the latter case, buffer components can crystallize in the frozen state, limiting the ability to maintain the pH, which in turn may perturb the protein's structure and render it more susceptible to deterioration. Thus, in formulating lyophilized IFN-γ, it is important to select a buffer in which the pH is reliably maintained during the freezing process. These observations have important implications for other proteins, depending on their pH sensitivity.

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