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Structural Studies on Acid Unfolding and Refolding of Recombinant Human Interferon γ

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ABSTRACT: Interferon γ is distinguished from other types of interferons in its instability upon acid treatment, as demonstrated by a loss of antiviral activity. Acid unfolding and refolding experiments were performed with recombinant DNA derived human interferon γ. When the protein was subjected to unfolding and refolding, the refolded protein showed two peaks (peaks I and II) in gel filtration, which have been shown to differ in size, structure, and antiviral activity. When the smaller, peak II, form was unfolded by dialysis against 0.01 M HCl containing 0.1 M NaCl (pH 2) and refolded by dialysis against various solvents at neutral pH, it re-formed as peak II but also generated peak I, and the ratio of the two forms was dependent on protein concentration and solvent conditions. Higher protein concentrations and higher ionic strength led to a greater ratio of peak I to peak II. Phosphate buffers caused precipitation of peak I. Since peak II is 4–8 times more active than peak I in the antiviral bioassay, generation of peak I by acid treatment of peak II should lead to a decrease in antiviral activity.

Immune interferon (IFN-γ)[1] differs from leukocyte (IFN-α) and fibroblast (IFN-β) interferons in a variety of biological and chemical properties (Wheelock, 1965; Green et al., 1969; Epstein et al., 1971; Falcoff, 1972; Stewart et al., 1980; Blalock et al., 1980; Rubin & Gupta, 1980; Yip et al., 1981a, 1982; Nakamura et al., 1984). In particular, instability of IFN-γ upon acid treatment distinguishes it from other IFNs (Wheelock, 1965; Green et al., 1969; Epstein et al., 1971; Yip et al., 1981a). For example, Yip et al. (1981b) observed that the antiviral activity of natural IFN-γ drops by approximately 10-fold upon dialysis against a pH 2 solution and then a neutral phosphate buffer. Conversely, other IFNs (IFN-α and IFN-β) maintain antiviral activity when treated in the same way. This suggests that IFN-γ is denatured in acid and cannot be refolded into the native structure. However, no structural studies of the protein have been carried out, in part due to difficulty in obtaining sufficient quantities of purified protein for structural studies.

We have constructed the gene for IFN-γ designed for expression in *Escherichia coli* (Alton et al., 1983), which lacks three N-terminal residues, Cys-Tyr-Cys, predicted from the originally published gene sequence (Gray et al., 1982; Devos et al., 1982). Recombinant IFN-γ, when expressed in *E. coli*, differs from natural IFN-γ (Rinderknect et al., 1984; Kelker et al., 1983, 1984) in that recombinant IFN-γ has an N-terminal Met residue (P. Lai, unpublished result) and glycosylation does not occur. In addition, the natural protein is known to undergo C-terminal processing (Rinderknecht et al., 1984). Therefore, natural IFN-γ usually lacks C-terminal residues whereas the amino acid sequence of recombinant IFN-γ is intact (P. Lai, unpublished result). In spite of these differences, structural studies on the recombinant protein should help elucidate the mechanism of acid instability of the natural protein.

We have carried out extensive chemical and physicochemical

[1] Abbreviations: IFN, interferon; Gdn·HCl, guanidine hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NH_4OAc, ammonium acetate; CD, circular dichroism; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane.

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characterizations of highly purified recombinant IFN-γ (Arakawa et al., 1985). It was observed that recombinant IFN-γ, when unfolded in urea and refolded, generates two forms, peak I and peak II (PI and PII), which can be separated by gel filtration. PI is heavily aggregated and less active than PII. In this study, the two forms were fractionated and subjected to acid treatment, and their acid unfolding and refolding were examined by various physicochemical techniques.

Materials and Methods

Materials. Ultrapure urea was obtained from Schwarz/Mann and ultrapure Gdn·HCl from Heico. All other chemicals used were of reagent grade.

IFN-γ Preparation. IFN-γ was purified from *E. coli* by a series of chromatographic procedures to greater than 95% purity as determined by SDS–PAGE analysis (Coomassie blue and silver staining). The IFN-γ preparation was shown to react with an antibody raised against the natural protein in Western blot analysis and was also characterized by amino acid composition and sequence analyses. These results will be published elsewhere. The purified IFN-γ was further treated to obtain two structurally different forms, PI and PII, as described previously (Arakawa et al., 1985). Briefly, solid urea was added to the protein solution (~1 mg/mL) in 0.1 M NH_4OAc to a final concentration of 7 M, which fully unfolds the protein. The protein solution was diluted 10-fold with 0.01 M NH_4OAc, which resulted primarily in formation of PII, followed by dialysis against 0.1 M NH_4OAc for storage. Alternatively, the urea-unfolded protein (~2 mg/mL) was dialyzed against 0.1 M NH_4OAc to allow formation of PI. The two forms were either used as obtained or chromatographically purified on a Sephadex G-75 column and stored in 0.1 M NH_4OAc. It was shown previously (Arakawa et al., 1985) that both PI and PII are stable in 0.1 M NH_4OAc, although PI may be converted very slowly to PII.

Acid Treatment. IFN-γ in either the PI or the PII form was dialyzed against 0.01 M HCl containing 0.1 M NaCl (pH 2.0) overnight at 4 °C and then against a variety of solvents at neutral pH overnight. The pH 2 solvent contained 0.1 M NaCl to minimize the Donnan effect. The pH of the dialyzed protein solution was directly checked and found to be 2. The protein concentrations were adjusted between 0.5 and 4 mg/mL, as indicated in each experiment.

Gel Filtration. Analytical gel filtration was performed on a Sephadex G-75 column (1 × 120 cm) in 0.1 M NH_4OAc containing 1 M urea (pH 7) at 4 °C. The inclusion of 1 M urea was necessary to prevent IFN-γ from binding to the column. Samples not containing urea were dialyzed against 0.1 M NH_4OAc and 1 M urea for 2 h immediately before the column was loaded. It was found previously that the above treatment does not affect the elution behavior of the sample.

Spectroscopy. CD spectra were determined at room temperature on a Jasco J-500C spectropolarimeter. The spectral bandwidth was set at 1 nm. Cuvettes used were 0.1 and 1 cm in light pass length for 190–260 and 240–340 nm, respectively. For each sample, the solvent spectrum was obtained and subtracted from the protein spectrum. The mean residue ellipticity, $[\theta]$, was calculated from the mean residue weight of 117 for IFN-γ. UV absorbance spectra were determined at room temperature on a Hewlett Packard Model 8451A diode spectrophotometer with an external plotter.

Protein Concentration. Protein concentration was determined spectrophotometrically in 3 M Gdn·HCl using the extinction coefficient of 0.65 mL/(mg·cm) at 280 nm. Occasionally, the protein concentration was also determined by a dye binding technique using Bio-Rad protein assay reagent with IFN-γ solution as standard. The protein concentration of the standard was determined by absorbance measurements in 3 M Gdn·HCl as described previously.

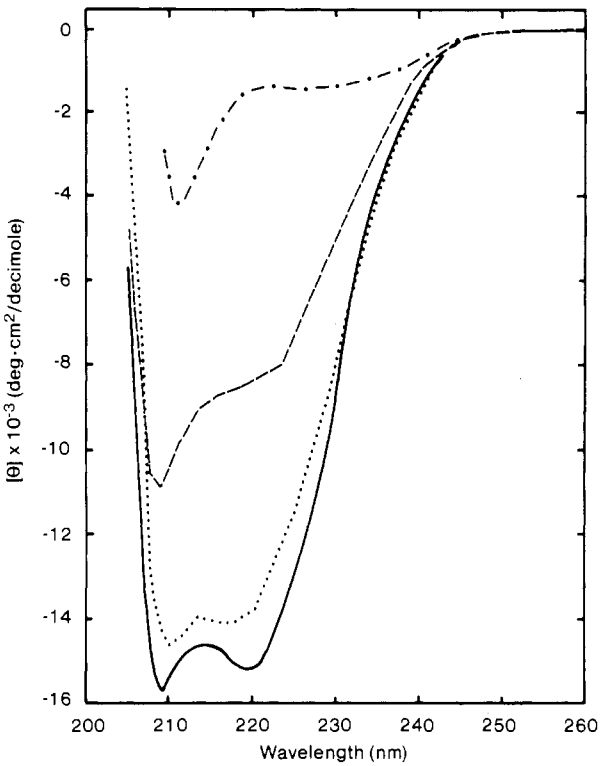

FIGURE 1: Far-UV CD spectra of IFN-γ under different conditions. Starting PII sample in 0.1 M NH_4OAc (—); acid (pH 2) (– –); 0.1 M NH_4OAc after acid treatment (···); 7 M urea (–·–).

Results and Discussion

Structure of IFN-γ under Different Conditions. IFN-γ assumes two forms in 0.1 M NH_4OAc, an aggregated form (PI) and a nonaggregated form (PII), when unfolded in urea and refolded in aqueous solutions at neutral pH (Arakawa et al., 1985). Sedimentation velocity and gel filtration experiments have suggested that PII is a dimer. It was shown that PII is stable over a long period and does not convert to the PI form. However, PI converts extremely slowly to PII in 0.1 M NH_4OAc, and this conversion could be accelerated in the presence of 1 M urea or at lower ionic strength. Figure 1 shows the far-UV CD spectrum of PII in 0.1 M NH_4OAc. The spectrum is characterized by two minima, at 209 and 220 nm, indicating the presence of ordered structures. Rough calculations according to the method of Greenfield & Fasman (1969) gave an estimate of 40% α-helix, 20% β-sheet, and 40% random coil. PII at 1 mg/mL in 0.1 M NH_4OAc was dialyzed against pH 2 buffer, and its CD spectrum in acid is shown in Figure 1 (dashed curve). The spectrum shows a minimum at 209 nm, a shoulder around 216 nm, and a substantial decrease in the ellipticity, $[\theta]$, over the wavelength examined relative to the values in 0.1 M NH_4OAc (solid curve). The amount of α-helix in acid was estimated to be ~20%. These changes in the CD spectrum indicate that PII IFN-γ is unfolded in acid (pH 2). However, the spectrum in acid is also different from the PII spectrum obtained in 7 M urea (–·–), which shows little presence of ordered structures. It is concluded that ordered structures, such as α-helix and β-sheet, still exist in acid although these structures, in particular α-helix, are significantly lost in acid as compared with the native state. Thus, the unfolding of IFN-γ in acid is partial relative to the unfolding which occurs in 7 M urea. If native PII is dimer, then

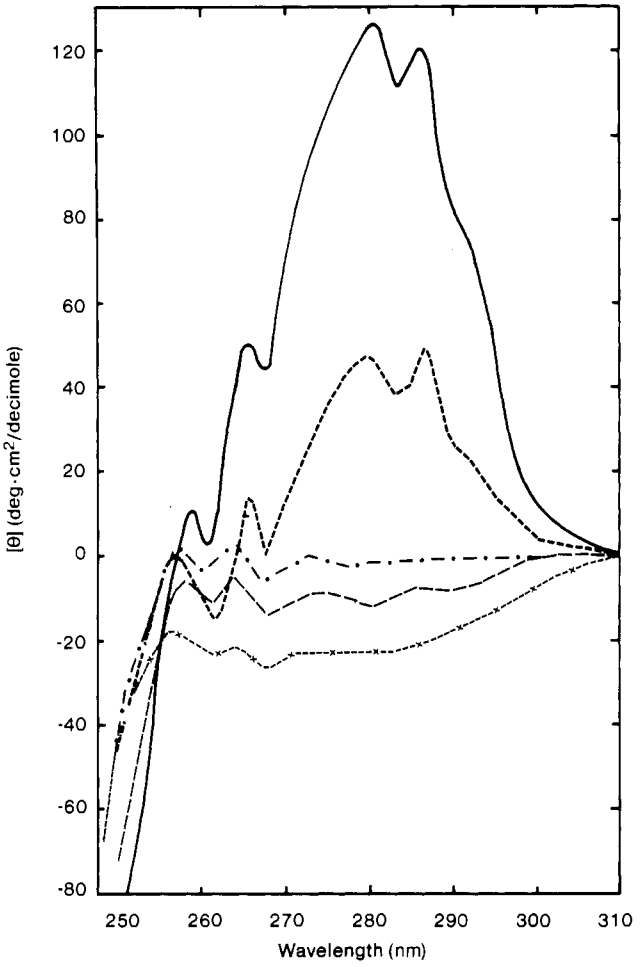

FIGURE 2: Near-UV CD spectra of IFN-γ under different conditions. Starting PII sample in 0.1 M NH_4OAc (—); acid (pH 2) (– –); 0.1 M NH_4OAc after acid treatment (- - -); 7 M urea (–·–); PI in 0.1 M NH_4OAc (×- - -×).

the dimeric structure may be lost in acid due to unfolding and electrostatic repulsion, since the dimer is not formed by covalent bonds. The acid-unfolded sample was renatured by dialysis against 0.1 M NH_4OAc and examined by far-UV CD. The result, shown in Figure 1 (dotted curve), indicates that IFN-γ when partially unfolded in acid is refolded into a structure similar to the initial PII material (i.e., before acid treatment), although small differences exist before and after acid treatment in the magnitude of $[\theta]$ and peak positions.

Near-UV CD spectra of these samples are given in Figure 2. The native PII in 0.1 M NH_4OAc is characterized by several positive peaks and shoulders (solid curve). This suggests that the aromatic residues in PII are incorporated into a rigid tertiary structure, which provides asymmetric environments for those residues giving rise to aromatic CD signals (Timasheff, 1970). The spectrum of IFN-γ in acid, shown by the dashed curve (Figure 2), is entirely different from the native spectrum and exhibits little distinct CD signals in the near-UV region, similar to the spectrum of IFN-γ unfolded in 7 M urea (–·–). Thus, PII, when dialyzed against acid, loses rigid tertiary structure, although the secondary structure is only partially unfolded as described above. The near-UV CD spectrum of PII in 0.1 M NH_4OAc after acid treatment is shown in Figure 2 (dotted curve). This spectrum is intermediate between the native PII and acid-unfolded structures. The result is in contrast to the far-UV CD result of the same sample, which suggests almost complete recovery to the native spectrum. These results suggest that the acid-unfolded IFN-γ

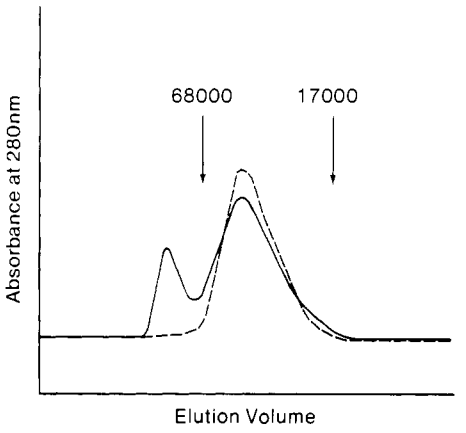

FIGURE 3: Gel filtration analysis of IFN-γ under different conditions. Starting PII sample (- - -); after acid treatment (—). PII at 1 mg/mL in 0.1 M NH_4OAc was dialyzed first against the pH 2 solvent and then against 0.1 M NH_4OAc as described in the text. Molecular weight standards are bovine serum (68 000) and myoglobin (17 000) shown by arrows.

Table I: Formation of PI and PII upon Acid Treatment of PII at Different Protein Concentrations[a]

protein concn (mg/mL)	appearance[b]	PII formed (%)	$[\theta]$ at 280 nm (deg·cm²/dmol)
0.5[c]	clear	100	125
0.5	clear	80	66
1	clear	56	24
2	clear	28	−36
4	pptn[d]		

[a] Acid-denatured PII was refolded by dialysis against 0.1 M NH_4OAc. [b] Appearance of the solution after acid treatment. [c] Untreated PII. [d] After centrifugation, no protein was observed in the supernatant.

can refold into a secondary structure similar to PII but the tertiary structure may be only partially restored. Alternatively, it is possible that the refolding results in formation of both PI and PII, as described previously. The observed near-UV CD spectrum is then the average of the spectra of the two forms, since PI gives small negative CD signals as shown by the curve (×- - -×), while PII has large positive signals (Figure 2).

Formation of both PI and PII after refolding of acid-treated PII was confirmed by analytical gel filtration on a Sephadex G-75 column, as shown in Figure 3. Native PII eluted between bovine serum albumin (M_r 68 000) and myoglobin (M_r 17 000) in a single peak. Conversely, the PII sample after acid treatment gave two peaks (solid curve), one at the void volume and the other at the same elution position as observed for the native PII. The percentage of PII formed was 75% under these conditions, which agrees qualitatively with the observed intermediate values of ellipticity, $[\theta]$, in the near-UV region for the refolded sample. The above results are similar to the results observed when IFN-γ in the PII form was unfolded in 7 M urea and refolded by lowering the urea concentration.

It has been consistently observed that PI is 4–8-fold less active than PII (Arakawa et al., 1985). Thus, generation of PI from PII should yield a drop of the specific activity of IFN-γ. If it is assumed that natural IFN-γ behaves similarly to PII, then a PI form may also be produced upon acid treatment by the same mechanism involved in the recombinant DNA derived protein described here. Therefore, effects of solution conditions on the refolding of PII were investigated to understand the mechanism involved in PI formation.

Solution Condition Dependence of Refolding. The ratio of PI to PII formed after acid treatment was examined at

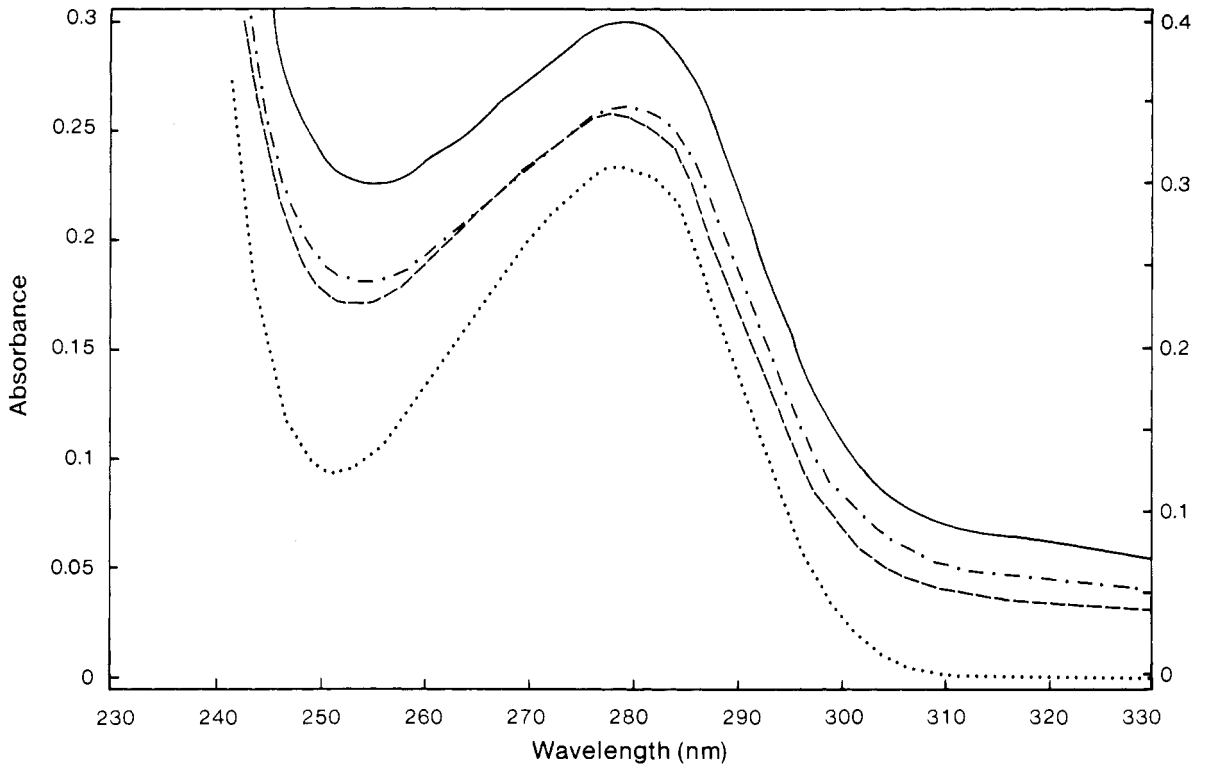

FIGURE 4: UV absorbance spectra of IFN-γ under different conditions. PI at 0.3 mg/mL in 0.1 M NH_4OAc was unfolded in acid and refolded in 0.01 M NH_4OAc. Starting PI sample (—) (left ordinate); pH 2 (– –) (left); 0.01 M NH_4OAc after acid treatment (–·–) (left); PII at pH 2 (···) (right).

different protein concentrations. PII solutions at 0.5–4 mg/mL in 0.1 M NH_4OAc were treated as described above and analyzed by near-UV CD spectra and gel filtration. The results are summarized in Table I. The 4 mg/mL sample showed extensive precipitation after dialysis back to 0.1 M NH_4OAc, although the solution was clear at pH 2. This indicates that the aggregation resulting in the precipitation occurred during the refolding process. After centrifugation of the above sample, no protein was detectable in the supernatant. The samples at 0.5–2 mg/mL were clear and showed no precipitation in either acid or refolding solvents. The CD results are shown in Table I as the mean residue ellipticity, $[\theta]$, at 280 nm, since at this wavelength PI and PII exhibited entirely different values of $[\theta]$. The gradual decrease in $[\theta]$ at 280 nm and the gel filtration results indicate that higher protein concentration led to greater formation of PI (20–100%). Since the formation of PI is due to aggregation of the protein, the complete precipitation which occurred at 4 mg/mL could be due to extremely enhanced protein aggregation, which resulted in PI aggregates large enough to precipitate. The result at 1 mg/mL (Table I) shows a 44% formation of PI whereas it was 25% in the previous case, suggesting that the formation depends slightly on the exact procedure for unfolding and refolding.

IFN-γ refolding was also studied in phosphate (pH 7) and 0.01 M NH_4OAc. The gel filtration and CD results demonstrated that formation of PII was strongly dependent on the ionic strength of the refolding solvents. Lower ionic strength favored PII formation in both phosphate and NH_4OAc. Both 5 mM phosphate and 0.01 M NH_4OAc showed a protein concentration dependence of refolding similar to that observed with 0.1 M NH_4OAc. In 5 and 40 mM phosphate, the refolded protein showed precipitation, and the protein remaining in the supernatant was in the PII form as determined by analytical gel filtration. It was hypothesized that the precipitation corresponded to the amount of PI formed. This possibility was tested by dialyzing a PI preparation in 0.1 M NH_4OAc, which contained 20% PII, against various solvents. The results showed that approximately 80% of the protein precipitated in 5 and 40 mM phosphate buffers while no precipitation occurred in aqueous NH_4OAc. Gel filtration analysis showed that the protein in the supernatant, which was ~20% of the total, was in the PII form. This clearly indicated that PI precipitated in these phosphate buffers while PII did not, supporting the above conclusions.

It appeared from these results that the solubility of PI depended on the kind of ionic species present in the solvents. Therefore, the following solvent systems were tested: 5 mM Tris–borate buffer (pH 7.5), 0.1 M NH_4OAc containing 5 mM $CaCl_2$, 5 mM carbonate (pH 7.5) titrated with HCl, and 0.01 M NH_4OAc containing 5 mM Na_2SO_4 (pH 7.0). Of these solvents, only 0.01 M NH_4OAc containing 5 mM Na_2SO_4 caused precipitation. At the pH used, borate and carbonate are mainly monovalent ions, suggesting that monovalent anions in general do not cause PI precipitation. The divalent cation, Ca^{2+}, did not have such an effect. The Na_2SO_4-containing solvent caused approximately 85% precipitation which was equivalent to the amount of PI present in the starting material. Therefore, the observed PI precipitation may be related to the presence of divalent anions.

Acid Treatment of PI. The results described above revealed that formation of PI occurred when PII was unfolded by increasing the pH to neutrality. It was of interest, therefore, to determine if PII formation occurred when PI was used as the starting material. This was studied by using a PI preparation in 0.1 M NH_4OAc (0.3 mg/mL). The UV absorbance spectrum of this sample (Figure 4, solid curve) showed large light scattering, demonstrating that the sample was heavily aggregated. Gel filtration analysis indicated the presence of 60% PI in this sample. This PI sample was dialyzed against acid and then 0.01 M NH_4OAc. Since conversion of PII from PI occurs much faster in 0.01 than 0.1 M NH_4OAc, spectroscopic and gel filtration experiments were carried out im-

mediately after overnight dialysis against 0.01 M NH_4OAc. The UV absorbance spectrum of PI in 0.01 M NH_4OAc after acid treatment shown in Figure 4 (–·–) exhibits light scattering comparable to the starting material. The gel filtration showed that ~60% PI was re-formed. This indicates that acid treatment has no effect on the ratio of PI to PII. If acid treatment can lead to the same state of unfolding regardless of whether PI or PII was used as starting material, one would expect 100% PII formation at such low protein concentration (0.3 mg/mL) using 0.01 M NH_4OAc as the refolding solvent. Therefore, the states of PI and PII at pH 2 were compared by UV absorbance spectra. It was shown in Figure 4 that PII shows no light scattering in acid while PI still has larage light scattering. This suggests that acid cannot disaggregate PI, which should lead to the same aggregated form upon refolding, i.e., PI.

Conclusions

It was shown in this paper that PI is formed, in addition to re-formation of PII, when PII is unfolded in acid and refolded in a variety of neutral solvents, depending on the protein concentration and ionic strength. The structure and gel filtration analyses indicated that the re-formed PII is indistinguishable from the starting material. Since the PI form can slowly convert to the PII form in neutral solvents, the acid unfolding and refolding of PII are essentially reversible processes. Therefore, PI is considered as an intermediate trapped during the refolding process from acid. There may be two possibilities for formation of PI. First, the acid-unfolded PII, in either monomeric or dimeric form, folds in the secondary structure but not in the tertiary structure and aggregates due to more exposed hydrophobic sites. Second, the acid-unfolded PII folds also in tertiary structure but differently from folding into the native PII, which resulted in nonspecific protein–protein contacts and hence aggregation. In either case, the direct conversion of PI to PII requires conformational changes as well as disaggregation. The nonspecific aggregation was decreased by lower ionic strength, which increases the repulsion of protein molecules. It is evident that IFN-γ has a strong self-associating tendency, since the aggregation should overcome strong electrostatic repulsion forces due to the high isoelectric point of the protein.

The mechanism of acid instability of natural IFN-γ may be inferred from the results obtained with recombinant IFN-γ. If one assumes that the natural protein behaves in the same way as the recombinant protein, then the natural protein may yield both PI and PII forms after acid treatment, even though the starting sample may have contained only PII. Although the acid treatments of the natural protein have been done below the lowest concentration examined here, the preparations contained high molecular weight impurities, which might enhance the formation of PI due to the excluded volume effect known to enhance protein aggregation (Minton, 1981; Lee & Lee, 1979; Arakawa & Timasheff, 1985). It is also possible that the acid-unfolded IFN-γ aggregated with the impurities due to its strong associating tendency during the refolding process. It has been consistently observed that PII is 4–8 times more active than PI in a standard CPE assay. Therefore, formation of PI or other types of aggregation should lead to a decrease in the antiviral activity. Since phosphate buffer has been used as a refolding solvent for the natural protein, PI, if formed, would precipitate, leading to a complete loss of biological activity for PI. However, the possibility cannot be excluded that natural IFN-γ behaves differently from the recombinant protein due to such factors as glycosylation, different isoelectric points (Yip et al., 1981b), and C-terminal processing.

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