Acid Unfolding and Self-Association of Recombinant *Escherichia coli* Derived Human Interferon γ†

Tsutomu Arakawa* and Yeuh-Rong Hsu
Protein Biophysics, Amgen, Thousand Oaks, California 91320
David A. Yphantis*
Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06268, and Department of Biology and Institute of Molecular Biology and Biotechnology, University of Crete, Iraklion, Crete
Received November 21, 1986; Revised Manuscript Received April 9, 1987

ABSTRACT: The secondary and tertiary structure of recombinant human interferon γ, determined by far- and near-UV circular dichroism, showed a transition from the native state to an unfolded state below pH 4.5. The acid unfolding was extensively studied at pH 3.5 as a function of NaCl concentration. Addition of 0.05–0.2 M NaCl to a pH 3.5 sample increased the amount of β-sheet structure with no change in the amount of α-helix and also induced reversible self-association of interferon γ to form large aggregates from the monomer. When samples at pH 3.5 were dialyzed against 0.1 M ammonium acetate (pH 6.9) to refold interferon γ, the samples that contained NaCl in acid formed aggregates upon dialysis while those without NaCl formed a dimer apparently identical with the starting protein (i.e., before acid treatment). Thus, the self-association of interferon γ in acid is closely correlated with its aggregation behavior in 0.1 M ammonium acetate after removal of acid.

It has been shown, using natural interferon γ (IFN-γ)[1] preparations, that IFN-γ is unstable against acid treatment; i.e., the protein loses antiviral activity when exposed to acid and then neutralized (Wheelock, 1965; Green et al., 1969; Epstein et al., 1971; Yip et al., 1981). However, the mechanism of the acid instability of IFN-γ has not been fully understood, in part because insufficient quantities of purified natural IFN-γ were available for structure analyses. We have prepared highly purified recombinant human IFN-γ derived from *Escherichia coli* in large quantities (Arakawa et al., 1985) and reported initial results on the acid unfolding and refolding of the *E. coli* derived material (Hsu & Arakawa, 1985). Although the *E. coli* derived IFN-γ differs from the natural protein in that it has an intact C-terminus and is not glycosylated, the structural study of *E. coli* IFN-γ should shed light on the mechanism of the observed acid instability of natural IFN-γ preparations.

We have shown in a previous paper (Hsu & Arakawa, 1985) that *E. coli* derived IFN-γ partially unfolds in acid and that during dialysis against neutral solvents it refolds into a structure apparently identical with the starting material. However, this process also generates an aggregated material. This aggregated material, apparently similar to a form generated by urea unfolding and refolding, is 4–8-fold less active than the nonaggregated form (Arakawa et al., 1985). CD analysis indicated that the aggregated form of *E. coli* derived IFN-γ has a secondary structure almost identical with that of the starting IFN-γ sample but is unfolded with regard to tertiary structure. It was thought, therefore, that the recombinant IFN-γ first forms a secondary structure similar to the native molecule during refolding from acid and then folds into the same tertiary structure as either dimer or aggregates. In order to understand more about acid unfolding and refolding, we have examined the structure of IFN-γ in acid as a function of NaCl concentration and have found that acid unfolding of IFN-γ is a complex phenomenon, as we report in this paper.

Materials and Methods

Recombinant human IFN-γ was purified from *E. coli* by a series of chromatographic procedures and was prepared as a nonaggregated form according to the procedure previously described (Arakawa et al., 1985). The sample was stored at −20 °C in 0.1 M NH_4OAc and thawed immediately before use.

A pH titration experiment was carried out by dialyzing an IFN-γ solution in 0.1 M NH_4OAc against 20 mM NaOAc buffer (20 mM in acetate and acetic acid) or 20 mM glycine hydrochloride buffer (20 mM in glycine). Samples at pH 2 were prepared by dialysis against aqueous HCl. Samples containing differing concentrations of NaCl were prepared by directly adding solid NaCl to an acidic protein solution obtained by dialysis against 20 mM NaOAc buffer (pH 3.5) or aqueous HCl (pH 2) without NaCl. Alternatively, solutions were prepared by dialyzing the protein against acidic solvents containing NaCl. Both methods gave identical results. The reversibility of the structure changes of IFN-γ in acid that were induced by NaCl was examined by dialyzing samples against acidic buffers without NaCl. Reversibility of acid unfolding with or without NaCl was examined by dialyzing the samples, exposed to acid, against 0.1 M NH_4OAc (pH 6.9). Dialysis was carried out for at least 18 h in dialysis tubing with a molecular weight cutoff of 3500 and a diameter of 1.1 cm.

Circular dichroic spectra were measured on a Jasco Model J-500C spectropolarimeter at room temperature. Cuvettes of 0.1- and 1-cm path length were used for the ranges of 190–260 nm and 260–340 nm, respectively. The solvent spectrum was determined and subtracted from the protein spectrum. The

† A preliminary report of part of this work has been presented at the 30th annual meeting of the Biophysical Society, San Francisco, CA, Feb 12, 1986.

* Address correspondence to this author.

[1] Abbreviations: IFN-γ, interferon γ; NH_4OAc, ammonium acetate; NaOAc, sodium acetate; CD, circular dichroism; RMS, root mean square; rpm, revolutions per minute. Unless otherwise specified, IFN-γ refers to the protein derived from *E. coli*.

0006-2960/87/0426-5428$01.50/0

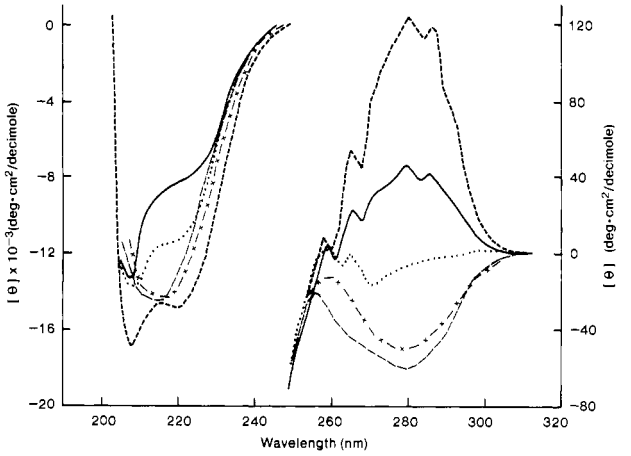

FIGURE 1: CD spectra of IFN-γ at pH 3.5. NaCl concentration: 0 (—), 0.05 (···), 0.1 (×), and 0.2 M (– –). For comparison, the spectra at pH 5.5 are shown (heavy dashed line).

data were expressed as the mean residue ellipticity [θ] calculated from a mean residue weight of 117. UV absorbance spectra were determined on a Hewlett-Packard Model 8451A diode array spectrophotometer. This device subtracts the solvent base line and generates a derivative of absorbance with regard to wavelength. The second derivative is known to be sensitive to conformational changes of proteins. Gel filtration was carried out on a Sephadex G-75 column (1 × 100 cm) equilibrated with the appropriate buffer. At neutral pH we found it necessary to equilibrate the column with 0.1 M NH_4OAc containing 1 M urea so as to prevent IFN-γ from binding to the column.

The sedimentation equilibrium procedures have been described in detail (Yphantis & Arakawa, 1987).

Results

The near-UV CD spectrum of IFN-γ at neutral pH shows a strong positive band at 280 nm that disappears and becomes negative when the protein is unfolded by urea or acid (Hsu & Arakawa, 1985). When the ellipticity at 280 nm was plotted vs. pH in NaOAc (≥pH 3.5) or glycine hydrochloride buffer (≤pH 3) containing 0.1 M NaCl, it showed a large change between pH 3.5 and pH 4.5 (data not shown), indicating an alteration in tertiary structure. The far-UV CD also showed a transition below pH 4.5 reflecting a change in secondary structure. However, the pH-induced secondary structure transition was strongly dependent on the ionic strength of the solvent. Therefore, acid denaturation was studied more extensively at constant pH as a function of NaCl concentration.

Near- and far-UV CD spectra at pH 3.5 in 20 mM NaOAc are shown in Figure 1 as a function of NaCl concentration. The results clearly indicate that the addition of NaCl alters the secondary and tertiary (or quatenary) structure of the protein at this pH. The far-UV CD spectra show an isosbestic point at approximately 208 nm. The α-helix content, calculated according to Greenfield and Fasman (1969), is given in Table I. The results show a nearly constant α-helix content of about $^1/_3$, implying that the spectral changes reflect transitions from random-coil to β-sheet configurations. This conclusion is reinforced by the presence of the isosbestic point at 208 nm, since, according to Greenfield and Fasman (1969), β-sheet and random coil have the same molar ellipticity at 208 nm and this ellipticity differs strongly from that of the α-helix. The near- and far-UV CD spectra at high salt were entirely different from the spectra of pH 5.5 or 7 (Figure 1), indicating that the NaCl-induced transition is not a transition from the acid-denaturated to the native state.

Table I: α-Helix Content of IFN-γ

[NaCl] (M)	α-helix (%) pH 2	pH 3.5
0	18	32
0.05	29	33
0.1	36	29
0.2	37	32

Table II: Aggregation of IFN-γ in Acid

	gel filtration, P_{15} (%)[a]	monomer estimated from equilibrium sedimentation (%)
pH 3.5, no NaCl	100	99[b]
pH 3.5, 0.05 M NaCl	>90	
pH 3.5, 0.1 M NaCl	<20	12
pH 3.5, 0.1 M NaCl → no NaCl	100	98.5[c]

[a] IFN-γ showed two peaks at tube number 10 (P_{10}, corresponding to the excluded volume of the column) and at tube number 15 (P_{15}). Results are given as percent of protein eluted at P_{15}. Blue dextran and bovine serum albumin eluted at the excluded volume of the column, and myoglobin eluted at tube number 16 in 20 mM NaOAc, pH 3.5. [b] The fraction of aggregate present appears to be concentration dependent, ranging from 1% in the lower loading concentration (below 0.3 g L^{-1}) to 6% at the highest concentration used (1 g L^{-1}). [c] Determined for loading concentrations below 0.3 g L^{-1}.

Table III: Apparent z-Average Molecular Weights for IFN-γ at pH 3.5 and 0.1 M NaCl[a]

loading concn (g/L)	$M_z \times 10^6$ at 7200 rpm	$M_z \times 10^5$ at 10000 rpm	$M_z \times 10^5$ at 15000 rpm	$M_z \times 10^5$ at 30000 rpm
0.25	1.15 ± 0.03	8.14 ± 0.24	5.25 ± 0.14	
0.5	1.22 ± 0.02	7.88 ± 0.18	5.40 ± 0.10	1.78 ± 0.06
0.75	1.26 ± 0.02	8.19 ± 0.17	5.30 ± 0.06	2.21 ± 0.08
1.0	1.23 ± 0.01	7.79 ± 0.08	4.85 ± 0.06	2.07 ± 0.06

[a] The ranges indicated only reflect fitting uncertainties.

The molecular size of IFN-γ at pH 3.5 was examined by gel filtration, and the results are summarized in Table II. A sample without NaCl showed an elution peak at tube 15 (P_{15}) with all the material eluting in this peak. Calibration of the column at pH 3.5 indicated that blue dextran and bovine serum albumin gave peaks at tube 10 and myoglobin at tube 16. The elution position for IFN-γ relative to myoglobin suggests that IFN-γ may dissociate to monomer at pH 3.5. This dissociation was confirmed by sedimentation equilibrium (Yphantis & Arakawa, 1987). The sample at pH 3.5 in 0.1 M NaCl showed two peaks, at tube 10 and at tube 15, with 80% of the protein eluting in the first peak; i.e., 80% or more of the loaded protein on the column was aggregated in this solvent system. About 10% aggregate was seen at pH 3.5 in 0.05 M NaCl by gel filtration.

A sample in 0.1 M NaCl at pH 3.5 was further examined in short-column (0.75 mm) sedimentation equilibrium experiments. The apparent z-average molecular weights observed at four loading concentrations are presented in Table III. Complete solution columns were visible only at the lowest speed used, 7200 rpm. At all other speeds, at least part of each channel could not be observed because of extreme concentration gradients. Accordingly, the apparent z-average molecular weights at speeds above 7200 rpm reflect the behavior of only *part* of the sample. The range of these molecular

weight estimates confirms the marked heterogeneity of the sample under these conditions. Our estimate of the z-average molecular weight of the sample as a whole is about 1.2×10^6, the value indicated by the values of 7200 rpm.

The fringe displacements at 30 000 rpm were analyzed by least-squares procedures to estimate the amount of monomer present. We assumed that the effective reduced molecular weight of the monomer was the same as that found without salt (Yphantis & Arakawa, 1987), that the solutions behaved ideally, and that all the protein loaded into the channels was conserved. Under these assumptions, the fits of the fringe displacement in terms of monomer and of the best fitting polymer in each channel (with estimated degree of polymerization varying from 17 to 28 from one observation channel to another) gave estimates of 12 ± 5% monomer present. The assumption of ideality is not crucial: assuming the second viral coefficient of IFN-γ in this solvent to be tenfold greater than the second viral coefficient corresponding to a charge of 11+ (the effective charge per IFN-γ molecule at this pH in the absence of salt), estimate the amount of monomer present to be 14.4 ± 7%. These values are in agreement with the results of gel filtration.

Removal of 0.1 M NaCl by extensive dialysis against 20 mM NaOAc, pH 3.5, resulted in dissociation of aggregated protein, as demonstrated by gel filtration in the absence of NaCl, which showed that all the protein eluted at P_{15} upon removal of NaCl (Table II). Equilibrium ultracentrifugation of this sample was carried out at 40 000 rpm with initial concentrations of 0.1, 0.2, and 0.3 g L^{-1} in the three sets of channels of a 30-mm path length external loading cell (Ansevin et al., 1970). Figure 2 presents values of the reciprocals of the apparent weight-average molecular weights observed as a function of observation concentration for loading concentrations of 0.1 and 0.3 g L^{-1}. As in the case of samples not exposed to 0.1 M salt at this pH (Yphantis & Arakawa, 1987), the sample shows extensive nonideality and almost complete dissociation to monomer with some heterogeneity evident. Direct nonlinear least-squares fitting of the measured fringe displacements to the model of a single nonideal component was strongly influenced by the small amounts of aggregates present. The effect of these aggregates was circumvented, as before (Yphantis & Arakawa, 1987), by appropriate truncation: we discarded all data points that corresponded to concentrations greater than 1 fringe in the two channels with initial concentrations of 0.1 g L^{-1} and greater than 7.5 fringes in the channel with loading concentration of 0.3 g L^{-1}. The fits with these truncated data sets provided our estimates of the reduced molecular weight $M(1 - \bar{v}\rho)$ as 4186 ± 240 kg mol^{-1} and of the second viral coefficient B as (8.6 ± 0.74) × 10^{-2} mol mL g^{-2}. The associated fitting residuals had an RMS value of 0.034 fringe. As before, these estimates were not critically dependent on the specific data truncations chosen.

Using the partial specific volume of 0.752 mL g^{-1} obtained previously for the Scatchard definition component and assuming that all of the nonideality can be ascribed to the Donnan effect, we estimate the molecular weight of the Scatchard component to be 16 780 ± 960 and the effective charge to be 11.8+ ± 0.5+. Estimates were also made of the size and extent of the single component assumed to be responsible for the observed heterogeneity with the complete, untruncated fringe measurements as described previously (Yphantis & Arakawa, 1987). The nonlinear least-squares fits indicated this component to be a 16-mer of IFN-γ present to the extent of 1.6 ± 0.4% of the protein observed in each of the three channels. Thus, this sample behaved quite similarly to samples that had not been exposed to 0.1 M NaCl at pH 3.5.

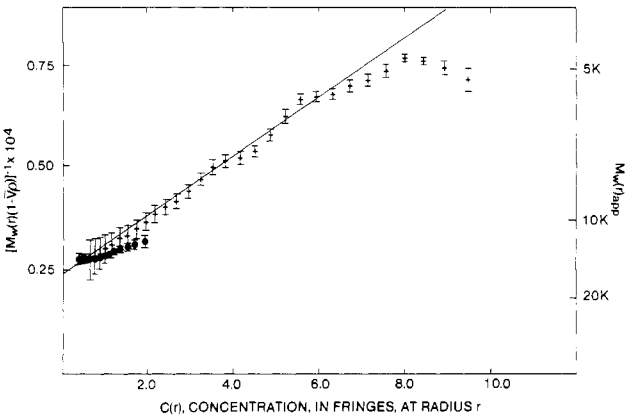

FIGURE 2: Molecular weight determination of a sample of IFN-γ dialyzed back to low salt after exposure to 0.1 M NaCl at pH 3.5. Reciprocals of the apparent reduced weight-average molecular weights, $M_w(r)$, of IFN-γ at pH 3.5 and an ionic strength of 0.0012 M are presented as a function of observation concentration, $C(r)$. Three solutions of IFN-γ at concentrations of 0.1 (●), 0.1 (data not shown), and 0.3 g L^{-1} (+) were equilibrated simultaneously at 40 000 rpm in a pH 3.5 buffer containing 0.020 M buffer (acetic acid + sodium acetate). The reduced weight-average molecular weights shown were obtained with meniscus concentrations estimated from nonlinear least-squares fitting of truncated data sets to the model of a simple nonideal system (see text). This was necessary since complete meniscus depletion was not achieved in all channels of this experiment because of the extreme nonideality and the limitation to a maximum operating speed of 40 000 rpm for the 30-mm optical path centerpieces [see Yphantis and Arakawa (1987)]. For clarity, only one out of four points obtained is shown here. To avoid overcrowding, the points corresponding to one of the channels with an initial loading concentration of 0.1 g L^{-1} were also omitted. The straight line corresponds to the parameters of the "best fit" (see text). The partial specific volume corresponding to the Scatchard component was used to fix the auxiliary molecular weight scale shown.

These results indicate that the unfolded form of IFN-γ at pH 3.5 reversibly self-associates into aggregates in a salt-dependent manner, favoring aggregation at higher salt concentration. We conclude that the observed conformational transition of IFN-γ at pH 3.5 on addition of NaCl, as demonstrated by near- and far-UV CD, is closely related to the self-association of the protein. In fact, the sample containing 0.1 M NaCl at pH 3.5 showed, after removal of NaCl, spectra apparently identical with those (at pH 3.5) before NaCl addition. This observation supports a close relation between aggregation and conformational change of the protein at this pH.

IFN-γ unfolded at pH 3.5 was refolded by dialyzing the protein solutions against 0.1 M NH_4OAc (pH 6.9). The refolded protein was examined by CD or UV absorbance, gel filtration, and sedimentation equilibrium (Table IV). Samples dialyzed from pH 3.5 without NaCl exhibited a conformation apparently identical with that of the starting material (i.e., before acid treatment) and appeared to be completely in the dimer form with no aggregated protein detectable when examined by gel filtration and equilibrium sedimentation (experiment 1).

Samples of IFN-γ exposed to pH 3.5 in the presence of 0.1 M NaCl were first dialyzed against pH 3.5 buffer in the absence of NaCl. These samples were then neutralized by dialysis against pH 6.9, 0.1 M NH_4OAc (experiment 3). These samples also showed no detectable aggregation, reflecting the reversibility of the NaCl-induced conformation change at pH 3.5. On the other hand, samples of IFN-γ dialyzed *directly* to neutrality from 0.1 M NaCl, pH 3.5

Table IV: Results of CD, Gel Filtration, and Apparent *z*-Average Molecular Weights for IFN-γ after Return to pH 6.9

conditions[a]	CD (or UV)[b]	dimer (%)[c]	concn (g/L)	apparent *z*-average molecular weight ×10⁶ at 7200 rpm	×10⁶ at 12000 rpm	×10³ at 24000 rpm	×10³ at 36000 rpm
expt 1, low salt → neutral pH	native (native)	100	0.25			36 ± 1.3	35.4 ± 0.5
			0.5				33.9 ± 0.7
			0.75			34.7 ± 0.7	34.4 ± 0.3
			1.0			36.0 ± 1.3	34.6 ± 0.5
av						35.0 ± 0.8	
expt 2, 0.1 M NaCl → neutral pH	aggregates	10	0.25	2.21 ± 0.08	1.08 ± 0.04	83 ± 6	
			0.5	1.51 ± 0.07	0.65 ± 0.04	42.5 ± 2.0	
			1.0	1.88 ± 0.07	0.91 ± 0.05	49.2 ± 2.8	
expt 3, 0.1 M NaCl → acid low salt → neutral pH	(native)	100	0.25			37.5 ± 2.5	
			0.5			38.0 ± 1.8	
			0.75			36.4 ± 0.9	
			1.0			34.3 ± 0.4	
av						36.6 ± 1.6	

[a] IFN-γ was dialyzed into a neutral pH solvent (0.1 M NH_4OAc) from the solvents listed. [b] Conformational information given in parentheses was evaluated from second derivatives of the UV absorbance. The CD and second-derivative spectra were compared with those for the native structure and for the aggregated form at neutral pH (Arakawa et al., 1985). [c] Determined by gel filtration, which showed two peaks in many cases.

(experiment 2), showed the re-formation of only 10% dimer by gel filtration. Sedimentation equilibrium of this sample (experiment 2) showed extensive heterogeneity: The *z*-average molecular weight corresponding to all of this sample that was visible at 7200 rpm approached 2 million, and species ranging down in size to the dimer were present. These results show a close correlation between the association state of IFN-γ in acid and aggregate formation upon removal of acid.

Similar but less extensive experiments were carried out at pH 2.0. The near- and far-UV CD spectra, shown in Figure 3, indicate a NaCl-induced structure transition in the secondary and tertiary (or quaternary) structure at this pH that is similar to the transition observed at pH 3.5. Without NaCl, the secondary structure is greatly reduced, as demonstrated by a calculated α-helix content of only 18% (Table I). As the NaCl concentration is increased, the α-helix content increases as shown in Table I. In addition, the far-UV CD shows an increase in the shoulder around 215 nm with increasing NaCl concentration and exhibits a negative peak at 214 nm in the presence of 0.2 M NaCl. These results suggest an increase in the amount of β-sheet structure in the presence of salt. Therefore, the NaCl-induced transition of the secondary structure at pH 2.0 may be characterized by a large increase in the α-helix content with a minor contribution from increased β-sheet structure. This is supported by the presence of an isosbestic point at approximately 205 nm, since random structure and α-helix have an identical molar ellipticity around 204 nm that is markedly different from the ellipticity of β-sheet structures. Comparison of the spectra at pH 2.0 with those at pH 3.5 shows that although the secondary and tertiary (or quaternary) structures at pH 2.0 differ from those at pH 3.5 in the absence of NaCl, they do become similar at higher salt concentrations. This suggests that the NaCl-induced transition at pH 2.0 may also be associated with self-association such as that observed at pH 3.5. This is supported by the observations that IFN-γ at pH 2.0 without NaCl, when dialzyed vs. 0.1 M NH_4OAc, formed 100% dimer as determined by gel filtration while samples at pH 2.0 containing 0.1 M NaCl showed 25% aggregates after the same dialysis.

For comparison, similar experiments were also carried out at pH 5.5. Far- and near-UV CD spectra at this pH were identical with those at pH 7.0 (Figure 1) and did not change upon addition of NaCl, indicating no effect of NaCl addition on the structure of IFN-γ. In agreement with this, IFN-γ

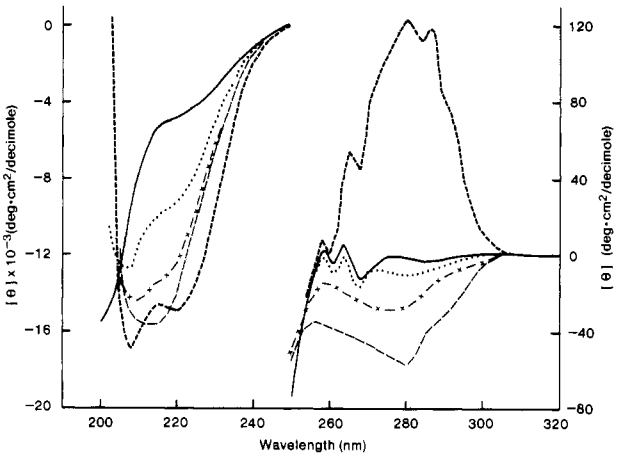

FIGURE 3: CD spectra of IFN-γ at pH 2.0. NaCl concentration: 0 (—), 0.05 (···), 0.1 (×), and 0.2 M (– –). For comparison, the spectra at pH 5.5 are shown (heavy dashed line).

samples at pH 5.5 with NaCl ranging from 0 to 0.2 M NaCl formed only dimers upon dialysis against 0.1 M NH_4OAc.

Discussion

One of the important findings in this study is that IFN-γ dimer dissociates into monomer at pH 3.5 in the absence of salt. CD analysis of this associated sample showed a conformational change in the IFN-γ. If dimer formation requires a definite conformation of IFN-γ, the acid-induced unfolding could be responsible for the observed dissociation. In addition, IFN-γ possesses large net positive charge at pH 3.5 (our estimates of the effective charge are 11+ to 12+, from the observed nonideality) from its high content of basic residues (Devos et al., 1982; Rinderknecht et al., 1984). This should result in strong electrostatic repulsion between protein molecules at such low ionic strengths. It is likely that the IFN-γ dimer also dissociates at pH 2 in the absence of NaCl, since protein unfolding is further enhanced by lowering the pH (Figures 1 and 3) at the same time that the net electrostatic charge is increased.

Sedimentation equilibrium, gel filtration, and UV absorbance spectra showed that when monomeric IFN-γ was dialyzed against 0.1 M NH_4OAc (pH 6.9), it re-forms a dimeric from apparently identical in conformation with the starting

material. Thus, it appears that the dimeric structure at neutral pH is obtained by direct association (upon neutralization) of the monomers that are present in acid.

In acid, IFN-γ monomer was observed to self-associate into large aggregates on addition of NaCl. This addition is accompanied by a significant increase in the β-sheet content at pH 3.5 and by an increase in both α-helix and β-sheet content at pH 2.0. The observed increase in the amount of β-sheet structure suggests that the self-association may be due to hydrogen bonds between β-sheets. Since the aggregates readily dissociate into monomer upon removal of NaCl, this aggregation is a reversible process. It was shown that when IFN-γ in acid containing NaCl was dialyzed against 0.1 M NH_4OAc (pH 6.9), both dimeric and aggregated forms were obtained. Some of the aggregates that were formed in acid containing NaCl apparently dissociate into monomer during dialysis against 0.1 M NH_4OAc, resulting in dimer formation. The rest of the protein appears to remain aggregated. The two most likely origins of the aggregates present after neutralization are (a) that during the transition to neutrality the monomer refolds into an "unnatural conformation" which then aggregates or (b) that some of the aggregates present at low pH remain aggregated with only (relatively) small configurational changes. The direct correlation of the presence of the neutral-pH aggregates with the NaCl-induced transition and the state of aggregation at pH 3.5 strongly supports the latter alternative. Thus, we propose that the apparent instability of IFN-γ on acid treatment arises from the aggregation in acid.

A major transition in tertiary structure of IFN-γ occurs between pH 3.5 and pH 4.5, while much of the secondary structure still largely remains at pH 3.5 in the absence of NaCl. This remaining secondary structure is further partially destroyed by lowering the pH to 2. This suggests that titration of additional carboxyl groups increases the electrostatic repulsions of these groups and destabilizes the ordered structures. Alternatively, it is possible that salt linkages stabilizing the ordered structure are destroyed by protonation of the carboxyl groups involved in the salt linkage. It may be of interest to point out that there are sequences of high α-helical potential (Chou & Fasman, 1978), which contain charged residues, such as Trp(36) to Phe(52) and Lys(86) to Leu(95).[2] If these sequences indeed form α-helix, then some positive charges may come close to negative charges because of their distribution in these sequences. Such a configuration would stabilize the α-helices and cause an abnormal pK for the carboxyl groups. Thus, the observed loss of α-helix content by lowering the pH from 3.5 to 2 may be explained by titration of these abnormal carboxyl groups and, hence, destabilization of α-helix forms. The observed increase in α-helix content at pH 2 by addition of NaCl most likely arises from charge shielding of positive charges, thus decreasing repulsive electrostatic free energy.

In conclusion, IFN-γ at low pH forms an increasing amount of aggregates on addition of NaCl. Incomplete dissociation of these salt-induced aggregates appears to be the most likely source of the extensive and heterogeneous aggregates that are observed when such solutions are dialzyed back to neutrality against 0.1 M NH_4OAc. In the absence of salt, no aggregates are seen at low pH, and only dimers are observed after dialysis against 0.1 M NH_4OAc.

Registry No. NaCl, 7647-14-5; NH_4OAc, 631-61-8.

[2] Amino acid residues are numbered as Met(−1)-Gln(1)-Asp(2) for IFN-γ with a total of 144 amino acid residues.

REFERENCES

Ansevin, A. T., Roark, D. E., & Yphantis, D. A. (1970) *Anal. Biochem. 34*, 237–261.

Arakawa, T., Alton, N. K., & Hsu, Y.-R. (1985) *J. Biol. Chem. 260*, 14435–14439.

Arakawa, T., Hsu, Y.-R., Chang, D., Stebbing, N., & Altrock, B. (1986) *J. IFN Res. 6*, 687–695.

Chou, P. Y., & Fasman, G. D. (1978) *Annu. Rev. Biochem. 47*, 251–276.

Devos, R., Cheroutre, H., Toya, H., Degrave, W., Van Heuverswyn, H., & Fiers, W. (1982) *Nature (London) 259*, 503–508.

Epstein, L. B., Cline, M. J., & Merigam, T. C. (1971) *Cell. Immunol. 2*, 602–613.

Green, J. A., Cooperband, S. R., & Kibrick, S. (1969) *Science (Washington, D.C.) 164*, 1415–1417.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Hsu, Y.-R., & Arakawa, T. (1985) *Biochemistry 24*, 7959–7963.

Rinderknecht, E., O'Connor, B. H., & Rodriguez, H. (1984) *J. Biol. Chem. 259*, 6790–6797.

Wheelock, E. F. (1965) *Science (Washington, D.C.) 149*, 310–311.

Yip, Y. K., Pang, R. H. L., Oppenheim, J. D., Nachbar, M. S., Henriksen, D., Zerebeckyj-Eckhardt, I., & Vilcek, J. (1981) *Infect. Immun. 34*, 131–139.

Yphantis, D. A., & Arakawa, T. (1987) *Biochemistry* (preceding paper in this issue).