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Sedimentation Equilibrium Measurements of Recombinant DNA Derived Human Interferon γ[†]

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ABSTRACT: Recombinant DNA derived human interferon γ (IFN-γ) from *Escherichia coli* was examined by equilibrium ultracentrifugation. Short-column equilibrium experiments at pH 6.9 in 0.1 M ammonium acetate buffer gave a *z*-average molecular weight of 33 500 ± 1400 at infinite dilution, corresponding to 1.98 ± 0.08 times the formula weight. Long- (2.6 mm) column experiments at pH 7.5 in 0.04 M imidazole buffer gave a molecular weight of 33 400 ± 500. Under the latter conditions IFN-γ behaves somewhat nonideally, with the departure from ideality accounted for by an effective (Donnan) charge of about 6+. No association of this dimer to form tetramer or higher polymers was observed, with the association constant for formation of tetramer from dimer K_{24} found to be less than 34 L mol^{-1}. Similarly, no dissociation to monomers was observable, with the dissociation constant to monomer K_{21} being less than 5×10^{-8} mol L^{-1}. At pH 3.55 in 0.02 M buffer (acetate plus acetic acid), there was virtually complete dissociation of the dimer to monomer. Extreme nonideality was seen in this low ionic strength system, and the effective charge on the protein was estimated to be about 11+. The reduced molecular weight $M(1 - \bar{v}\rho)$ of the monomer was found to be about 4.09 ± 0.20 kg mol^{-1}; this corresponds to a molecular weight of 16 410 ± 820, with the Scatchard definition of components. A small amount of a polymer with a molecular weight of about 0.5 $\times 10^6$ was detected under these conditions.

Although the amino acid sequence of human interferon γ (IFN-γ)[1] has been reported (Rinderknecht et al., 1984), there have been several conflicting reports on the molecular weight of IFN-γ. Values of 20 000 and 25 000 (along with a value of about 45 000 for a minor active component) have been observed by SDS-polyacrylamide gel electrophoresis for natural IFN-γ [see Kelker et al. (1983)]. The lower values are consistent with the expected monomer molecular weight for this glycoprotein. Treatment with glycosidases decreased the SDS gel estimates of sizes for these two components to values comparable to the sequence molecular weight with a possible C-terminal processing. Thus, the apparent subunit in SDS is a monomer. Gel filtration of either natural IFN-γ or recombinant *E. coli* derived IFN-γ in aqueous solution near neutral pH has indicated molecular weight values ranging from 34 000 to 70 000 [Rinderknecht et al., 1984; Devos et al., 1984; see Yip et al. (1982) for a review of earlier reports]. Target molecular weights ranging from 63 000 to 73 000 were found for similar IFN-γ preparations (Pestka et al., 1983) frozen at −135 °C in an unspecified solvent matrix when irradiated with 10-MeV electrons.

Accordingly, the size of the molecule in aqueous solution remains unknown, with the number of subunits variously es-

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[1] Abbreviations: IFN-γ, interferon γ; SDS, sodium dodecyl sulfate; DEC, Digital Equipment Co.; rpm, revolutions per minute; RMS, root mean square.

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timated from two to four. This paper presents sedimentation equilibrium determinations of the molecular weight of recombinant human IFN-γ from *E. coli* near neutral conditions where the molecule is stable and also under acidic conditions where the molecule has been reported to be partially denatured (Hsu & Arakawa, 1985).

Materials and Methods

Recombinant human IFN-γ was obtained as a nonaggregated form from *E. coli* by the chromatographic procedure described by Arakawa et al. (1985). Long-term storage was at −20 °C in 0.1 M NH_4OAc. Samples for examination were dialzyed against a several hundred fold excess of buffer and kept cold until used.

Sedimentation equilibrium experiments were performed in a Model E ultracentrifuge (Beckman Instruments Co.) equipped with a pulsed argon ion laser (Paul & Yphantis, 1972), a digital laser controller (Yphantis et al., 1984), and an automated control system for photography (Laue et al., 1984). Rayleigh interferograms were taken on Technical Pan 35-mm film (Kodak 2415) and were measured with a Digital Equipment Co. (DEC) PDP-8/L minicomputer controlled system [Laue & Yphantis, 1979; Laue and Yphantis, unpublished results; Laue, 1981) that provides estimates of fringe displacement as a function of position. Typically, the images were measured every 8 μm (corresponding to about every 4 μm in the ultracentrifuge cell).

Routine data analyses were performed by nonlinear least-squares approaches (Johnson et al., 1981) with various assumed models. The programs used were derived from a version of the NONLIN programming system written in FORTRAN for a DEC PDP 11/23 microcomputer system (M. L. Johnson private communication). Several of these programs have also been adapted for use under VMS on a DEC VAX 11/780 system. The indicated ranges of the fitting parameters normally returned by these programs correspond to a confidence range about 65% probability. (For Gaussian error distributions these 65% confidence regions are essentially equivalent to ranges of the mean plus or minus one standard deviation.) When the data from several channels were fit jointly to a common model, the data sets were thinned out to about 100 points per channel so as to keep the number of data points down to a manageable level. Apparent weight average molecular weights were calculated as a function of position with a variant of the BIOSPIN program (Roark & Yphantis, 1969; Roark, 1971).

Short-column equilibrium experiments were carried out in multichannel centerpieces (Yphantis, 1960) loaded with 15–20 μL of each solution. In these experiments, values of $M_{z,app}$, the apparent z-average molecular weights, were determined from nonlinear least-squares fits of the fringe displacements in a solution channel to a model of a single ideal component. This procedure is similar to an orthogonal linear least-squares procedure (Correia, 1981, 1983; Correia and Yphantis, unpublished results) and can be shown to be equivalent to determination of the $M_{z,app}$ from the ratio of the second derivative to the first derivative of the fringe displacement (Yphantis, 1964). Short-column experiments were run for a minimum time of 70 min. No differences in apparent molecular weights (nor in concentration distributions) were seen on prolonging the centrifugation time threefold.

Long-column experiments at both high and low speeds were carried out in "external loading cells" (Ansevin et al., 1970) with three pairs of channels and with centerpieces of either 12- or 30-mm optical path length. Blanks were run both before and after each set of runs at all the speeds used in the runs

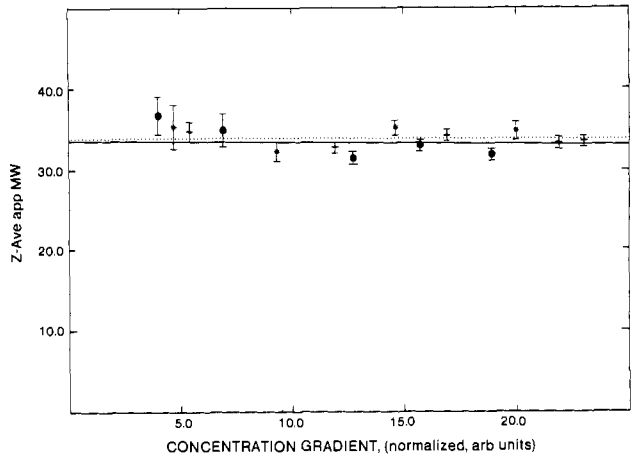

FIGURE 1: Short-column equilibrium ultracentrifugation of IFN-γ in pH 6.9 0.1 M ammonium acetate at 23.2 °C. The loading concentrations were 1.47, 1.11, 0.74, and 0.37 g L^{-1}, and equilibrium speeds were (*) 24 000, (+) 30 000, and (●) 36 000 rpm. Values of $M_{z,app}$ are presented as a function of the normalized concentration gradient, $(dc/dr)/(r\omega^2) = [dc/d(r^2/2)]/\omega^2$, at which they were observed. The dashed line represents the weighted average of the apparent z-average molecular weights and the solid line the weighted least-squares fit of the apparent z-average estimates to a straight line.

to correct for optical distortions. The centrifugation times used at each speed exceeded, in many cases severalfold, estimates of the time required to attain equilibrium. These estimates were made by use of the relation of van Holde and Baldwin (1958) with their ϵ parameter taken as 0.001 while assuming a (worst case) diffusion coefficient of 6×10^{-7} cm^2 s^{-1}.

The partial specific volume $\bar{v}$ of isoionic recombinant IFN-γ protein was estimated as 0.737_3 mL g^{-1} from the known sequence according to the method of Traube as detailed by Cohn and Edsall (1943). Partial specific volumes and expected monomer molecular weights for the polyelectrolyte salts of the protein both as usually defined (titration component) and as defined by Scatchard were estimated from this isoionic value, from the known sequence molecular weight (16 908), and from the effective (Donnan) charge as outlined by Johnson and Yphantis (1978) and by Szuchet and Yphantis (1976). Wherever possible, the auxiliary data needed for such estimations, including solvent densities, were obtained from the *International Critical Tables*, from Harned and Owen (1958), or from the *Handbook of Chemistry and Physics*. Molar volumes for unlisted components were approximated according to the method of Traube to obtain estimates of $\bar{v}$ (and hence density increments). Rough values of titration charge, without correction for nonidealities, were estimated at the pH of each experiment assuming that the protein had 20 carboxyl groups of $pK = 4.6$, 2 histidyl groups with $pK = 7.0$, 1 α-amino group with $pK = 7.8$, 4 tyrosyl groups with $pK = 9.8$, 20 lysyl groups of $pK = 10.2$, and 8 arginyl groups with pK above 12.

Results

A short-column equilibrium sedimentation experiment showed that IFN-γ exists as a dimer at pH 6.9 in 0.1 M NH_4OAc as was conjectured earlier (Arakawa et al., 1985). Figure 1 presents values of the $M_{z,app}$ as a function of the normalized concentration gradient $(dc/dr)/(r\omega^2) = [dc/d(r^2/2)]/\omega^2$, at which they were observed. (This graph is essentially equivalent to a presentation of the apparent z-average molecular weight as a function of observation concentration.) The dashed line is the weighted average, and the solid line is the (weighted) least-squares fit of the apparent z-average estimates to a straight line. It can be seen that the $M_{z,app}$ values are sensibly independent of the normalized gradient, [dc/(d-

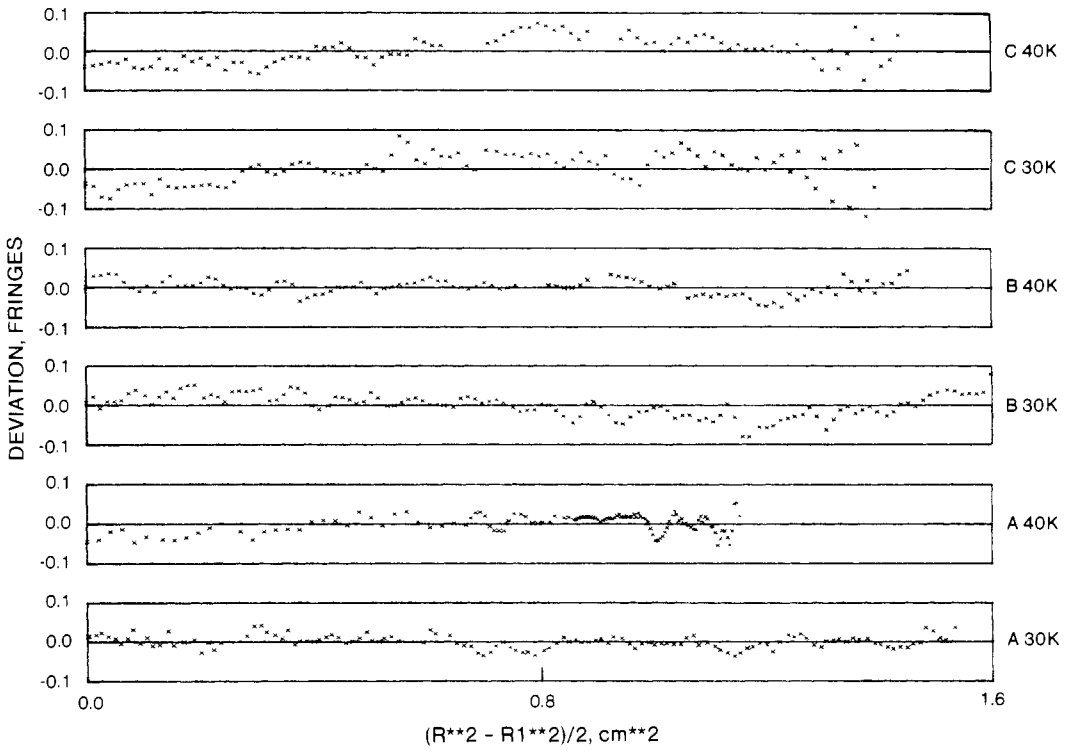

FIGURE 2: Fitting deviations as a function of position for IFN-γ in 0.040 M imidazole buffer, pH 7.5. One set of fringe displacements measured for six channels (three loading concentrations at two equilibrium speeds) was jointly fit to the model of a single solute component with simple nonideality so as to estimate a common value of the reduced molecular weight $M(1 - \bar{v}\rho)$, and of the second viral coefficient. The residuals from this fit are presented for each of these channels as a function of position. The loading concentrations used in the three-channel centerpiece were (channel A) 0.14, (channel B) 0.42, and (channel C) 1.4 g L^{-1}. Equilibrium speeds were 30000 and 40000 rpm. One fringe in the 12-mm optical path centerpiece used here corresponds to a concentration of about 0.25 g L^{-1}. The radius to an observation point is R, and the radius corresponding to the first point used in the fits is R_1.

$(r^2/2)]/\omega^2$ (and therefore of concentration). Similarly, there is no significant dependence on equilibrium speed. From the intercepts of both lines, we estimate the molecular weight at infinite dilution to be 33500 ± 1400. This value corresponds to 1.98 ± 0.08 times the formula molecular weight of the monomer and was obtained assuming the protein to be isoionic. Essentially, the same ratio (1.98 ± 0.08 and 2.02 ± 0.08) was found from calculations using component definitions corresponding (respectively) to the usual macromolecular salt (titration component) and to the macromolecular component defined according to Scatchard.

Equilibrium ultracentrifugation of IFN-γ was carried out at pH 7.5 in 0.04 M imidazole buffer. The initial concentrations loaded into the 12-mm optical path centerpiece were 0.14, 0.42, and 1.42 g L^{-1}, and the ultracentrifuge was run at 30000 and 40000 rpm at 29.5 °C. Two independent sets of measurements of blank corrected displacements were obtained for each of the three channels (loading concentrations) of both 30000 and 40000 rpm. The system was analyzed by fitting each of the two sets of six blank corrected measurements separately to a number of models. It was found necessary to include simple nonideality, but there was no significant improvement in fits using models that included more complex nonideality, dissociation, self-association, or heterogeneity. The fits reported correspond to the model of a single nonideal component, expressing all the nonideality through the second viral coefficient.

Figure 2 presents the residuals from one such fit. The fitting procedure returned the values 8.836 ± 0.130 and 8.782 ± 0.125 kg mol^{-1} for the reduced molecular weight and (1.01 ± 0.15) × 10^{-3} and (0.89 ± 0.14) × 10^{-3} mol mL g^{-2} for the viral coefficient B_2, with RMS residuals of 0.033 and 0.027 fringe, respectively, for the two data sets. [The reduced molecular weight is given by $M(1 - \bar{v}\rho)$, where M is the molecular weight, $\bar{v}$ the partial specific volume, and ρ the density.] The corresponding molecular weights are 33690 ± 490 and 33480 ± 480 with the isoionic $\bar{v}$ and 33550 ± 490 and 33340 ± 480 with the value of $\bar{v}$ estimated for the Scatchard component. For comparison, the dimer formula weight is 33816 and the predicted Scatchard molecular weight is 33501 with the estimated titration charge (33627 with the inferred Donnan charge). Making the assumption that all the observed nonideality arises from the Donnan effect, the values estimated for the second viral coefficient correspond to an effective charge z of 6.3 ± 0.5 and 5.9 ± 0.5, about 60% of the estimated titration charge.

Attempts were made to set limits on potential dissociation or association of the dimer in this experiment. Both of the data sets (each consisting of three channels of data at 30000 rpm and three channels at 40000 rpm) were separately fit to models of reversible chemical equilibrium with simple nonideality; in looking for dissociation, the data sets were fit as a monomer–dimer system, and when examining the possibility of association, the data sets were fit as a dimer–tetramer system. In these searches, we set the dimer molecular weight and the second viral coefficient for all species equal to the mean of the two sets of values already estimated; i.e., we made the assumption that is often made (vide infra) that the nonideality is the same for all species of the putative equilibria. In addition, we assumed that all species shared the same partial specific volume and refractive increment. The decrease in the RMS of the fitting deviations upon inclusion of the assumed equilibria was less than 2 × 10^{-4} fringes, a completely negligible decrease. We found that we could set the upper limit of the dissociation constant for the dimer as 5 × 10^{-8} mol L^{-1} and the upper limit of the equilibrium constant for dimerization

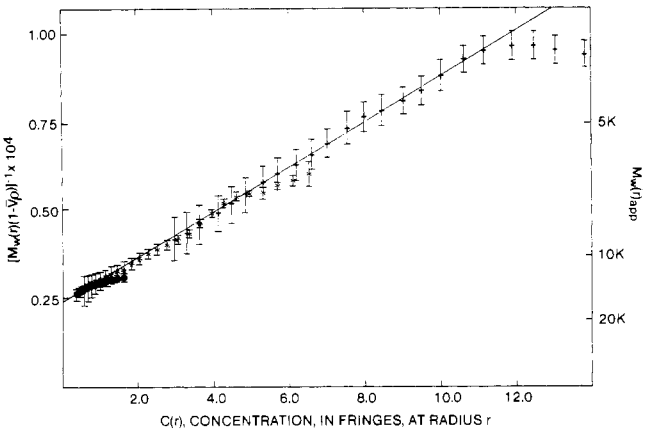

FIGURE 3: Reciprocals of the apparent reduced weight-average molecular weights of IFN-γ at pH 3.55 and an ionic strength of 0.0012 M. Three solutions of IFN-γ at concentrations of 0.097 (●), 0.29 (*), and 0.97 g L^{-1} (+) were equilibrated simultaneously at 40 000 rpm in a pH 3.55 buffer containing 0.020 M buffer (acetic acid + sodium acetate). The reduced weight-average molecular weights shown were obtained by 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 because of the extreme nonideality and the limitation to a maximum operating speed of 40 000 rpm for the 30-mm optical path centerpieces. For clarity, only one out of every four points obtained is shown here. The straight line corresponds to the parameters of the "best fit" (see text). The isoionic partial specific volume was used to fix the auxiliary molecular weight scale shown.

of the dimer (to form tetramer) as 34 L mol^{-1}. These values correspond, roughly, to the experimental error of the determinations; at a dimer concentration of about 2 g L^{-1}, they each represent a concentration of monomer (or tetramer) that is about the same as the RMS of the residuals of the fits.

Out of concern that we were restricting unduly the search for association of the dimers to tetramers by fixing the nonideality at the mean of the values estimated for the two data sets, we repeated these searches, fixing only the dimer molecular weight and converging on K_{24} (the association constant to form tetramer from dimer) and on the second viral coefficient, along with the 12 other parameters needed for the fits of the six channels in each data set. Again we were able to set the same limits for the association of the dimer (corresponding to the 65% confidence level). Attempts to fix molecular weight, nonideality, and association simultaneously were only partially successful; although the least-squares procedures yielded essentially the same values of the fitting parameters indicating completely negligible association, the data were insufficiently robust to properly define the error ranges of the K_{24}.

Acetate buffers near pH 3.5 were used as dissociating solvents for IFN-γ. In one experiment the protein was examined at a low ionic strength (about 0.0012 M) at pH 3.55 in 0.02 M buffer (acetic acid + sodium acetate). We used an external-loading multichannel centerpiece with a 30-mm optical path to facilitate measurements at the low observation concentrations needed with the large expected nonideality of this system. The loading concentrations used were 0.097, 0.29, and 0.97 g L^{-1}, and the experiment was performed at a speed of 40 000 rpm and at 24.7 °C.

The reciprocals of the apparent weight molecular weights determined for this system are presented in Figure 3 as a function of observation concentration. This system appears to be extremely nonideal, with nonideality comparable to that of aldolase (Szuchet & Yphantis, 1973) or β-lactoglobulin (Szuchet & Yphantis, 1976) in 0.1 M acetic acid. The lack of strict overlap of the curves for the three loading concentrations indicates that the system cannot be described as a single-component system. The limited divergence of the curves near the base of each channel suggests that the heterogeneity is caused by the presence of small amounts of aggregates much larger than the principal component.

Least-squares fitting for estimation of the size and nonideality of the principal component in this system requires either a complex model (that includes the presence of the aggregates as well as a description of the nonideality) or the exclusion of regions with significant amounts of aggregates from the fits. Preliminary attempts to fit the complete distributions as a heterogeneous system with two molecular species and either simple or complex nonideality were unsatisfactory. Accordingly, as in experiments with β-lactoglobulin (Szuchet & Yphantis, 1976), we fit truncated data sets with the simple model of a single nonideal component with a single (second) viral coefficient. It was found adequate to truncate the original data sets at the points suggested by Figure 3; data were discarded for fringe displacements corresponding to concentrations greater than 1 fringe for channel A, 4 fringes for channel B, and 11 fringes for channel C. This truncation of the data gave what we consider to be our best estimate of the parameters of the main component of IFN-γ at pH 3.55 at low ionic strength: reduced molecular weight = 4.09 ± 0.20 kg mol^{-1} and B_2 = (7.4 ± 0.5) × 10^{-2} mol^{-1} mL g^{-2}. The associated fitting deviations were 0.051 fringe RMS. The values of the fitting parameters returned by NONLIN are not critically dependent on the exact truncation limits used. More extensive pruning of the data yielded essentially the same values of the fitting parameters (with larger uncertainties in the fitting parameters and lower values of the fitting deviations). On the other hand, less truncation resulted in systematically lowered estimates of both the apparent reduced molecular weight and the second viral coefficient (with lower uncertainties and larger values of the RMS fitting deviations) (data not shown) with the complete original data set giving values of reduced molecular weight of 3.17 ± 0.21 kg mol^{-1} and B_2 of (4.9 ± 0.5) × 10^{-2} mol mL g^{-2}.

The simple procedure of using the native value of the partial specific volume for estimation of molecular weights in acetic acid solutions gives results that appear to be reliable to within 10% (Szuchet & Yphantis, 1973, 1976; Szuchet, 1976). With this procedure, our best estimate of the reduced molecular weight, 4.09 ± 0.20 kg mol^{-1}, corresponds to a molecular weight of 15 460, about 91% of the formula weight of the monomer. Making the assumption that all observed nonideality arises from the Donnan effect, we calculate the effective charge on the monomer to be 11.0 ± 0.3. This corresponds to about 38% of the maximum tritration charge on IFN-γ under these conditions. Values in the range 30–60% have been observed for the effective Donnan charge in other protein systems in similar solvents (Szuchet & Yphantis, 1973, 1976; Szuchet, 1976).

The generalized Scatchard definition (including preferential interactions with solvent components) is the appropriate component definition for charged macromolecules in sedimentation equilibrium experiments using refractometric optics without any assumptions of mass conservation (Szuchet & Yphantis, 1973). The only change needed here in the equations previously presented is the minor one of properly accounting for the two-component supporting electrolyte used here. Instead of subtracting $z/2$ moles of a single salt component, we subtract $\alpha(z/2)$ moles of the salt present at a mole fraction of α and $(1 - \alpha)(z/2)$ moles of the remaining uni-

univalent supporting electrolyte. (This is feasible because the counterion to the macroion is common to both salts.) Using this modificaion of the equations of Szuchet and Yphantis (1976) and taking the effective charge as 11 and the number of cationic groups that become charged between pH 9.8 (the estimated isoionic point of IFN-γ) and pH 3.55 and 7.7, we obtain a molecular weight 17 145, a molar volume of 12 904 L mol^{-1}, and a partial specific volume of 0.752_6 mL g^{-1} for the Scatchard component corresponding to the known composition of IFN-γ. These values assume that there are no preferential interactions with any of the solent components and that there is no change in the volume of the protein species, above and beyond any expected changes in the electrostriction associated with titration from the isoionic point to pH 3.55 [see Kauzmann (1958), Rasper and Kauzmann (1962), and Kauzmann et al. (1962)]. The former assumption seems reasonable at such low acetate concentration (Arakawa & Timasheff, 1982). Using the above estimate for $\bar{v}$, our "best estimate" of the reduced molecular weight corresponds to a molecular weight of 16 410 ± 820 for the Scatchard component; this is 95.7 ± 4.8% of the predicted value.

The degree of polymerization and the amounts of aggregates seen in this experiment were examined with nonlinear least-squares fitting procedures under the following assumptions: (a) the main component is characterized by our "best estimate" of the parameters; (b) the aggregates present all have the same degree of polymerization, j; (c) all species have the same partial specific volume and refractive increment; (d) all species share the same value for the second viral coefficient. The latter is a common assumption, equivalent to the assumption that the activity coefficients of all species (j-mers) are equal to j times the activity coefficient of the monomer (Adams & Williams, 1964; Roark & Yphantis, 1969). If the nonideality is primarily due to charge (Donnan) effects, it is equivalent to the assumption that all species have the same charge to mass ratio (Roark & Yphantis, 1971). Under these assumptions, fitting of the original untruncated data set to the model of a heterogeneous nonideal system of two molecular species with a common second viral coefficient indicated the aggregate to be about a 33-mer, with a molecular weight just over a half million. The amount of aggregate estimated in the three channels is (A) 1.0%, (B) 1.0%, and (C) 6.3% of the total protein in each channel. These estimates depend only weakly on the size of the aggregate. They should be considered as rough estimates only, good to within, perhaps, a factor of 2 in view of the assumptions made.

Discussion

It is well-known that association and nonideality can mask each other's presence no matter what thermodynamic or semithermodynamic observation technique is used. Thus, there is always some question about the uniqueness of descriptions obtained in terms of the extent of nonideality and association, notably dimerization. This masking can be completely effective, however, only at very low levels of dimerization. Self-association may be expressed as a viral coefficient expansion valid for low fractions of polymer (Stafford & Yphantis, 1972) and is reflected in the second viral coefficient for dimerization only; the effects of all other associations are reflected solely in higher viral coefficients. The viral coefficient expansion for dimerization may be written as $M_1/M_w = 1 - \alpha + 3\alpha^2 - \ldots$, where α is the weight fraction of dimer. Thus, a small enough extent of dimerization would be directly and fully compensated for by a second viral contribution from, say, excluded volume or Donnan effect. The nonlinear least-squares approach used here reflects the existence of such potential compensation in a large cross-correlation coefficient (>0.998 in the fits of the pH 7.5 data) between the second viral coefficient and the dimerization constant; the effects of the associated uncertainty are included in the estimated confidence ranges of both these variables. Consequently, the estimates of the limits of association of the dimer to form tetramer are believed to be valid.

The molecular weight of IFN-γ at neutral pH established in this paper is inconsistent with the value determined by radiation inactivation (Pestka et al., 1983). The latter method may be complicated for self-associating proteins by a number of factors that can affect self-association. Although the dimeric structure was established with the recombinant *E. coli* derived IFN-γ, it is very likely that the natural, glycosylated protein with C-terminal processing is also a dimer. This speculation is based on the observations that neither glycosylation nor C-terminal processing apparently alters the protein conformation (Arakawa et al., 1986b) and that the self-association of *E. coli* derived IFN-γ occurs without 13 C-terminal residues (Arakawa et al., 1986a). These results suggest that the binding site to form the dimer is identical in recombinant *E. coli* derived and natural IFN-γ preparations. The dimeric molecular weight has been suggested for the natural IFN-γ preparations with gel filtration technique (Devos et al., 1984; Rinderknecht et al., 1984; Miyata et al., 1986). However, the elution volume of proteins in gel filtration is a function of their Stokes radii rather than their molecular weights. Glycoproteins such as natural IFN-γ can have an anomolous Stokes radius, as observed for erythropoietin (Davis et al., 1987).

This appears to be the first rigorous demonstration of dissociation of IFN-γ dimers to monomers under acidic conditions. This dissociation seems to be correlated with denaturation of the protein under the same conditions (Arakawa et al., 1987). Thus, increased electrostatic repulsion at low pH and ionic strength resulted in the denaturation and dissociation. Miyata et al. (1986) suggested a dissociation of natural IFN-γ at pH 11 using a gel filtration technique. However, gel filtration under such alkaline condition is confounded by the possibility that standard proteins and the protein in question may behave differently. For example, a standard protein that fully denatures at pH 11 could have the same elution position as a more stable protein at that pH with a molecular weight 2 times that of the standard protein, since the denatured protein has a larger Stokes radius.

The nature of the aggregates observed at pH 3.55 is of interest. These aggregates are not in rapid (within a time scale of several hours) chemical equilibrium with monomer since the apparent molecular weights are not single-valued functions of observation concentration. The (remote) possibility that the apparent heterogeneity arises from volume changes on association can be ruled out since the correlation between the logarithm of the apparent association constant needed to describe the aggregation in each channel with the hydrostatic pressure near the base of each channel is poor (data not shown). Thus, these aggregates must reflect heterogeneity.

The amount (6%) of the large aggregate (about a 33-mer) seen in the channel (C) with the highest loading concentraction appears to be significantly higher than the amounts present (1%) at the two lower lower loading concentrations where the maximum concentrations are less than half those in channel C. Small amounts (>2%) of a similar aggregate (about a 16-mer) were found at loading cncentrations comparable to the two lower loading concentrations used here in another sample of IFN-γ with a different history but under similar

solvent conditions (Arakawa et al., 1987). These observations suggest the possibility of increased aggregation at higher concentrations, perhaps related to the aggregation observed in the same solvent but with 0.1 M salt added (Arakawa et al., 1987). Further experiments are needed to establish if the concentration dependence of this aggregation is reproducible and, if so, whether any part of this aggregation is reversible and under what conditions.

Knowledge of the effective charge at pH 3.55 enables us to estimate the extent of acetate binding to IFN-γ under our experimental conditions. Assuming that the molecule can be represented by a compact uniformly charged sphere (Edsall & Wyman, 1958) with a radius estimated from $\bar{v}$ and M as 19.3 Å and with a distance of closest approach of small ions of 21.3 Å, we calculate an electrostatic "w factor" of 0.153. At an effective charge of 11+, the effective pK of the carboxyl groups is decreased by $-(0.868wz)$ to an effective pK of 3.1. The net titration charge on the protein is then estimated to be about 16.6+. The difference of 5.6 between this value and the observed effective (Donnan) charge is our estimate of the acetate binding under these conditions. This must be considered only a rough estimate since it is subject to several assumptions made both directly in selecting model parameters and indirectly through the use of simple nonspecific equations for electrostatic nonideality.

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