The Effect of pH and Temperature on the Self-Association of Recombinant Human Interleukin-2 as Studied by Equilibrium Sedimentation

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The self-association of recombinant human interleukin-2 (rhIL-2) in solution was investigated as a function of pH and temperature using equilibrium sedimentation. Studies were performed at pH 3.6, 6.5 and 8.2, at 1°C and 20°C. A model assuming an ideal single molecular species describes the data observed at pH 6.5 at both temperatures. At pH 8.2, the data from both temperatures can be better described by a weak monomer-dimer association equilibrium. The values of the association constants obtained indicate the presence of less than 10% dimer at a concentration of 1 mg/ml at both temperatures. At pH 3.6, aggregates with a Z average molecular weight of over 35 times that of monomeric rhIL-2 were formed. The smallest associating species present under these conditions corresponds to the monomer, which produces aggregates with a wide range of molecular weights. The monomer appears to be in equilibrium with the smallest aggregates, in that a model describing an indefinite association fits the data obtained at the highest centrifugal speed. No model was found to successfully describe the association of the monomer into the much larger aggregates observed at lower speeds. This may be the result of the lack of rapid thermodynamic reversibility of the larger aggregates. Temperature was found to have no significant effect on the largest aggregates that were formed at pH 3.6.

KEY WORDS: interleukin-2; self-association; aggregation; analytical ultracentrifuge.

INTRODUCTION

In recent years, commercial production of therapeutic proteins has been made possible due to advances in recombinant DNA technology (1,2). Most commercially available proteins are lyophilized for increased shelf-life (3), which have to be reconstituted into solution prior to clinical use (4). The process of lyophilization involves freezing the solution prior to the removal of water, which can concentrate the protein, buffer salts, electrolytes. During this process of freezing, the solution pH is known to change (5), which causes inactivation of enzymes and proteins (6,7), due to irreversible denaturation leading to aggregation and precipitation.

Human interleukin-2 (IL-2), a therapeutic protein was the first hormone of the immune system to be discovered and characterized (8,9). Recently, recombinant human interleukin-2 (rhIL-2) was approved for treatment in early stages of kidney cancer, and is being evaluated for its use in AIDS and other immunological dysfunctions (10).

The native structure of rhIL-2 is important for the interaction with its specific receptors on cells (11). Changes in the solution environment have been reported to alter the structure of rhIL-2. Between pH 4 and 2, loosening of the tertiary structure without a significant change in the secondary structure of rhIL-2 was reported (12). The self-association of rhIL-2 was studied at pH 7.0 using indirect methods such as fluorescence intensity dependance on rhIL-2 concentration, collisional quenching of tryptophan fluorescence and by monitoring rhIL-2 effects on membrane fusion (13). In these studies rhIL-2 was found to exist as a dimer at concentrations greater than 0.4 micromolar.

We report here the effect of pH and temperature on the self-association of rhIL-2 as studied by equilibrium sedimentation.

MATERIALS AND METHODS

Recombinant human interleukin-2 (rhIL-2) was a gift from Cetus Corporation (now Chiron Corporation). Its sequence is identical to that of human IL-2 (14) with the following exceptions: (i) it consists of 132 amino acids, the N-terminal residue being proline, (ii) cysteine at position 125 in the amino acid sequence is replaced by serine and (iii) the molecule is not glycosylated. Based on this amino acid composition, the molecular weight of rhIL-2 is 15.3 kDa (kilo Daltons). The protein was supplied as a frozen aqueous solution (at −80°C) in 0.01M citrate buffer, at pH 6.5, containing 0.15M NaCl. This solution was subdivided into small vials and frozen again. The vials were used as needed.

Sodium citrate and sodium chloride were obtained from Sigma Chemicals and J. T. Baker Inc. respectively, sodium hydroxide and hydrochloric acid were obtained from Fisher Scientific Company. These chemicals were used without further purification.

Dialysis

Typically 200μl of rhIL-2 was exhaustively dialyzed against 0.01M citrate buffer containing 0.15M NaCl at different pHs (3.6, 6.5 and 8.2), with the pH being adjusted by adding either 0.1N NaOH or 0.1N HCl. The final ionic strength of the buffer (due to the presence of salt and citrate species) was 0.16 M at pH 3.6 and 0.2 M at pH 6.5 and 8.2 respectively, and did not change with the addition of the acid or base. Although at pH 8.2, the buffer capacity of citrate buffer is low ($pK_{a,3}$ = 6.4), the purpose of this investigation was to study the effect of citrate species on the self-association of rhIL-2. All dialysis experiments were performed at 2°C using a DiaCell™ assembly manufactured by Instrumed Company.

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Ultracentrifuge Studies

For all experiments, a six channel exterior loading analytical ultracentrifuge cell was used (15). The cell was loaded such that the top channel contained 0.14 mg/ml, middle channel 0.5 mg/ml and the bottom channel 1.4 mg/ml of rhIL-2. Appropriate dilutions were made with the dialyzed buffer such that the final volume in each of the three channels was 100μl. Prior to the loading of the solution, 10μl of FC-43 oil was added to improve the visibility of the bottom of the cell. The reference channels were loaded with 120μl of the dialyzed buffer.

The cell containing the solution-solvent pairs was centrifuged in a Beckmann Model E analytical ultracentrifuge at a given rotor speed and controlled temperature. The rotor speeds were 7.2 k (1°C and 20°C), 14 k and 28 k RPM (1°C) at pH 3.6, 28 k and 36 k (1°C) and 36 k and 48 k RPM (20°C) at pH 6.5 and 8.2. Images of Rayleigh interferograms were recorded using a TV camera and digitized using a PC controlled system (16), every 2 hours starting 18 hours after the run began. Equilibrium was said to have been attained when two images recorded two hours apart were indistinguishable. Similar procedure was followed at different speeds and temperatures. The specific volume of rhIL-2 (0.75 ml/gm) was calculated from the amino acid composition data using the method of Cohn and Edsall (17). The density of the dialyzed solvent was measured with a Paar DMA 602 densitometer at 20°C (1.0064 g/ml) which was corrected to 1°C (1.0081 g/ml).

Data Analysis

For an ideal system, under the influence of centrifugal force, the molecular weight M_i of the ith component in solution can be related to the effective reduced molecular weight σ_i (18) by the equation,

$$\frac{\partial \ln C_{r,i}}{\partial(r^2/2)} = \sigma_i = \frac{M_i(1 - \bar{v}\rho)\omega^2}{RT} \quad (1)$$

where $C_{r,i}$ = concentration of ith component at radius r: R = Gas constant, T = Absolute temperature, $\bar{v}$ = partial specific volume, ρ = density, ω = angular velocity, and r = radius of the cell from the center of rotation. Thus, the concentration distribution of the ith species for σ_i, is given by,

$$C_{r,i} = C_{0,i}\exp\sigma_i\left(\frac{r^2 - r_0^2}{2}\right) \quad (2)$$

where $C_{o,i}$ = concentration of the ith component at radius r_o.

For a monomer-nmer (n = 2,3 etc.) association, the total concentration $C_{r,t}$ at any radius r, can be expressed in terms of the monomer concentration $C_{r,1}$ and an association constant K_n by,

$$C_{r,t} = C_{r,1} + K_n(C_{r,1})^n \quad (3)$$

Since the Rayleigh interferometer measures relative refractive indices (proportional to concentration) as a function of distance across the cell, another parameter δy is introduced for each channel. This represents the y-axis offset of the interference fringes. Therefore the total concentration (expressed as fringe shift) at any position in the cell can be represented as,

$$C_{obs} = C_{true} + \delta y \quad (4)$$

If one knows the value of dn/dc (change in refractive index with concentration), the fringe shifts and equilibrium constants can be converted to any concentration units needed.

With the assumption that no volume change occurs on association, equations (2) through (4) are combined into one equation which is used to globally fit the experimental data from all channels and at all speeds simultaneously by means of a non-linear least squares program, "NONLIN" (18). Each attempted fit results in a root mean square (RMS) error and a graph of residuals. Quality of fit for a particular model is determined by the following criteria: (i) the value of RMS error should be small and similar to that obtained from the optical system (the average RMS value for a blank is in the range of 0.01 to 0.02 fringes) and (ii) the scatter of residuals should be random.

Initially, an ideal single molecular weight species (ISS) model (Equation 1) is used to fit the data which gives the Z average molecular weight (M_z) (19). If the fit is good, this value is the molecular weight (M_1) of the species. If the fit is bad, it suggests the presence of one or more of the following factors: a) self-association (negative virial coefficient), b) repulsive interactions (positive virial coefficient), or c) some non interactive species (solution is a mixture). Subsequently, attempts are made to fit a series of ideal and non-ideal association models to the data. For each model fitted, the molecular weight of the monomer (M_1), the value of ln K, (where K is the association constant) and the value of B (the second virial coefficient) are produced. If the fits are materially improved by allowing the data from each loading concentration and centrifuge speed to produce an independent value for ln K, this indicates the presence of species that are not in reversible equilibrium (20).

RESULTS AND DISCUSSION

The first study was conducted to investigate the self-association of rhIL-2 at pH 6.5 and 20°C. It was found that the ISS model successfully describes the data under these conditions, resulting in a value for M_z (and hence probably M_1 because of the good fit, i.e. RMS error of 0.013 fringes) of 16.0 ± 0.1 kDa. Figure 1 shows the concentration as a function of distance from the meniscus and the inset shows a plot of residuals as a function of distance. The latter does not display any significant systematic error. An ideal monomer-dimer model (with a dimerization constant which was within error limits equal to zero) also fit the data well (low RMS error of 0.013 fringes and residuals essentially identical with the ISS) yielding the same value for the monomer molecular weight as the ISS fit. It is therefore clear that under these conditions rhIL-2 exists largely as a monomer. The ideal monomer-trimer model fit to the data yielded reasonable values for M_1 and the RMS error (16.2 kDa and 0.02 fringes), however the residuals showed considerable systematic error at the higher concentrations (Fig 2). Attempts to fit other models were unsuccessful, in that they displayed consider-

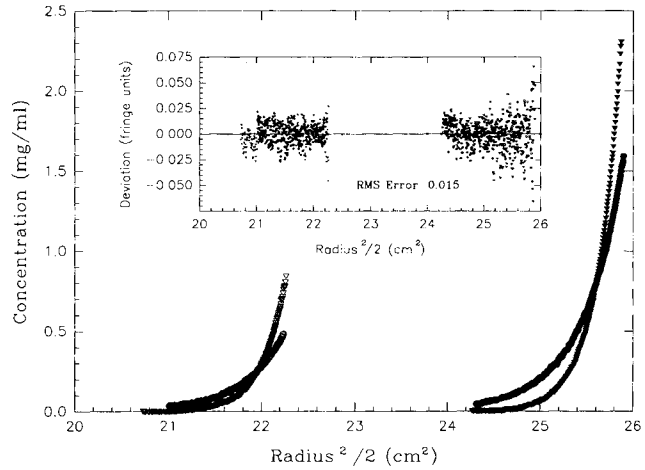

Fig. 1. Plot of concentration versus the distance from the center of the meniscus for an ideal single species model at pH 6.5, 20°C. Key: (○) channel 1, 36 k RPM; (▽) channel 1, 48 k RPM; (●) channel 2, 36 k RPM; and (▼) channel 2, 48 k RPM. Inset shows a plot of the scatter of residuals versus distance for an ideal single species model at pH 6.5, 20°C.

able systematic error and usually large RMS error. In the interest of brevity, unless necessary for clarity of the presentation, only those fits which do not show noticeable systematic error will be discussed.

Since a change in temperature can alter association, the self-association of rhIL-2 was investigated at 1°C. The value of M_z found from the ISS model at this temperature was slightly higher (16.8 ± 0.2 kDa) than at 20°C, perhaps indicating the presence of higher molecular weight aggregates. An ideal monomer-dimer model could also be fitted to the data, and as at 20°C, the RMS error for this model and the scatter of residuals were similar to that of the ISS model. The resulting value of M_1 (16.0 ± 0.2 kDa) for the monomer-dimer model is slightly closer to the reported value than that of the ISS fit. The dimerization constant (4 ± 1 × 10^2 l/mole, RMS error 0.011 fringes) indicated that a small amount of self-association might be present. In order to see if the apparent change in the dimerization constant with temperature is real, the data from the two temperatures were fitted simultaneously to the monomer-dimer model, after introducing corrections for the change in the values of the density and RT (gas constant and temperature). The appearance of systematic errors and/or a segregation of the residuals by temperature, with the equilibrium constant constrained to a single value would indicate a significant change in the association with temperature. If such is observed and then the equilibrium constants are released (unconstrained) they should then fall into two sets of values corresponding to the two temperatures. This technique of fitting forces the value of M_1 to have the same value at both temperatures. The fits for both the ISS and the dimerization model with the equilibrium constant constrained were good and essentially identical thus indicating no change in association with temperature. A value of M_z for rhIL-2 of 16.2 ± 0.1 kDa was found from both models with reasonable RMS error (i.e. 0.014 and 0.015 fringes). The resulting constrained dimerization constant was within error limits equal to zero indicating very little dimerization at the two temperatures. These results are included in Table I. The combined data could not be successfully fit by any other association model. Thus it appears that at pH 6.5 rhIL-2 exists largely as an ideal monomer at both temperatures.

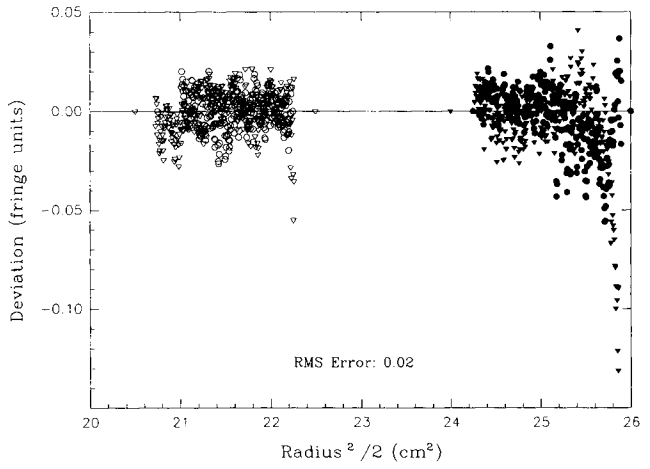

Fig. 2. Plot of scatter of residuals versus distance for a monomer-trimer model at pH 6.5, 20°C.

At pH 8.2, at 1°C and 20°C the M_z of rhIL-2 resulting from the ISS model were determined to be 16.9 ± 0.1 kDa and 19.2 ± 0.1 kDa respectively. These values are larger than those measured at pH 6.5, possibly due to the presence of higher molecular weight aggregates at pH 8.2. The association model that best described the data at both temperatures separately, was the ideal monomer-dimer, with moderately different values of the dimerization constant at the two temperatures. On fitting as ISS model to the data from the two temperatures simultaneously, a value of M_z of 17.8 ± 0.2 kDa was obtained. However the fit was bad, with an RMS error of 0.053 and the presence of systematic error. The combined data could be best described by an ideal monomer-dimer model, in which the equilibrium constants were unconstrained, and M_1 had a value of 16.1 ± 0.2 kDa. The resulting values of K_2 fell into size ranges corresponding to the two temperatures. The average values were 9.4 ± 2 × 10^2 l/mole at 1°C and 2.8 ± 0.5 × 10^2 l/mole at 20°C. The RMS error for this fit was 0.018 which was a significant improvement over that of the ISS model. The scatter of residuals for this fit was good, being similar to that of Fig. 1. It therefore appears that at this pH the protein engages in a weak monomer-dimer self-association in which the value of K_2 increases as the temperature is lowered.

At pH 3.6, the value of M_z (determined from the ISS fit) was found to be 20.1 ± 0.4 kDa at 1°C and 28 k RPM. The only association model which described the data successfully was that of the isodesmic indefinite association model. The assumptions behind this model (21) are that; any monomer or assembly of monomers can associate with any other monomer or assembly of monomers, and all of the interactions involve the same free energy and therefore may be expressed with one association constant. The value of this association constant was found to be 4.6 × 10^2 l/mole. The molecular weight calculated for the associating monomer (17.6 ± 0.2 kDa) is higher than that expected from the composition (15.3 kDa) and somewhat higher than that found from the ISS model at pH 6.5 (16.2 ± 0.1 kDa) or for the

Table I. The effect of pH and temperature on the self-association of rhIL-2[1]

Experimental[2] Conditions	Ideal Model(ISS)		Specific Association Model			
	M_z (kDa)[3]	RMS error	Model	M_1 (kDa)	K (l/mole)	RMS error
pH 6.5, 1°C and 20°C	16.2 ± 0.1	0.014	Monomer-Dimer	16.2 ± 0.1	0[a]	0.015
pH 8.2, 1°C and 20°C	17.8 ± 0.2	0.053	Monomer-Dimer	16.1 ± 0.2	$9.4 \pm 2 \times 10^2$ at (1°C)[b] $2.8 \pm .5 \times 10^2$ at (20°C)[b]	0.018
pH 3.6, 1°C, 28 k RPM	20.1 ± 0.4	0.020	Indefinite Association	17.6 ± 0.2	463	0.019
pH 3.6, 1°C, 14 k RPM	112.3 ± 6.7	0.038	No association model which included the monomer fit the data			
pH 3.6, 1°C and 20°C, 7.2 k RPM	582.7 ± 8.4	0.084	No association model which included the monomer fit the data			

[1] Only the best fits are included, except for the ISS fits which are necessary in order to provide a value for M_z.
[2] Where two temperatures are listed, the data from the two temperatures were combined.
[a] The single constrained dimerization constant yielded as good a fit as the unconstrained.
[b] The unconstrained dimerization constant yielded the best fit.
[3] $M_z = M_1$ if the ideal model is a good fit.

associating monomer at pH 8.2 (16.1 ± 0.2 kDa). These higher values may be due to the uncertainty of the value calculated for the specific volume as the effects of molecular conformation and solvation are not taken into account by the calculation. Since a 1% error in the evaluation of the specific volume leads to an almost 3% error in the calculated molecular weight, one might expect such changes as solvent conditions are varied. The even larger value of M_1 observed at pH 3.6 may be due to larger changes in the specific volume under these more extreme solvent conditions. The only way one can confirm this is by measuring the density of the solution to at least six significant figures in the various solvents.

The higher value observed for M_z suggested that higher aggregates may be present that were not being observed at 28 k RPM. This prompted us to perform experiments at lower speeds at this pH in order to observe larger aggregates. At 14 k RPM and 1°C, the M_z was found to be 112 ± 7 kDa. At 7.2 k RPM the data taken at the two temperatures (1° and 20°C) yielded similar values for M_z, the average of which was 583 ± 8 k Da, thus indicating the presence of large aggregates. The bad fit obtained indicates that a wide range of molecular sizes are present. It was not possible to describe either the separate or the combined data from the 7.2 k and 14 k RPM runs with any specific association model using the monomer. This may indicate a complicated association path above an aggregate of a few monomers and/or thermodynamic irreversibility (or perhaps a kinetically impeded equilibrium) of the higher aggregates. It is interesting to note that the larger aggregates (those observed at 7.2 k RPM) displayed no temperature sensitivity.

From these studies it can be concluded that rhIL-2 exists largely as an ideal homogenous monomeric species at pH 6.5, and not a dimer as reported in the literature (13). At pH 8.2, the best fitting model is that of a monomer-dimer association. The values of K_2 indicate that the dimerization is weak and gets weaker with increasing temperature. Strong self-association is observed at pH 3.6 in which aggregates of rhIL-2 are formed. The very different values of M_z observed by centrifugation at various speeds indicate that a wide range of aggregates are present at this pH. Although there is a reversible equilibrium (indefinite association) involving the monomer for the smaller aggregates (those observed at the higher speed) this is not so for the larger aggregates. Our inability to find an association model for these larger aggregates involving the monomer indicates that either the larger aggregates are formed through a more complex pathway and/or there is little (or slow) reversibility of such equilibria. The lack of sensitivity of the size of the larger aggregates to temperature change reinforces this interpretation.

These findings are important in the formulation of protein drugs. The side chains of proteins usually undergo deamidation, oxidation and disulfide exchange reactions at physiological pH. Therefore, a lower pH would be suitable to minimize these reactions in protein formulations. Our results indicate that proteins may aggregate at lower pH, and therefore stability consideration should include the possibility of pH induced aggregation during formulation development.

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