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High Concentration Formulations of Recombinant Human Interleukin-1 Receptor Antagonist: I. Physical Characterization

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Abstract

At relatively high protein concentrations (i.e., up to 100 mg/mL), recombinant human interleukin-1 receptor antagonist (rhIL-1ra) was found to exist in a monomer–dimer equilibrium controlled by solution ionic strength. Sedimentation equilibrium at 25°C was used to measure the increase in the dimer dissociation constant (K_d) as a function of ionic strength. K_d increased from 2.0 to 12.6 mM as the solution ionic strength was increased from 0.011 to 0.184 molal. These K_d values were used with both static light scattering and membrane osmometry data collected over a protein concentration range of 1–100 mg/mL to determine second osmotic virial coefficients. Expanding the second osmotic virial coefficient model to account for separate monomer–monomer (B_{22}), monomer–dimer (B_{23}), and dimer–dimer (B_{33}) interactions reveals net monomer–dimer interactions are attractive, whereas the others are repulsive. Lastly, isothermal titration calorimetry dilution experiments showed that rhIL-1ra dimerization is enthalpically driven ($H_{\text{dimerization}} \ll 0$), which is consistent with intermolecular cation– π interactions previously proposed as the monomer–monomer contact sites in dimers.

Keywords

static light scattering; second osmotic cross virial coefficient; dimer; IL-1ra; ionic strength; sedimentation equilibrium; membrane osmometry; isothermal titration calorimetry

INTRODUCTION

The biopharmaceutical need for high concentration protein formulations required to achieve acceptable efficacy (>25 mg/mL)—within the volume limit for subcutaneous delivery (<1.5 mL)—has grown with increased development of therapeutics such as monoclonal antibody (MAb)-based products, as well as other proteins requiring relatively high doses. In addition to typical stability challenges,¹ high protein concentrations present unique obstacles during formulation, production and administration.^{2,3} For example, Donnan effects during processing are more prominent in highly concentrated solutions,⁴ whereas increases in viscosity due to reversible selfassociation can complicate tangential flow filtration operations² and make solutions impractical for subcutaneous injection.⁵

At high protein concentrations, macromolecular crowding due to excluded volume favors selfassociation between protein molecules.⁶ Whereas the rate of diffusion-limited association decreases at higher protein concentrations, transitionstate limited association rates may increase due to excluded volume stabilization of the transition state.⁷ Electrostatic effects are also more pronounced at high protein concentrations, where, at low solution ionic strengths, intermolecular separation distances may approach the Debye screening length. In addition, increased solution nonideality from excluded volume and protein-protein interactions is cause for concern when extrapolating results from typical analytical methods at low protein concentrations. It is possible to examine concentrated formulations directly using spectroscopic techniques with very small pathlengths; indeed, several proteins in concentrated formulations have shown conformational differences and decreased thermodynamic stabilities in comparison to those measured at low protein concentrations.⁸ Furthermore, at relatively high protein concentrations, a nonlinear increase in protein activity coefficient⁹ may lead to a rate of aggregation of protein molecules that far exceeds what is expected based on behavior of the protein in more dilute solutions.^{10,11} Thus, an incremental increase in protein concentration (e.g., from 125 to 150 mg/mL) may cause a dramatic increase in aggregation rate.¹²

To prevent aggregation during long-term storage, therapeutic protein formulations must stabilize both the protein native state (i.e., conformational stability) and stabilize the protein against attractive intermolecular interactions (i.e., colloidal stability).¹³ In this manuscript, we have characterized the effect of ionic strength on solution nonideality for highly concentrated solutions of recombinant human interleukin-1 receptor antagonist (rhIL-1ra), a 17 kDa cytokine inhibitor commercially formulated at 150 mg/mL. Data from 1D NMR experiments and dynamic light scattering revealed protein concentration-dependent self-association, which sedimentation equilibrium studies confirmed to be a monomer–dimer equilibrium. Sedimentation equilibrium was also used to measure the dimer dissociation constant (K_d) as a function of solution ionic strength. These measured K_d values were used to fit membrane osmometry and static light scattering data to an expanded second osmotic virial coefficient model accounting for separate monomer–monomer (B_{22}), monomer–dimer (B_{23}), and dimer–dimer (B_{33}) interactions. Lastly, isothermal titration calorimetry experiments were used to further quantify the energetics (H , T , S) of rhIL-1ra reversible dimerization.

THEORY

Donnan Effect

At low solution ionic strengths, unequal electrolyte partitioning during dialysis due to the Donnan effect becomes significant.^{4,14} Solution ionic strength is defined by

$$I(\text{mol kg}^{-1}) = \frac{1}{2} \sum_i m_i z_i^2 \quad (1)$$

where m_i is the molal (m) ion concentration and z_i is the ion charge. Following the approach of Stoner et al.,⁴ an iterative computation was used to calculate buffering anion concentrations, pH and the consequent protein charge determined from the amino acid sequence and average pK_a values of charged residues.¹⁵ The effect of ionic strength on the ion activity coefficients was calculated using the extended Debye–Hückel equation and tabulated values for each effective hydrated ion radius.¹⁶ The calculated activity coefficients were used to correct the pK_a values of the respective buffers at each ionic strength (Tab. 1). For a protein concentration of 100 mg/mL it was assumed that the effect on each anion–cation pair was proportional to the molar ratio of buffering anions. At pH 6.5, the following protein concentrations were used in calculations for citrate and phosphate buffer: 56 mg/mL for $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ (–3 charge), 44 mg/mL for $\text{Na}_2\text{C}_6\text{H}_6\text{O}_7$ (–2 charge), 83 mg/mL for NaH_2PO_4 (–2 charge), and 17 mg/mL for Na_2HPO_4 (–2 charge). The derived equations for adjusted ion molar concentrations (C_3) caused by the Donnan effect are as follows:

1 : 1 electrolyte (NaH_2PO_4 , NaCl)

$$\begin{aligned} C_{3+} &= \frac{-zC_2 + \sqrt{(zC_2)^2 + 4((C'_3 / \rho')(\rho - C_2 M_2 / 1000))^2}}{2}, \\ C_{3-} &= \frac{zC_2 + \sqrt{(zC_2)^2 + 4((C'_3 / \rho')(\rho - C_2 M_2 / 1000))^2}}{2} \end{aligned} \quad (2a,b)$$

1 : 2 electrolyte ($\text{Na}_2\text{C}_6\text{H}_6\text{O}_7$, Na_2HPO_4)

$$\begin{aligned} C_{3+} &= \frac{-zC_2 + \sqrt{(zC_2)^2 + 16((C'_3 / \rho')(\rho - C_2 M_2 / 1000))^2}}{2}, \\ C_{3-} &= \frac{zC_2 + \sqrt{(zC_2)^2 + 16((C'_3 / \rho')(\rho - C_2 M_2 / 1000))^2}}{4} \end{aligned} \quad (2c,d)$$

1 : 3 electrolyte ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$)

$$C_{3+} = \frac{-zC_2 + \sqrt{(zC_2)^2 + 36((C'_3 / \rho')(\rho - C_2M_2 / 1000))^2}}{6},$$

$$C_{3-} = \frac{zC_2 + \sqrt{(zC_2)^2 + 36((C'_3 / \rho')(\rho - C_2M_2 / 1000))^2}}{6} \quad (2e,f)$$

Primed quantities denote the permeate side of the dialysis membrane, unprimed quantities are on the protein (retentate) side of the membrane, C_2 is protein molarity, M_2 is protein molecular weight, C_3 is ion molarity, and ρ is the solution density calculated as a function of rhIL-1ra concentration:⁴

$$\rho = 0.000265c_2 + 1.00656 \quad (3)$$

Membrane Osmometry

One method to determine the second osmotic virial coefficient is membrane osmometry, which measures osmotic pressure directly and can accommodate very high protein concentrations. This approach has successfully been used to observe nonideality of bovine serum albumin (BSA) at protein concentrations up to 475 mg/mL.¹⁷

The virial equation for osmotic pressure (Π) of a single protein species is

$$\frac{\Pi}{RT} = \frac{c}{M_n} + Bc^2 \quad (4)$$

where c is the total protein concentration (g/mL), M_n the number average protein molecular weight, and B is the second osmotic virial coefficient (mol mLg^{-2}). Accounting for a monomer–dimer equilibrium, the monomer and dimer quantities are denoted by subscripts 2 and 3, respectively:

$$\frac{\Pi}{RT} = \frac{c_2}{M_2} + \frac{c_3}{M_3} + Bc^2 \quad (5)$$

In Eq. (5), B represents the total nonideality of the system. The second osmotic virial coefficient may be expanded to account for separate monomer–monomer (B_{22}), monomer–dimer (B_{23}), and dimer–dimer (B_{33}) interactions according to the monomer or dimer mol fraction (y_i) as follows:

$$B = y_2^2 B_{22} + y_3^2 B_{33} + 2y_2 y_3 B_{23} \quad (6)$$

Using the expansion of B shown in Eq. (6), Eq. (5) becomes

$$\frac{\Pi}{RT} = \frac{c_2}{M_2} + \frac{c_3}{M_3} + B_{22}c_2^2 + B_{33}c_3^2 + 2B_{23}c_2c_3 \quad (7)$$

From statistical mechanics, the second osmotic virial coefficient for two-body interactions assuming spherically symmetric forces is defined as¹⁸

$$B_{ij} = \frac{2\pi}{M^2} \int_0^\infty r^2 \left(1 - e^{-u(r)/kT} \right) dr \quad (8)$$

The subscripts i and j represent either monomer (2) or dimer (3). M is the protein molecular weight, r is the intermolecular separation distance, $u(r)$ is the interaction potential describing all interaction energies between two molecules, k is the Boltzmann constant, and T is the absolute temperature.

The second osmotic virial coefficient of a hard sphere with radius R is calculated on a molar basis by using Avogadro's Number (N) as follows:¹⁹

$$B^{\text{HS}} = \frac{16\pi NR^3}{3M} \quad (9)$$

Static Light Scattering

Static light scattering (SLS) measurements, which require less protein than membrane osmometry experiments, are often used to determine second osmotic virial coefficients, though preparation of dust-free samples is time-intensive. Traditionally, SLS is used at dilute protein concentrations below 5 mg/mL where the Rayleigh scattering theory for liquid solutions is most applicable. Two critical concerns when applying Rayleigh scattering theory at higher solute concentrations are independent scattering and multiple scattering. The commonly applied light scattering theory was developed for independent scattering, which assumes scattering from one particle may be modeled without reference to any other particles or regard for mutual polarization effects.¹⁴ Particle separation distances of 3 times the radius are generally sufficient for this assumption to be valid.²⁰ Multiple scattering, due to extinction of the incident light from re-scattering, is apparent if the scattering intensity is not directly proportional to the particle concentration.²⁰ When performing SLS experiments at low protein concentrations, the protein is usually assumed to exist as a single nonassociating species or a combination of assembly states independent of protein concentration.

The derivation of the Rayleigh equation for static light scattering of a single solute species substitutes the osmotic pressure gradient, (Π/c) , for the solvent chemical potential gradient (μ_1/c) , and is calculated from Eq. (4) as follows:²¹

$$\frac{Kc}{R_\theta} = \frac{-(\partial\mu_1/\partial c)}{RTV_1} = \frac{(\partial\Pi/\partial c)}{RT} = \frac{1}{M_w} + 2Bc \quad (10)$$

where R_θ is the excess Rayleigh ratio, $\bar{V}_1$ the solvent partial molar volume, and K is an optical constant defined as

$$K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{N_A \lambda_0^4} \quad (11)$$

where n_0 is the solvent refractive index, dn/dc the protein refractive index gradient, N_A the Avagadro's number, and λ_0 is the wavelength of the incident laser light. Similar to Eq. (5) for membrane osmometry, the protein concentration can be expanded to account for monomer and dimer species (denoted by subscripts 2 and 3) as a function of the total protein concentration. The monomer and dimer protein concentrations can be expressed as a function of the total protein concentration and the dimer dissociation constant ($K_d = [M]^2/[D]$) while converting K_d from molarity to g/mL as follows:

$$c_2 = \frac{M_2}{4000} \left(\left(K_d^2 + 8000 \frac{K_d c}{M_2} \right)^{1/2} - K_d \right) \quad (12)$$

$$c_3 = \frac{M_3}{16000 K_d} \left(\left(K_d^2 + 8000 \frac{K_d c}{M_3} \right)^{1/2} - K_d \right)^2 \quad (13)$$

For a single second osmotic virial coefficient (B), the Rayleigh equation (Eq. (10)) for a monomer–dimer equilibrium becomes

$$\begin{aligned} \frac{Kc}{R_\theta} &= \frac{K_d}{2M_2(K_d^2 + (8000K_d c)/M_2)^{1/2}} \\ &+ \frac{1}{2M_2} + 2Bc = \frac{1}{M_w} + 2Bc \end{aligned} \quad (14)$$

The simplified form of Eq. (14) uses the weight average molecular weight M_w , calculated from the value of K_d as a function of c . Substitution of the osmotic pressure gradient calculated from Eq. (7) into Eq. (10) gives the expanded Rayleigh equation accounting for separate monomer–monomer (B_{22}), monomer–dimer (B_{23}), and dimer–dimer (B_{33}) interactions:

$$\frac{Kc}{R_0} = \frac{(\partial \Pi / \partial c)}{RT} = \frac{\left(\begin{aligned} &2000K_d M_2 + 2000M_2(K_d^2 + 8000K_d c / M_2)^{1/2} \\ &+ 2B_{22}M_2^3 K_d(K_d^2 + 8000K_d c / M_2)^{1/2} - 2B_{22}M_2^3 K_d^2 \\ &+ B_{33}M_3^2 K_d M_2(K_d^2 + 8000K_d c / M_2)^{1/2} \\ &+ 2000B_{33}M_3^2(K_d^2 + 8000K_d c / M_2)^{1/2} \\ &- B_{33}M_3^2 K_d^2 M_2 - 6000B_{33}M_3^2 K_d c + 3B_{23}M_3 K_d^2 M_2^2 \\ &+ 12000B_{23}M_3 K_d M_2 c - 3B_{23}M_3^2(K_d^2 + 8000K_d c / M_2)^{1/2} \end{aligned} \right)}{4000M_2^2(K_d^2 + 8000K_d c / M_2)^{1/2}} \quad (15)$$

Sedimentation Equilibrium

Sedimentation profiles for a single ideal species may be modeled by the following equation:

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$$c_r = c_{r0} e^{\left[\left((1 - \nu\rho)\omega^2 / 2RT \right) M_w (r^2 - r_0^2) \right]} \quad (16)$$

where c_r is the protein concentration at radius r , c_{r0} the protein concentration at reference radius r_0 , ω the angular velocity, M_w the weight average molecular weight, ν the solute partial specific volume, and ρ is the solvent density. The specific volume of rhIL-1ra is 0.752 mL/g.²³ Solvent densities were calculated using SEDNTERP software (v. 1.08) and were within a range of 1.0001–1.01 g/mL. The following equation was used to fit c_{r0} , $K_{a,2}$, B and E accounting for self-association:²²

$$\begin{aligned} c_{r, \text{total}} = & e^{\left[\ln(c_{\text{monomer}, r_0}) + \left((1 - \nu\rho)\omega^2 / 2RT \right) M(r^2 - r_0^2) - BM_w(c_{\text{total}, r} - c_{\text{total}, r_0}) \right]} \\ & + e^{\left[\ln(c_{\text{monomer}, r_0}) + \ln(K_{a,2}) + \left((1 - \nu\rho)\omega^2 / 2RT \right) 2M(r^2 - r_0^2) - BM_w(c_{\text{total}, r} - c_{\text{total}, r_0}) \right]} + E \end{aligned} \quad (17)$$

where $K_{a,2}$ is the equilibrium dimer association constant, E the baseline offset, and B is the second osmotic virial coefficient. For an associating system, this second osmotic virial coefficient is an 'average' measure of nonideality over the radial protein distribution in the cell.²⁴ This radial dependence is due to differences in B for monomer and dimer species, whose equilibrium protein concentrations change as total protein concentration varies with radial position in the cell.²⁴ The equilibrium constant was converted from fringe units to units of L mol⁻¹ using:

$$K_a = K_{\text{fringe}} \left(\frac{(dn/dc)l}{\lambda} \right)^{n-1} \frac{M^{n-1}}{n} \quad (18)$$

where dn/dc is the protein refractive index (determined to be 0.173 mL/g, see Materials and Methods Section), l the pathlength (1.2 cm), λ the laser wavelength (675 nm), M the protein molecular weight (17.3 kDa), and n is the degree of association (2). Similarly, B values were converted to units of L/g with Eq. (19) before final conversion to mol mLg⁻²

$$B = B_{\text{fringe}} \left(\frac{(dn/dc)l}{\lambda} \right)^{n-1} \frac{M^{n-1}}{n} \quad (19)$$

Isothermal Titration Calorimetry

The exothermic enthalpy of dimerization ($H_{\text{dimerization}}$) was determined by fitting calorimetry data for the endothermic enthalpy of dimer dissociation upon dilution to the following equation for H_{dilution} and $H_{\text{dimerization}}$.²⁵

$$\delta q_i = \Delta H_{\text{dimerization}} \{ V_0 ([D]_i - [D]_{i-1}) - V ([D]_0 - [D]_{i-1}) \} \quad (20)$$

$$\delta q_i = \delta q_{i,\text{raw}} - \Delta H_{\text{dilution}} \quad (21)$$

where V_0 is the cell volume (1.4 mL), V the injection volume (25 μ L), $[D]$ the reversible dimer protein concentration calculated from K_d values determined by sedimentation equilibrium, δq_i the heat change following each injection, and $H_{\text{dimerization}}$ is the enthalpy of dimerization. The fit value of H_{dilution} for monomeric rhIL-1ra was adjusted to account for the increasing protein concentration in the cell for $i > 1$. The protein concentration of dimer was determined from K_d values determined by sedimentation equilibrium. All other thermodynamic parameters were determined using the standard relationship:

$$\Delta G = -RT \ln K = \Delta H - T\Delta S \quad (22)$$

MATERIALS AND METHODS

Materials

Pharmaceutical grade rhIL-1ra was manufactured and purified (>99%) at Amgen Inc. The stock formulation contained 220 mg/mL protein in citrate buffered saline (CSE): 10 mM sodium citrate buffer pH 6.5, 140 mM sodium chloride, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). rhIL-1ra stock solution was maintained at -80°C

until needed. All other chemicals were purchased from Sigma or Fisher Scientific and were of reagent grade or higher quality.

Dialysis and Sample Preparation

Dialysis for buffer exchange from the stock formulation was performed against excess solvent overnight using Pierce 10000 MWCO Slide-A-Lyzer Dialysis Cassettes. A final protein concentration after dialysis of approximately 156 mg/mL was used for 1D NMR, viscosity and dynamic light scattering experiments, and a final protein concentration after dialysis of approximately 100 mg/mL for all other experiments. It was assumed all EDTA was removed during dialysis. Samples at lower protein concentrations were prepared by dilution with buffer at the corresponding retentate ionic strength accounting for the Donnan effect (Tab. 1). The Donnan effect was also taken into account at the higher protein concentrations used for viscosity, dynamic light scattering, and 1D NMR experiments. All samples were filtered with Millex-GV 0.22 μ m PVDF syringe filters prior to experiments. Protein concentrations were measured using a Perkin-Elmer Lambda 35 UV/VIS Spectrophotometer with an extinction coefficient of 0.77 L/g cm for rhIL-1ra.²⁶ All experiments were performed at pH 6.5 and no excipients other than buffer ions were present in solutions tested.

Preparation of Irreversible Dimer

A dimer of rhIL-1ra that did not dissociate during SE-HPLC (“irreversible rhIL-1ra dimer”) was generated by incubation of the protein in CSE at 37°C for 2 weeks. Purified irreversible dimer was collected following elution from a SE-HPLC Tosoh Biosep GSK-2000SWXL column on a Beckman Gold HPLC system using a SWXL guard column, 0.2- μ m prefilter and CSE running buffer at 0.5 mL/min while monitoring the absorbance at 280 nm. Mass percent dimer protein concentrations were determined from UV absorbance at 280 nm combined with SE-HPLC chromatograms assuming equal extinction coefficients for both native protein and irreversible dimer.

Dynamic Light Scattering

The hydrodynamic diameter of rhIL-1ra was determined by photon correlation spectroscopy using a Brookhaven particle size analyzer equipped with a He–Ne laser (633 nm), BI-9000AT digital correlator, BI-APD avalanche photodiode, and BI-200SM goniometer. Each sample was measured in triplicate for 1 min with the detector at a 90° angle, a 400 nm pinhole size, and the sample in a 25°C circulating water bath. The nonnegatively constrained least-squares (CONTIN) algorithm in Brookhaven 9KDLSW dynamic light scattering software was used to determine intensity-weighted hydrodynamic diameter distributions. Changes in the solution viscosity and refractive index were accounted for at each protein concentration. The refractive index for each protein solution was measured using a Bausch and Lomb refractometer. Viscosity measurements were made at 25°C using 4.8 mL of protein solution in a size 25 Cannon–Fennske Routine capillary flow viscometer.⁵ The kinematic viscosity was determined by efflux time multiplied by the viscometer constant of 0.002008 cSt/s before final conversion to centipoise (cP) using the protein solution density calculated from Eq. (3).

NMR Spectroscopy

1D ^1H NMR experiments were performed on rhIL-1ra samples in 90% H_2O and 10% $^2\text{H}_2\text{O}$. Spectra were acquired at 25°C on a Varian INOVA 500 MHz spectrometer and processed using NMRPipe software.²⁷ To test for reversible self-association, spectra were first measured for a sample at 156 mg/mL protein concentration. Dissociation was initiated by dilution of the sample to 25 mg/mL protein concentration, and the sample was analyzed immediately (ca. 15 min delay time). Spectra for both samples were collected using 64 transients, 6 kHz spectral width and 8000 points. Spectra for both samples were apodized with a $\cos^2$ function during processing.

Static Light Scattering

The protein refractive index gradient (dn/dc) for each solution was measured with a Wyatt Optilab interferometric refractometer. Using the gradient method, injections were made with a BAS Beehive syringe pump and a 1 mL syringe operating at 0.1 mL/min with protein concentrations of 0.6, 0.52, 0.42, 0.30, 0.18, 0.06 mg/mL. The value of dn/dc for rhIL-1ra was determined to be 0.173 ± 0.003 mL/g for the entire range of solution ionic strengths. Second osmotic virial coefficients were measured using a Brookhaven light scattering system from Brookhaven Instrument Corporation, consisting of a BI-200SM goniometer, 633 nm vertically polarized He–Ne laser, BI-9000AT correlator, and a BI-APD avalanche photodiode. Each B_{ij} measurement was made at 90° using a 2 mm pinhole size and a rhIL-1ra concentration range of 1–100 mg/mL. Quartz cuvettes were washed extensively with 0.02 mm filtered water and dried overnight in a dust-free vacuum chamber. At least 200 acceptable count rates (photons/second) were recorded for each protein concentration using the software's statistical dust-rejection cutoff ratio of 1.3. Measurements were performed at 25°C . DOS version BI-ZP software was used to record photon counts for each protein concentration and SigmaPlot was used for least-square linear regression to determine virial coefficients. Toluene was used to calibrate the instrument. The refractive index for each buffer solution was measured using a Bausch and Lomb refractometer.

Sedimentation Equilibrium

A Beckman XL-I analytical ultracentrifuge was used to measure dimer dissociation constants (K_d) at 25°C . The XL-I was chosen for the ability of the interference optics to accommodate high protein concentrations, in addition to superior signal/noise ratio in comparison to the absorbance optics of the XL-A. Samples were prepared with protein concentrations of approximately 1, 2.5, and 5 mg/mL. Using six sector cells, 110 μL rhIL-1ra solution was loaded in each sample sector, and 115 μL buffer was loaded into each reference cell. Spin rates of 14k, 16k, and 18k rpm were used with respective equilibrium times of 44, 38, and 32 h. Equilibrium was confirmed by overlaying scans from the final time with a scan 6 h prior. Least-squares regression for Eqs. (16) and (17) was completed using Microcal Origin 6.0 to globally fit the nine sedimentation profiles at each solution ionic strength.

Membrane Osmometry

Direct measurement of osmotic pressure was performed using a Wescor 4420 Colloid Osmometer in Manual Mode with a 10000 MWCO polyethersulfone membrane at 25°C. Calibration of the instrument was performed with lysozyme in NaCl solutions (2–8 mg/mL protein concentration) of known B_{22} values (0.1 M NaCl, pH 3: $B_{22} = 3.95 \times 10^4 \text{ mol mLg}^{-2}$; 0.1 M NaCl, pH 7.5: $B_{22} = -3.65 \times 10^4 \text{ mol mLg}^{-2}$; 0.005 M NaCl, pH 6: $B_{22} = 28.5 \times 10^4 \text{ mol mLg}^{-2}$).²⁸ Osmotic pressures for rhIL-1ra concentrations of 2–100 mg/mL protein concentrations were measured in duplicate. After an initial 300 μL protein sample injection, the pressure reading deflected downward before increasing to a stable reading. Once this stable reading was attained, an additional 100 μL protein sample was injected. After this sample produced a stable reading, a third 100 μL protein sample was injected. The third stable reading was considered final in accordance with the operator manual,²⁹ and the osmotic pressure was recorded.

Isothermal Titration Calorimetry

An Omega Isothermal Titration Calorimeter was used to record thermograms for dilution of 100–110 mg/mL rhIL-1ra at 25°C and pH 6.5. Ten equal 25 μL protein injections were used with a delay of 180 s and a stir rate of 250 rpm. The same buffers used as the retentate during dialysis were used in the cell for protein injections and no change in pH was observed following titrations for any of the solution conditions examined. ‘Blank’ injections were also collected by injection of buffer alone into buffer and the resulting heat measurement was subtracted from sample thermograms. Data collection and peak integration were performed using MicroCal ORIGIN ITC Software.

RESULTS

Donnan Effects

Results of the Donnan effect calculations using Eqs. (2a–f) are summarized in Tab 1 for equilibrium dialysis of citrate and phosphate buffered solutions at pH 6.5, 25°C, and a protein concentration of 100 mg/mL. The retentate ionic strength is altered from the permeate ionic strength by as much as 32% for 4.5 mM citrate solutions and 26% for 16.0 mM phosphate solutions. The effect is larger in citrate solutions compared to phosphate solutions of comparable ionic strength due to the greater charge of the anions (ca. –3 for citrate vs. ca. –1 for phosphate at pH 6.5). The Debye length also increases by a factor of 3 from 0.7 to 2.0 nm as solution ionic strength is reduced from 0.184 to 0.025 m.

NMR Spectroscopy

Self-association was observed by 1D ^1H NMR, indicated by peak width broadening and a loss of resolution at high protein concentration as shown in the left spectrum in Figure 1. At 156 mg/mL and an ionic strength of 0.031 m, the poor resolution of the spectrum reflects increased relaxation times consistent with self-association. Dilution of this sample to 25 mg/mL protein concentration and immediate analysis showed that spectral resolution was greatly increased due to dissociation of the reversible dimer (right spectrum in Fig. 1).

Dynamic Light Scattering

Dynamic light scattering measurements at increasing protein concentration in Figure 2 show a clear increase in the apparent rhIL-1ra hydrodynamic diameter from the measured value for monomer (3.4 ± 0.3 nm for a 95:5 monomer: reversible dimer sample by mass at $I = 0.181$ m) to that measured for dimer (5.7 ± 0.3 nm for a 15:85 monomer: irreversible dimer sample by mass at $I = 0.181$ m). The difference in diameter between solution ionic strengths of 0.031 and 0.091 m is also statistically significant (e.g., using the two-sample t -test, the difference in mean hydrodynamic diameter at 106 mg/mL has a p -value of 0.0142). Thus, the observed size increase in Figure 2 is best explained by equilibrium dimerization. However, the contribution to the light scattering signal from dimer is much greater than that from monomer due to the sixth-power dependence of light scattering intensity on the size of the scatterer. This strong size dependence precludes quantifying the respective monomer and dimer levels at each concentration in Figure 2. Measurements of viscosity in Figure 2 for dynamic light scattering show a slightly larger increase with increasing protein concentration at the lower solution ionic strength (e.g., using the two-sample t -test, the difference in mean viscosity at 156 mg/mL protein concentration has a p -value of 0.0002).

Sedimentation Equilibrium

Sedimentation profiles at 18k rpm for 1, 2.5, and 5 mg/mL protein concentrations are displayed in Figure 3. As the ionic strength decreases from 0.184 to 0.011 m for the three protein concentrations, the increase in dimerization caused increased curvature indicated by the direction of the arrows. Each curve contains approximately 250 data points. A summary of the regression results in Table 2 compares the global fit results at each ionic strength using Eq. (16) with both a fixed and floating molecular weight, and Eq. (17) fitting self-association and nonideality. The fit as a single ideal species using Eq. (16) and residuals (data not shown) were improved by allowing the molecular weight to float, instead of fixing it at the value for monomer (17.3 kDa). The increase in the molecular weight above the value for monomer fit using Eq. (16) shows the presence of higher molecular weight species. The fit using Eq. (17) accounting for self-association and nonideality shows the dimer dissociation constant increases from 2.0 to 12.6 mM as the ionic strength increases from 0.011 to 0.184 m. 95% confidence intervals for K_d values were estimated from corresponding intervals for the molecular weight fit using Eq. (16). The errors for the molecular weight are the same for all ionic strengths (± 0.2 kDa), but the effect of small uncertainty is larger at larger K_d values. The second osmotic virial coefficient fit using Eq. (17) approaches the excluded volume limit ($B^{HS} = 2.9$ mLg⁻¹ for rhIL-1ra monomer calculated using Eq. (9) with a hard sphere radius of 1.7 nm observed using dynamic light scattering) at higher ionic strength due to increased screening of electrostatic interactions. A similar result has been observed for lysozyme in acetate buffer at pH 4.5.¹⁹

Static Light Scattering and Membrane Osmometry

Debye plots for static light scattering of rhIL-1ra at protein concentrations of 1–6 mg/mL are shown in Figure 4. From the results in Table 3 using Eq. (10), rhIL-1ra appears to be monomeric ($M = 17.3$ kDa) over the solution ionic strength range of 0.025–0.184 m, with protein–protein interactions becoming more attractive (increasingly negative B_{22} values) at

lower ionic strength. However, the full static light scattering data collected from 1 to 100 mg/mL displayed in Figure 5 do not match the aforementioned explanation. Instead of a linear plot as in Figure 4 that would be expected if the rhIL-1ra samples were either entirely monomeric or contained a protein concentration-independent fraction of oligomers, the curvature at increasing protein concentration is characteristic of equilibrium self-association. The greater curvature at low ionic strength from smaller values of Kc/R is caused by the increased presence of higher weight average molecular weight species. Based on observations from dynamic light scattering, this higher molecular weight species is an equilibrium dimer. To confirm the validity of using static light scattering at protein concentrations up to 100 mg/mL, the osmotic pressure was measured directly using membrane osmometry and the data is also displayed in Figure 5. The dashed lines in Figure 5 show the fit of Eqs. (7) and (15) to the osmometry and static light scattering data respectively, using values of K_d determined from sedimentation equilibrium as described in the previous section. Values for B_{22} , B_{33} and B_{23} normalized to the hard sphere excluded volume displayed in Figure 6 show monomer–monomer and dimer–dimer interactions are repulsive (positive values), whereas monomer–dimer interactions are attractive (negative values).

Isothermal Titration Calorimetry

A representative endothermic thermogram for the injection of concentrated rhIL-1ra into buffer is shown in Figure 7. The narrow thermogram heat pulses observed for all solution conditions tested indicated fast dimer dissociation kinetics. A ‘blank’ injection of buffer into buffer used for subtraction is also shown in Figure 7. The integrated thermogram peak areas from isothermal titration calorimetry dilution studies and the fit using Eq. (20) are displayed in Figure 8. The higher endothermic enthalpy of dilution at low ionic strength reflects the heat uptake necessary for dissociation of increased levels of reversible dimer. Successive injections are progressively less endothermic due to the rising protein concentration (and, hence, increasing amount of dimer) in the cell. Table 4 summarizes the thermodynamic parameters of rhIL-1ra dimerization using 95% confidence intervals from K_d values determined by sedimentation equilibrium.

DISCUSSION

rhIL-1ra reversible dimerization increases at low solution ionic strength, presumably due to reduced screening of attractive electrostatic interactions. This decrease in screening is manifested in the threefold increase in the Debye length over the ionic strength range of 0.184 to 0.025 m (Tab. 1). Cation– π interactions between monomers have been suggested as a possible origin of attractive electrostatic interactions that lead to dimerization,³⁰ and our results are not inconsistent with this interpretation. Self-association is reflected in the crystallographic asymmetric unit for rhIL-1ra,³¹ which contains two very similar independent monomers positioned in a way amenable to cation– π interactions between K93 and R97 of one monomer and W16 and Y34 of the other.³⁰

The association energetics displayed in Table 4 show rhIL-1ra dimerization is enthalpically controlled ($H_{\text{dimerization}} \ll 0$) rather than entropically driven. The enthalpic driving force is

compensated by an unfavorable entropy change ($T \Delta S_{\text{dimerization}} < 0$). This apparent phenomenon of enthalpy–entropy compensation is ubiquitous in aqueous systems involving noncovalent interactions.³² Hydrophobic interactions during protein association would contribute to positive enthalpy and entropy changes.³³ Therefore, the increasingly negative $\Delta H_{\text{dimerization}}$ and $\Delta S_{\text{dimerization}}$ for rhIL-1ra at decreasing ionic strength is consistent with the proposed role of cation– π interactions during dimerization instead of hydrophobic interactions.³⁴

In contrast to rhIL-1ra, reversible dimerization has been observed to increase at increasing solution ionic strength for several proteins. Light scattering has previously been used to observe self association for insulin³⁵ and bovine β -lactoglobulin (β -Lg),^{36,37} the latter at protein concentrations up to 56 mg/mL. Bovine β -lactoglobulin is now a well-known model protein for reversible dimerization, and the equilibrium is strongly dependent on solution ionic strength, temperature and solution pH. β -Lg has a β -barrel fold topology similar to the crystal structure of rhIL-1ra and is also similar in size (18.4 kDa).³⁸ β -Lg exists mostly as a dimer at neutral pH and monomer below pH 3, though increasing salt concentration stabilizes the dimer by reducing electrostatic repulsion through ion binding.³⁸ Dimerization buries 6% of the monomer surface area, which is stabilized by twelve intermolecular hydrogen bonds and two ion pairs.³⁹ Another self-associating protein is α -chymotrypsin, which exhibits macromolecular crowding enhanced dimerization in high concentrations of glucose, sucrose and raffinose.⁴⁰ Similar to β -Lg, increasing ionic strength increases α -chymotrypsin dimerization at acidic pH.⁴¹ Human spectrin^{42,43} and recombinant humanized antibody vascular endothelial growth factor (rhUMab VEGF)⁴⁴ have both also shown enhanced self-association at high ionic strength due to shielding of repulsive electrostatic interactions.

The second osmotic cross virial coefficients in Figure 6 show attractive monomer–dimer interactions (negative B_{23} values), whereas monomer–monomer and dimer–dimer interactions are repulsive (positive B_{22} and B_{33} values, respectively). The decrease in magnitude of all three coefficients with increasing ionic strength is due to screened electrostatic interactions. These interactions are similar in magnitude to the hard sphere excluded volume, as the ratio of the virial coefficients to the hard sphere excluded volume is close to unity within the calculated 95% confidence intervals. From aggregation studies of rhIL-1ra at high protein concentrations,¹² the agreement between activity coefficients for rhIL-1ra and a hard sphere fluid further indicates excluded volume effects are the dominant contribution to solution nonideality at high protein concentrations. The hard sphere fluid approximation is reasonable for rhIL-1ra, which is a small, globular protein with a calculated net charge of -1.9 at pH 6.5 used in these studies (Tab. 1). This result for rhIL-1ra is consistent with previous studies of the activity of hemoglobin at very high protein concentrations, which may be accurately modeled as a hard sphere fluid.¹⁰ The measured viscosity of rhIL-1ra also showed reasonable agreement with hard sphere predictions. At a protein concentration of 100 mg/mL, the observed solution viscosity shown in Figure 2 for rhIL-1ra is approximately 1.5 times the estimated value for a solution of nonassociating hard spheres ($\mu^{\text{HS}} = 1 + 2.5\phi$,²¹ where $\phi = v_c$; a specific volume, v_c , of 0.752 mL/g was used for rhIL-1ra²³) due to attractive interactions. This increase is less than values reported for self-associating MAb formulations at comparable high protein concentrations.⁵ However, self-

association of a spherical protein the size of rhIL-1ra is expected to have a smaller effect on viscosity than the self-association of a much larger, nonspherical antibody.

Net protein–protein interactions are not determined by uniform bulk colloidal properties; instead, second osmotic virial coefficients are governed by a few highly attractive or repulsive interactions resulting from configurations with high geometric complementarity.⁴⁵ Negative second osmotic cross virial coefficients for rhIL-1ra monomer–dimer interactions (B_{23}), in contrast to positive values observed for monomer–monomer (B_{22}) and dimer–dimer (B_{33}) interactions, support this contention. The existence of positive virial coefficients in an associating system is not unprecedented. Fitting static light scattering data to an equation analogous to Eq. (14), Aymard et al.⁴⁶ showed the dimer dissociation constant (K_d) for β -Lg at pH 2 decreases with increasing solution ionic strength from 0.013 to 0.2 m whereas the net B value remains positive for protein concentrations up to 35 mg/mL at 20°C. The increasingly positive B values at decreasing ionic strength determined by sedimentation equilibrium for rhIL-1ra (Tab. 2) reflect only protein–protein interactions without the protein-salt contribution inherent in virial coefficients determined by static light scattering.¹⁹ As stated in the Theory section, the B determined by sedimentation equilibrium is also an ‘average’ measure of nonideality from varying monomer and dimer contributions over the radial protein distribution in the cell.²⁴

CONCLUSIONS

With increased development of highly concentrated protein therapeutics, the potential for selfassociation during formulation is a more frequent concern. When screening potential formulations with self-association, correct analysis of static light scattering data to assess colloidal stability must account for the protein concentration-dependence of equilibrium association. In this manuscript we have developed an expanded second osmotic virial coefficient model accounting for a monomer–dimer equilibrium in order to successfully use static light scattering for rhIL-1ra protein concentrations up to 100 mg/mL. This expanded virial coefficient model elucidates the separate contributions from monomer–monomer (B_{22}), monomer–dimer (B_{23}), and dimer–dimer (B_{33}) interactions to solution nonideality. For rhIL-1ra, the magnitudes of these separate interactions are close to the respective excluded volumes. Consequently, only marginal increases in viscosity were observed for rhIL-1ra solutions at high protein concentrations in comparison to the estimated viscosity for a hard sphere fluid.

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Figure 1.

1D ^1H spectra collected at an ionic strength of 0.031 m (16.0 mM phosphate). Left spectrum: initial rhIL-1ra sample at 156 mg/mL protein concentration. Right spectrum: same rhIL-1ra sample following dilution to 25 mg/mL protein concentration. The vertical scale of the right spectrum was increased to match the scale of the left spectrum, which is at a sixfold higher protein concentration.

Figure 2.

(A) Viscosity of rhIL-1ra solutions. Ionic strength: ■, 0.091 m (20.8 mM citrate); Δ, 0.031 m (16.0 mM phosphate). Error bars are the standard deviation from triplicate samples at each concentration. Solid line is the polynomial fit used for calculations. Dashed line is an estimate of the hard sphere viscosity determined from solute volume fraction (ϕ) ($\mu^{\text{HS}} = 1 + 2.5\phi$,²⁰ where $\phi = vc$; a specific volume, v , of 0.752 mL/g was used for rhIL-1ra²²). (B) Dynamic light scattering results at 25°C. Ionic strength: ■, 0.091 m (20.8 mM citrate); Δ, 0.031 m (16.0 mM phosphate). Error bars are the standard deviation from triplicate samples at each concentration. Solid lines represent for the hydrodynamic diameter measured at $I = 0.181$ m for a 95:5 monomer:dimer sample by mass (3.4 nm) and 15:85 monomer: irreversible dimer sample by mass (5.7 nm) at $I = 0.181$ m. Dashed lines represent the standard deviation from triplicate measurements (± 0.3 nm).

Figure 3.

Sedimentation equilibrium profiles at 18k rpm for rhIL-1ra solutions at the following ionic strengths (and respective buffer): 0.184 m (46.5 mM citrate); 0.111 m (83.3 mM phosphate); 0.091 m (20.8 mM citrate); 0.060 m (42.7 mM phosphate); 0.031 m (16.0 mM phosphate); 0.025 m (4.5 mM citrate); 0.011 m (3.4 mM phosphate). Increasing curvature denoted by the arrows indicates greater dimer concentration and decreasing ionic strength. (A) 1 mg/mL rhIL-1ra loading concentration. (B) 2.5 mg/mL rhIL-1ra concentration. (C) 5 mg/mL rhIL-1ra concentration.

Figure 4.

Static light scattering data for rhIL-1ra at 25°C and pH 6.5 over a protein concentration range of 1–6 mg/mL. Solid lines represent the fit using Eq. (10). Ionic strength (and respective buffer): o, 0.184 m (46.5 mM citrate); ◆, 0.111 m (83.3 mM phosphate); ■, 0.091 (20.8 mM citrate); ◇, 0.060 (42.7 mM phosphate); △, 0.031 (16.0 mM phosphate); ●, 0.025 (4.5 mM citrate). Error bars are standard deviations from 200 acceptable measurements.

Figure 5.

(A) Static light scattering data at 25°C and pH 6.5 fit using Eq. (15) (dashed lines). Ionic strength (and respective buffer): o, 0.184 m (46.5 mM citrate); ◆, 0.111 m (83.3 mM phosphate); ■, 0.091 m (20.8 mM citrate); ◇, 0.060 m (42.7 mM phosphate); ▲, 0.031 m (16.0 mM phosphate); ●, 0.025 m (4.5 mM citrate). Error bars from the standard deviation are less than the symbol size. (B) Membrane osmometry data at 25°C and pH 6.5 fit using Eq. (7) (dashed lines). The symbol notation for ionic strength (and respective buffer) is the same as that for static light scattering. Error bars from the standard error are less than the symbol size.

Figure 6.

Virial coefficients calculated using Eqs. (7)) and (15) normalized to the hard sphere value for rhIL-1ra calculated from a hard sphere radius of 1.7 nm for monomer and 2.85 nm for dimer ($B_{22}^{HS}/M_2 = 1.66 \times 10^{-4} \text{ mol mLg}^{-2}$ for monomer-monomer interactions; $B_{33}^{HS}/M_3 = 1.95 \times 10^{-4} \text{ mol mLg}^{-2}$ for dimer-dimer interactions; $B_{23}^{HS}/M_{2.5} = 1.77 \times 10^{-4} \text{ mol mLg}^{-2}$ for monomer-dimer interactions using a hard sphere radius of 2.3 nm). $\diamond$: osmometry, $\square$: light scattering. Error bars are based on 95% confidence intervals from sedimentation equilibrium K_d values. (A) Monomer-monomer interactions (B_{22}). (B) Dimer-dimer interactions (B_{33}). The error bars below 0 on the y -axis extend to -0.04 for the

osmometry data at 0.111 m, -19.3 for light scattering data at 0.184 m, and -21.3 for osmometry data at 0.184 m. (C) Monomer-dimer interactions (B_{23}).

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Figure 7.

(A) Representative ITC thermogram for ten 25 μ L injections of 104 mg/mL rhIL-1ra in 4.5 mM citrate buffer, $I = 0.025$ m (syringe) into 1.4 mL 4.5 mM citrate buffer, pH 6.5 at 25°C (cell). (B) 'Blank' thermogram in the absence of protein (4.5 mM citrate buffer injected into 4.5 mM citrate buffer, pH 6.5 at 25°C).

Figure 8.

ITC integrated endothermic peak areas resulting from dilution at 25°C and pH 6.5 of concentrated rhIL-1ra injections and fit using Eq. (20) (dashed lines). Ionic strength (and respective buffer): o, 0.184 m (46.5 mM citrate); ◆, 0.111 m (83.3 mM phosphate); ■ 0.091 (20.8 mM citrate); ◇, 0.060 (42.7 mM phosphate); ▲, 0.031 (16.0 mM phosphate); ●, 0.025 (4.5 mM citrate). Error bars are the standard error from duplicate measurements.

Table 1.

Summary of the Donnan Effect Calculations Using Eqs. (2a–f) for Phosphate and Citrate Buffered Solutions at pH 6.5, 25°C, and 100 mg/mL Protein Concentration

Anion Concentration (mM)		Sodium Concentration (mM)			Ionic Strength (m)		
Permeate							
6.4 (citrate)		18.3			0.037		
25 (phosphate)		33.3			0.042		
50 (phosphate)		64.8			0.080		
25 (citrate)		71.6			0.124		
93 (phosphate)		111.1			0.144		
50 (citrate)		120.9			0.246		
Anion Concentration (mM)	Buffer p <i>K</i> _a	Sodium Concentration (mM)	Ionic Strengtd (m)	Difference From Permeate (%)	Debye Lengtd (nm)	Protein Charge	pH
Retentate							
4.5 (citrate)	6.77	20.9	0.025	32	2.0	−1.8	6.42
16.0 (phosphate)	7.48	36.0	0.031	26	1.7	−2.0	6.55
42.7 (phosphate)	7.56	57.0	0.060	25	1.3	−1.9	6.52
20.8 (citrate)	6.98	62.4	0.091	27	1.0	−1.8	6.42
83.3 (phosphate)	7.64	101.0	0.111	23	1.0	−1.9	6.52
46.5 (citrate)	7.12	112.3	0.184	25	0.7	−1.9	6.46

The protein is located on the retentate side of the membrane and the dialysis buffer is located on the permeate side.

Table 2.

Summary of Sedimentation Equilibrium Regression Results

Buffer	Ionic Strength (molal $\times 10^3$)	Single Ideal Species Fit			Single Ideal Species Fit	
		M_w (Floated)	(Variance) ^{1/2}		M_w (Fixed)	(Variance) ^{1/2}
A						
7.4 mM phosphate	11	19.2	0.014		17.3	0.026
6.4 mM citrate	25	18.4	0.014		17.3	0.017
25 mM phosphate	31	18.3	0.014		17.3	0.015
50 mM phosphate	60	18.1	0.014		17.3	0.014
25 mM citrate	91	17.9	0.012		17.3	0.012
93 mM phosphate	111	17.8	0.012		17.3	0.012
50 mM citrate	184	17.6	0.012		17.3	0.012
Buffer	Ionic Strength (molal $\times 10^3$)	K_a (Fringes)	K_a (M ⁻¹)	K_d (mM)	B (mLg ⁻¹)	(Variance) ^{1/2}
B						
7.4 mM phosphate	11	0.019	505	2.0 (1.7, 2.3)	6.1 $\pm$ 0.3	0.014
6.4 mM citrate	25	0.01	266	3.8 (2.9, 5.2)	5.2 $\pm$ 0.3	0.014
25 mM phosphate	31	0.009	239	4.2 (3.2, 6.0)	5.2 $\pm$ 0.3	0.014
50 mM phosphate	60	0.007	186	5.4 (3.9, 8.7)	4.5 $\pm$ 0.3	0.014
25 mM citrate	91	0.005	133	7.5 (4.9, 15.3)	4.1 $\pm$ 0.3	0.012
93 mM phosphate	111	0.0045	119	8.4 (5.4, 20.4)	3.6 $\pm$ 0.3	0.012
50 mM citrate	184	0.003	80	12.6 (6.8, 79.8)	3.4 $\pm$ 0.3	0.012

(A) Comparison of the fit as a single ideal species using Eq. (16) when fixing the protein molecular weight or allowing it to float. (B) Fit using Eq. (17) with a fixed protein molecular weight of 17.3 kDa accounting for self-association and nonideality. 95% confidence intervals (CI) are shown in parentheses for K_d values and $\pm$ CI for B values. The second osmotic virial coefficient, B , is shown to approach the hard sphere value at increasing ionic strength ($B^{\text{HS}} = 2.9 \text{ mLg}^{-1}$ for rhIL-1ra monomer calculated using Eq. (8) with a hard sphere radius of 1.7 nm).

Table 3.

Apparent Values Using Eq. (10) to Fit rhIL-1ra Light Scattering Data in Figure 4

Ionic Strength (m)	$B_{22} \times 10^4$	$B_{22}/B_{22}^{\text{HS}}$	M_w
0.025	-5.0 ± 0.2	-3.0 ± 0.1	17.2 ± 0.1
0.031	-4.5 ± 0.2	-2.7 ± 0.1	17.2 ± 0.1
0.060	-4.2 ± 0.2	-2.5 ± 0.1	17.2 ± 0.1
0.091	-3.0 ± 0.2	-1.8 ± 0.1	17.2 ± 0.1
0.111	-2.6 ± 0.1	-1.6 ± 0.1	17.2 ± 0.1
0.184	-2.5 ± 0.2	-1.5 ± 0.1	17.2 ± 0.1

$B_{22}^{\text{HS}} / M = 1.66 \times 10^{-4} \text{ mol mLg}^{-2}$ for monomer–monomer interactions calculated using Eq. (8) with a hard sphere radius of 1.7 nm.

Table 4.

rhIL-1ra Dimerization Energetics at 25°C and pH 6.5 Calculated Using Eqs. (20) and (22) With the Integrated Endothermic Peak Areas From ITC Dilution Experiments Shown in Figure 8

Ionic Strength (m)	K_d (mM)	$G_{\text{dimerization}}$ (kcal/mol)	$H_{\text{dimerization}}$ (kcal/mol)	$T S_{\text{dimerization}}$ (kcal/mol)	H_{dilution} (cal/mol)
0.025	3.8 (2.9, 5.2)	-3.3 (-3.5, -3.1)	-14.8 (-13.9, -16.5)	-11.5 (-10.5, -13.4)	-0.022 (-0.018, -0.029)
0.031	4.2 (3.2, 6.0)	-3.2 (-3.4, -3.0)	-14.1 (-13.1, -16.4)	-10.9 (-9.7, -13.4)	-0.019 (-0.014, -0.027)
0.060	5.4 (3.9, 8.7)	-3.1 (-3.3, -2.8)	-12.2 (-10.9, -15.5)	-9.1 (-7.6, -12.7)	-0.017 (-0.012, -0.028)
0.091	7.5 (4.9, 15.3)	-2.9 (-3.1, -2.5)	-11.1 (-9.0, -17.5)	-8.3 (-5.8, -15.0)	-0.018 (-0.011, -0.033)
0.111	8.4 (5.4, 20.4)	-2.8 (-3.1, -2.3)	-11.0 (-8.9, -21.0)	-8.1 (-5.8, -18.7)	-0.016 (-0.009, -0.037)
0.184	12.6 (6.8, 79.8)	-2.6 (-3.0, -1.5)	-8.1 (-5.6, -44.0)	-5.5 (-2.6, -42.5)	-0.011 (-0.005, -0.044)

Values in parentheses are 95% confidence intervals based on 95% confidence intervals for sedimentation equilibrium K_d values. The values for H_{dilution} were fit using Eq. (21).