Analyses of Thermodynamic Data for Concentrated Hemoglobin Solutions Using Scaled Particle Theory: Implications for a Simple Two-State Model of Water in Thermodynamic Analyses of Crowding In Vitro and In Vivo

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ABSTRACT Quantitative description of the thermodynamic consequences of macromolecular crowding (excluded volume nonideality) is an important component of analyses of the thermodynamics and kinetics of noncovalent interactions of biopolymers in vivo and in concentrated polymer solutions in vitro. By analyzing previously published thermodynamic data, we have investigated extensively the comparative applicability of two forms of scaled particle theory (SPT). In both forms, macromolecules are treated as hard spheres, but MSPT, introduced by Ross and Minton, treats the solvent as a structureless continuum, whereas bulk water molecules are included explicitly as hard spheres in BSPT, an approach developed by Berg. Here we use both MSPT and BSPT to calculate the excluded volume component of the macromolecular activity coefficient of hemoglobin (Hb) at concentrations up to 509 mg/ml by fitting osmotic pressure data for Hb and sedimentation equilibrium data for Hb and sickle-cell Hb (HbS). Both forms of SPT also are used here to analyze the effects of other globular proteins (BSA and Hb) on the solubility of HbS. In applying MSPT and BSPT to analyze macromolecular crowding, the extent of hydration δ_{Hb} (in $gH_2O/gprotein$) is introduced as an adjustable parameter to specify the effective (hard sphere) radius of hydrated Hb. In our nonlinear least-squares fittings based on BSPT, the hard sphere radius of bulk water molecules is either fixed at 1.375 Å or floated. Although both forms of SPT yield good fittings (with different values of δ_{Hb}) at Hb concentrations up to 350 mg/ml, only BSPT gives good fittings of all available Hb osmotic pressure data as well as of the sedimentation equilibrium and solubility data. Only BSPT predicts values for δ_{Hb} (~0.5–0.6 g/g) in the range obtained for Hb from hydrodynamic measurements (~0.36–0.78 g/g). These findings indicate the applicability, at least in the context of BSPT, of a simple two-state classification of water (bulk water and water of macromolecular hydration) as a basis for interpreting excluded volume nonideality in concentrated solutions of globular proteins.

INTRODUCTION

Scaled Particle Theory (SPT) provides a potentially powerful theoretical basis for analysis of the thermodynamic consequences of excluded volume under the crowded conditions of the intracellular environment (Fulton, 1982; Cayley et al., 1991; Zimmerman and Trach, 1991). In the cytoplasm of *Escherichia coli*, the macromolecule (i.e., protein, ribosome, RNA, and DNA) concentration increases from ~275 mg/ml to ~450 mg/ml as the osmolarity of the growth medium is increased from 0.1 Osm to 1.0 Osm (Cayley et al., 1991). Cayley et al. (1991) proposed that this general increase in macromolecule concentration greatly increases the effect of macromolecular crowding as a driving force for macromolecular association in vivo (e.g., binding of proteins to DNA) as the osmolarity of the growth medium increases. They proposed that the increase in macromolecular crowding with increasing external osmolarity compensates at a thermodynamic level for the concomitant increase in cytoplasmic K^+ concentration, resulting in an osmotically insensitive extent of binding of proteins to nucleic acids and, hence, a homeostasis of gene expression over a range of osmolarities. Alternatively, Zimmerman and Trach (1991) proposed that the effects of macromolecular crowding at any external osmolarity are so large that virtually all of these DNA-binding proteins are bound (either specifically or nonspecifically) to DNA. According to this proposal, homeostasis results from the constancy of the ratio of specific to nonspecific DNA sites at all external osmolarities. Both of these proposals are based on estimates of the macromolecular concentration dependence of thermodynamic nonideality in vivo (specifically, macromolecular activity coefficients) obtained by using Minton's (1983) version of scaled particle theory (here designated MSPT).

MSPT was introduced by Ross and Minton (1979) to calculate the variation in solubility of sickle-cell hemoglobin (HbS) as a function of added "inert" (non-HbS) protein concentration, for comparison with data published by Behe and Englander (1978). Ross and Minton assigned a radius for HbS of 28 Å (approximately equal to the unhydrated radius of a spherical model of HbS), which they (Ross and Minton, 1977) had obtained previously from a McMillan-Mayer hard sphere (MMHS) virial analysis of osmotic pressure measurements as a function of hemoglobin (Hb) concentration (Adair, 1928). The solubility data of Behe and Englander also were analyzed by Berg (1990), who developed an alternative version of SPT (here designated BSPT) that includes water explicitly as hard spheres to calculate macromolecular activity coefficients. Specifically, Berg (1990) used BSPT to predict the radius of HbS that provided the best (visual)

Received for publication 21 September 1994 and in final form 1 December 1994.

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0006-3495/95/03/835/12 $2.00

agreement with the solubility curve calculated by Ross and Minton (1979) using MSPT. From a semi-quantitative comparison of the MSPT- and BSPT-predicted solubility curves, Berg reported a radius of HbS (33 Å) that is close to the experimental hydrodynamic radius (~33.6 Å; Squire and Himmel, 1979).

One of the goals of this paper is to determine the utility of, and interpret the fitting parameters predicted by, MSPT and BSPT. No systematic comparisons of the applicability of these two theories to the existing body of diverse thermodynamic measurements on concentrated solutions of any macromolecule have been made to date. For this first quantitative comparison of MSPT and BSPT, we have analyzed the following sets of thermodynamic measurements on concentrated protein solutions containing Hb or HbS: 1) osmotic pressure data for Hb extending to 350 mg/ml (Adair, 1928); 2) osmotic pressure data for Hb up to 509 mg/ml (data collected by Adair, but published by Dick, 1967); and 3) sedimentation equilibrium data for carbon monoxide-saturated Hb (COHb) and for carbon monoxide-saturated sickle cell Hb (COHbS), which extend to ~350 mg/ml (Ross et al., 1978). To quantify the comparison of MSPT and BSPT made by Berg (1990), we also used both MSPT and BSPT to analyze the effects of Hb and BSA on the solubility for HbS, as reported by Behe and Englander (1978). These sets of thermodynamic data are expected to provide informative tests of MSPT and of BSPT, because they extend over an exceptionally wide range of Hb concentrations. Hb is relatively spherical (Squire and Himmel, 1979), nonaggregating in water (cf. the discussion of Kruger et al., 1990), and weakly charged in the range of pH of these experimental data (Adair, 1928). Moreover, the Hb data are the most appropriate available for evaluation of the applicability of BSPT and MSPT at macromolecular concentrations in the physiological range.

THEORETICAL BACKGROUND

Overview of applications of SPT to macromolecular solutions

Ross and Minton (1977) used a truncated virial expansion to calculate the mechanical pressure of a hard sphere model protein dissolved in continuum solvent to account for the contribution of intersolute interactions to the dependence of osmotic pressure on Hb concentration (Adair, 1928). On the basis of McMillan-Mayer theory (1945) (cf. Hill, 1960), the osmotic pressure of the protein solution was equated to the mechanical pressure of a model solution where only hard sphere interactions among the proteins were considered explicitly. (We designate this approach as MMHS: McMillan-Mayer with hard sphere virial coefficients.) Subsequently, Minton and others (e.g., Minton, 1981; Minton, 1983; Behe and Englander, 1978; Berg, 1990) used MMHS and/or SPT as alternative methods of predicting the excluded volume nonideality of aqueous solutions containing one or more (in the case of SPT) types of globular proteins as a function of macromolecular concentration. SPT has the distinct advantage of being readily applicable to calculate macromolecular activity coefficients in solutions containing spherical macromolecules of different radii, whereas applications of MMHS to solutions containing more than one macromolecular component do not appear to be practical. (To date, none has been reported.)

SPT was derived by Lebowitz and co-workers to describe thermodynamic properties of a hard sphere fluid containing one (Reiss et al., 1959) or more (Lebowitz et al., 1965) components. Analogous expressions for mixtures of particles of different shapes have not been derived using SPT. Two variants of SPT have been developed with the specific objective of calculating the contribution of excluded volume to macromolecular activity coefficients in concentrated protein solutions: the first by Minton and co-workers (Ross and Minton, 1979) and the second by Berg (1990). Neither of these forms of SPT is predicated on the virial expansion or entails the explicit evaluation of virial coefficients.

Minton's application of SPT (here designated MSPT) considers only interactions among the macromolecules, modeled as hard spheres, to calculate their activity coefficients due to excluded volume interactions. Water and all other low molecular weight species are not explicitly considered. The solution is therefore treated like a single-component system, in which the existence of solvent is recognized only insofar as it may affect the size (i.e., effective hard sphere radius) of the macromolecule of interest. Some implementations of MSPT to analyze and interpret data pertaining to thermodynamic nonideality in concentrated protein solutions in vitro have been summarized by Minton (1983).

Berg's (1990) later application of SPT (designated BSPT) explicitly includes water as a hard spherical particle that interacts with itself and with solute macromolecules. In this approach, the solute activity coefficient, a measure of the excess free energy of the solute due to hard particle interactions, is related to the isothermal, isobaric work of the hypothetical process of transferring that solute from pure water (represented as hard spheres) to the macromolecular solution (represented as a mixture of hard sphere water and hard sphere macromolecules).

Summary of SPT formulas

SPT can be used to predict the contribution of excluded volume effects (i.e., hard core interactions) to the excess chemical potential of a species k in a system comprised of one or more species modeled as hard spheres. The activity coefficient of species k (relative to the ideal gas reference state where the interaction potential of the fluid with species k is zero everywhere) can be expressed as

$$\ln \gamma_k^{SPT} = -\ln(1 - S_3) + \left[\frac{6S_2}{1 - S_3}\right] r_k + \left[\frac{12S_1}{1 - S_3} + \frac{18S_2^2}{(1 - S_3)^2}\right] r_k^2 + \left[\frac{8S_0}{1 - S_3} + \frac{24S_1S_2}{(1 - S_3)^2} + \frac{24S_2^3}{(1 - S_3)^3}\right] r_k^3. \quad (1)$$

In Eq. 1, each term of the form S_j ($0 \leq j \leq 3$) is given by

$$S_j = \frac{\pi}{6} \sum_i \nu_i (2r_i)^j, \tag{2}$$

where ν_i is the number density of species i characterized by a hard sphere radius r_i and $\pi = 3.14159 \cdots$. When all of the $r_i \to 0$, all of the $S_j \to 0$ and $\ln\gamma_k^{SPT} \to 0$, consistent with the fact that Eq. 1 was derived with respect to the ideal gas reference state.

MSPT formulation

Ross and Minton (1979) implicitly used a continuum solvent model (i.e. neglecting the molecularity of solvent and any low-molecular weight solutes) to treat the macromolecular solution as a single component macromolecular system. To use MSPT in fitting thermodynamic data, S_j (in Eq. 2) is given by

$$S_j^{MSPT} = \frac{\pi}{6} \nu_{Hb} (2r_{Hb})^j. \tag{3}$$

With this definition of S_j^{MSPT}, the quantity calculated from Eq. 1, $\ln \gamma_{Hb}^{MSPT} \to 0$ as the Hb concentration $\nu_{Hb} \to 0$.

BSPT formulation

Nonideality of a solute in solution is typically determined experimentally relative to an ideal dilute solution reference state, achieved by approaching the limit of infinite dilution of solute in solvent (i.e., where $\nu_w \to \nu_{w,0}$, the value of ν_w for pure water, and where all $\nu_{i \neq w} \to 0$). As noted by Berg (1990), the ideal dilute solution (designated by the superscript "ids") of Hb in water is obtained when S_j in Eq. 2 takes the form

$$S_j^{ids} = \frac{\pi}{6} \nu_{w,0} (2r_w)^j. \tag{4}$$

introducing Eq. 4 into Eq. 1 yields $\ln \gamma_{Hb}^{SPT,ids}$. The Hb concentration-dependent contribution from excluded volume effects to nonideality of the Hb-water mixture defined by BSPT, therefore, is

$$\ln \gamma_{Hb}^{BSPT} \equiv \ln \gamma_{Hb}^{SPT} - \ln \gamma_{Hb}^{SPT,ids}, \tag{5}$$

where $\gamma_{Hb}^{SPT,ids}$ is the activity coefficient of Hb in pure water relative to the ideal gas reference state (obtained using Eqs. 4 and 1), and γ_{Hb}^{SPT} is the activity coefficient of Hb in the Hb-water mixture (at ν_{Hb}), also relative to the ideal gas reference state. In all of the applications of BSPT presented in this paper, no more than two species of differing size are considered (Hb and water, w). Hence, γ_{Hb}^{SPT} in Eq. 5 is calculated from

$$S_j = \frac{\pi}{6} [\nu_w (2r_w)^j + \nu_{Hb} (2r_{Hb})^j], \tag{6}$$

where ν_w is calculated as described in the next section.

Variants of BSPT: approximate and exact calculations of the number density of the bulk solvent

When eqs. 1 and 6 are used to calculate the solute activity coefficient in the macromolecular solution, one of the necessary input variables not known a priori is ν_w. To calculate ν_w, Berg derived an approximate analytic expression by introducing the SPT expression for the solute activity coefficient into the Gibbs-Duhem equation and integrating at constant pressure and temperature. This approximate analytic expression (Eq. A9 in Berg, 1990) is accurate to within 2% at concentrations up to 200 mg/ml (according to our more exact numerical calculations, as described below), but becomes less accurate at the higher macromolecular concentrations in the physiological range that also are of interest here.[1]

In our adaptation of BSPT, the hard sphere mechanical pressure in the macromolecular solution (where the bulk water density is ν_w) is equated to the hard sphere mechanical pressure of pure water (of density ν_w^o) by using the following general SPT expression (Lebowitz et al., 1965):

$$\frac{\pi}{RT} P^{SPT} = \frac{6S_0}{1 - S_3} + \frac{18S_1S_2}{(1 - S_3)^2} + \frac{18S_2^3}{(1 - S_3)^3}, \tag{7}$$

where the S_j are given either by Eq. 4 for water or by Eq. 6 for the macromolecular solution. Our approach to the evaluation of ν_w is thermodynamically equivalent to Berg's integration of the Gibbs-Duhem relation at constant temperature and pressure, but is computationally preferable in situations where the approximation required for analytic integration of the Gibbs-Duhem equation ceases to be reliable. To derive an expression for the SPT activity coefficient of a component in a mixture of hard spheres (Lebowitz et al., 1965), the pressure is included initially as an unknown quantity in the excess free energy, and the derivation proceeds by integrating the isothermal, variable-pressure Gibbs-Duhem relation. Alternatively, holding the pressure constant yields the isobaric, isothermal Gibbs-Duhem equation used by Berg to derive his approximate analytic expression for ν_w. Specifically, Eq. 5.2 in Lebowitz et al. (1965) at constant pressure is equivalent to Eq. A.6 in Berg (1990).

Our numerical method of evaluating ν_w using Eq. 7 stipulates that the SPT-predicted hard sphere pressure of the macromolecular solution be equal (within a small fraction, $\sim 10^{-15}$) to the SPT-predicted hard sphere pressure of pure

[1] We find that the approximation in Berg's (1990) development of BSPT always overestimates the activity coefficient (γ_{Hb}) in a model Hb solution as compared with the exact value determined using our version of BSPT. The percent overestimation increases monotonically with increasing macromolecular concentration from ~2% at 200 mg/ml to ~8% at 400 mg/ml. In the analyses performed here, Berg's version of BSPT gives a smaller value for δ_{Hb}, although it always falls within 1 SD of the best-fitted value of δ_{Hb} obtained using our version of BSPT. The difference between the two versions of BSPT is negligible for the protein concentration range examined by Berg (1990), but becomes significant at higher concentrations of macromolecules, such as those encountered in vivo.

water (i.e., the ideal dilute solution state). This pressure is very large (~7800 atm) compared with typical experimental conditions (1 atm), because only hard sphere interactions are included in the SPT model. Any application of SPT wherein the particle density of the model system is equated to that in a real (liquid) system will necessarily predict an excessive pressure, because the attractive forces responsible for the volume of a system in the condensed state are not included in SPT.

It is noteworthy that, using Eqs. 2 and 7, one can derive an exact, analytic expression for the number density of bulk water in the macromolecular solution (ν_w) in which ν_w is a cubic polynomial (cf. Guttman, 1994). In all cases examined, we have found the solution to contain one real root (with a physically reasonable value) and two complex roots. Because the coefficients of the cubic equation do not indicate clearly the class of this cubic polynomial, it is possible that some cases yield three real roots.

Specification of hard sphere radii of Hb and water

Applications of MSPT and BSPT to describe the concentration dependence of Hb nonideality in water require specification of the radius of Hb (r_{Hb}) and, for BSPT, the radius of water (r_w), both of which are taken to be independent of the particle densities, ν_i. In general, the effective hard sphere volume of Hb appropriate for applications of SPT may differ from its partial molar volume. The hard sphere radius r_{Hb} (in Å) that appears in Eq. 3 or Eq. 6 is related to V^h_{Hb}, the hydrated macromolecular volume, by analogy with the classical expression used in analyzing transport data. (See, e.g., pp. 339–341 in Tanford (1961) and pp. 584–586 in Cantor and Schimmel (1980)):

$$r_{Hb} = \left(\frac{3V^h_{Hb}}{4\pi}\right)^{1/3}, \qquad (8)$$

$$\text{where} \quad V^h_{Hb} = M_{Hb}\frac{10^{24}}{N_A}(\bar{v}_{Hb} + \delta_{Hb}v^\circ_w).$$

Here V^h_{Hb} is the volume of the hydrated Hb particle (in Å^3/molecule), δ_{Hb} is the nondimensionalized increment of hydration (as conventionally expressed), in units of gH_2O/gprotein; v°_w is the specific volume of pure water (1.0 cm^3/g at 20°C); $\bar{v}_{Hb}$ is the partial specific volume of Hb (0.75 cm^3/g); M_{Hb} is the molecular mass of Hb (64,610), and N_A is Avogadro's number (Tanford, 1961; Squire and Himmel, 1979). The same values of the quantities M_{Hb}, $\bar{v}^\circ_{Hb}$, and δ_{Hb} also are applied in analyzing solubility data for the sickle-cell mutant Hb (HbS), because HbS differs from Hb by only one amino acid in each of the β subunits.

All of the quantities on the right-hand side of Eq. 8 are taken as known except δ_{Hb}, which is floated (rather than r_{Hb}) when MSPT or BSPT is used to calculate $\ln\gamma_2$ for the purpose of fitting a given set of data. A single value of δ_{Hb} is fitted over the entire Hb concentration range. Thus, r_{Hb} is assumed to be independent of Hb concentration. The data of Adair and Adair (1947) indicate that the partial specific volume of Hb is independent of Hb concentration, at least over much of the range of the data fitted here. (Consequently, for the purpose of analyzing osmotic pressure measurements using the equation presented below, $\bar{v}_{Hb}$ in Eq. 8 can be equated to $\bar{v}^\circ_{Hb}$, its value in the "ideal dilute solution" standard state.) It cannot be known a priori whether the effective hard sphere volume of Hb differs from its partial molar volume, or whether this difference is constant over the experimental range of Hb concentrations. If, however, the functional form of $\ln\gamma_2$ that results from assuming the constancy of δ_{Hb} (in either MSPT or BSPT) proves capable of fitting a given body of data over the entire range of Hb concentrations, then this assumption may at least be deemed consistent with the available experimental information. Estimates of δ for proteins in solution typically exhibit some dependence on the method of measurement (Kuntz and Kauzmann, 1974). Thus, best-fitted values of δ_{Hb} obtained by analyzing various kinds of thermodynamic data with the hard sphere model for Hb (MSPT), or for both Hb and H_2O (BSPT), may not be in close agreement with each other or with values obtained by analyses of transport experiments (0.36–0.74 gH_2O/gHb; Tanford, 1961; Kuntz and Kauzmann, 1974; Squire and Himmel, 1979).

For the BSPT calculations, a hard sphere radius for water (r_w) also must be specified. In most of our fittings, r_w was fixed at 1.375 Å (Pierotti, 1965); cases where both r_w and δ_{Hb} were fitted are discussed below. We use a single value of r_w to describe a given set of data over the entire Hb concentration range. Therefore, we implicitly assume that r_w is independent of Hb concentration over the range of data reported.

METHODS

To ascertain the extent to which MPST and BSPT can account for the concentration dependence of Hb nonideality in aqueous solutions, the theoretical dependences of γ^{MSPT}_{Hb} and γ^{BSPT}_{Hb} on Hb concentration are compared using fittings of experimental thermodynamic data from Hb osmotic pressure measurements, Hb sedimentation equilibrium measurements, and HbS solubility in solutions with non-HbS protein added.

All fittings were obtained using NONLIN (Johnson and Frasier, 1985; Straume et al., 1991). All errors reported from our fittings are 1 SD as obtained from NONLIN using a 67% confidence probability. Values of the reduced chi-squared (χ^2_r) are evaluated from the variance obtained from NONLIN.

Osmotic pressure fittings

Comparisons of the predictions of MSPT or BSPT with experimental measurements of osmotic pressure (Π) as a function of Hb concentration are obtained using the following general thermodynamic relationship between Π and the solute activity coefficient γ_{Hb} (derived in the Appendix).

$$\frac{\Pi}{RT}M_{Hb} = \int_0^{c_{Hb}} (1 - c_{Hb}\bar{v}^\circ_{Hb})^{-1}\left(1 + \left(\frac{\partial \ln \gamma_{Hb}}{\partial \ln c_{Hb}}\right)_{T,\mu_{i\neq Hb}}\right) dc_{Hb} \qquad (9)$$

$$= -\frac{1}{\bar{v}^\circ_{Hb}}\ln(1 - c_{Hb}\bar{v}^\circ_{Hb}) + \frac{c_{Hb}\ln\gamma_{Hb}}{1 - c_{Hb}\bar{v}^\circ_{Hb}} - \int_0^{c_{Hb}} \frac{\ln\gamma_{Hb}}{(1 - c_{Hb}\bar{v}^\circ_{Hb})^2} dc_{Hb}.$$

Here $\bar{v}^\circ_{Hb}$ (in ml/g) is the partial specific volume of Hb in its standard (ideal dilute solution) state, c_{Hb} is the Hb concentration (in g/ml), R is the gas

constant, T is the temperature, and M_{Hb} is fixed at 64,610 Da. For calculations using Eq. 9 to analyze the Hb osmotic pressure data reported by Dick (1967), we converted the Hb molalities to molar concentration units by using a partial specific volume of 0.75 ml/g for Hb and a density of water (bulk and hydration) equal to 1 g/ml. Further details on the introduction of the predictions of either MSPT or BSPT into Eq. 9 for comparisons with experimental data are given in the Appendix.

Using Eqs. 1, 3, and 9, we have derived an explicit analytic equation for the MSPT-predicted osmotic pressure. By analogy with Eq. 8, we define the hard sphere hydrated (h) and unhydrated ($\delta = 0$) volumes (u) of Hb (in Å^3/molecule) as

$$V^h_{Hb} \equiv \frac{4}{3}\pi r^3_{Hb} = M_{Hb}\frac{10^{24}}{N_A}(\bar{v}_{Hb} + \delta_{Hb}\bar{v}^o_w)$$

and

$$V^u_{Hb} \equiv M_{Hb}\frac{10^{24}}{N_A}\bar{v}_{Hb}.$$

With the Hb concentration, ν_{Hb}, in units of molecules/Å^3, we obtain

$$\Pi = \frac{RT10^{24}}{N_A}\left[\left(\frac{1}{V^h_{Hb} - V^u_{Hb}} - \frac{15(V^h_{Hb})^2 + 7V^h_{Hb}V^u_{Hb}}{V^u_{Hb}(V^h_{Hb} - V^u_{Hb})^2} + \frac{15(V^h_{Hb})^4 - 24(V^h_{Hb})^3V^u_{Hb}}{V^u_{Hb}(V^h_{Hb} - V^u_{Hb})^4}\right)\ln\left(\frac{1 - V^u_{Hb}\nu_{Hb}}{1 - V^h_{Hb}\nu_{Hb}}\right) + \frac{22 + (\alpha_3/V^h_{Hb})(V^h_{Hb} - V^u_{Hb})}{(V^h_{Hb} - V^u_{Hb})(1 - V^h_{Hb}\nu_{Hb})} + \frac{\alpha_4}{2V^h_{Hb}(1 - V^h_{Hb}\nu_{Hb})^2} + \frac{\alpha_5}{3V^h_{Hb}(1 - V^h_{Hb}\nu_{Hb})^3}\right], \quad (10)$$

where, in Eq. 10,

$$\alpha_1 = \frac{24(V^u_{Hb}/V^h_{Hb}) - 15}{(1 - V^u_{Hb}/V^h_{Hb})^4}, \qquad \alpha_2 = -\frac{V^h_{Hb}}{V^u_{Hb}}(15 + \alpha_1), \qquad \alpha_3 = \frac{V^h_{Hb}}{V^u_{Hb}}\left((15 + \alpha_1)\frac{V^h_{Hb}}{V^u_{Hb}} - \alpha_1 + 21\right)$$

$$\alpha_4 = -(\alpha_1 + \alpha_2 + \alpha_3 + \alpha_5), \quad \text{and} \quad \alpha_5 = \frac{9}{1 - V^u_{Hb}/V^h_{Hb}}.$$

A weighted nonlinear least-squares method (Straume et al., 1991; Johnson and Frasier, 1985) was used to fit Eq. 10 to the osmotic pressure data.

The predictions of BSPT are introduced into Eq. 9 as described in the Appendix, and the integral specified in Eq. 9 is evaluated numerically using standard routines from the IMSL library.

Sedimentation equilibrium fittings

The apparent molecular weight of Hb, $M_{Hb,app}$, evaluated from sedimentation equilibrium experiments, can be expressed in terms of directly measurable quantities as

$$M_{Hb,app} = \frac{2RT}{\omega^2(1 - \bar{v}_{Hb}\rho)}\frac{d\ln c_{Hb}}{d(x^2)}, \quad (11)$$

where ω is the rotor speed, ρ is the solution density, and x is the axial distance from the center of the rotor (see, e.g., pp. 254–262 of Tanford, 1961). The experimental quantity $M_{Hb,app}$ also is related to the dependence of $\ln \gamma_{Hb}$ on $\ln c_{Hb}$ by the thermodynamic expression

$$M_{Hb,app} \equiv \frac{M_{Hb}}{1 + (\partial \ln \gamma_{Hb}/\partial \ln c_{Hb})_{T,P}}, \quad (12)$$

where M_{Hb} = 64,610 g mol^{-1}. To obtain a theoretical prediction of $M_{Hb,app}$, the activity coefficients, γ_{Hb}, are calculated using either Eqs. 1–3 for MSPT or Eqs. 1, 2, and 4–6 for BSPT, and the derivative $(\partial \ln \gamma_{Hb}/\partial \ln c_{Hb})_{T,P}$ is evaluated numerically using standard routines from the IMSL library. An unweighted nonlinear least-squares method (Straume et al., 1991; Johnson and Frasier, 1985) was used for all of the sedimentation equilibrium fittings.

Solubility fittings

For the analysis of aqueous solubility data of HbS as a function of non-HbS protein (typically called "inert" and designated here NHbS) added to the aqueous phase, the activity of HbS in the gel phase is assumed to be independent of the amount of added NHbS protein, either Hb or BSA (Behe and Englander, 1978; Ross and Minton, 1979). Because of the similar molecular weights and near spherical shapes of Hb, HbS, and BSA, the radius of the NHbS protein is assumed to be equal to that of HbS in our SPT analyses. The condition of phase equilibrium for the solubility experiment is that the thermodynamic activity of HbS in the saturated solution (ss) is equal to that in the gel phase at a given T and P. Because it is assumed that the gel phase HbS activity is unaffected by NHbS protein, the activity of HbS in a saturated solution in the absence of NHbS protein, $a^{ss,0}_{HbS}$, is equal to $a^{ss,cNHbS}_{HbS}$, the activity of the HbS component in a saturated solution in the presence of NHbS protein at concentration c_{NHbS}. (Note that the superscript zero, used here to denote the absence of NHbS, differs from the symbol used elsewhere in this paper to denote the standard state value of the indicated thermodynamic property.) Therefore,

$$c^{ss,cNHbS}_{HbS} = c^{ss,0}_{HbS}\frac{\gamma^{ss,0}_{HbS}}{\gamma^{ss,cNHbS}_{HbS}}. \quad (13)$$

Theoretical calculations of the HbS activity coefficients are obtained using either MSPT Eqs. 1–3 or BSPT Eqs. 1, 2, and 4–6. To determine numerically the functional relationship between $c^{ss,cNHbS}_{HbS}$ and c_{NHbS}, we first calculate $\gamma^{ss,0}_{HbS}$ from $c^{ss,0}_{HbS}$. We then specify c_{NHbS} and search iteratively for the value of $c^{ss,cNHbS}_{HbS}$ (recalculating $\gamma^{ss,cNHbS}_{HbS}$ with each iteration of $c^{ss,cNHbS}_{HbS}$) that satisfies Eq 13. For the fitting procedure, $c^{ss,0}_{HbS}$ and δ_{Hb} are floated simultaneously. In our fittings, we have combined the solubility data of three separate experiments, the same as those analyzed by Minton (1980) and considered subsequently by Berg (1990). Each data set, extracted from Fig. 2 of Behe and Englander (1978), was corrected for the reported 5% artifactual decrease in the supernatant protein concentration. An unweighted nonlinear least-squares method (Straume et al., 1991; Johnson and Frasier, 1985) was used in the solubility fittings.

RESULTS

SPT-based analysis of osmotic pressure data for Hb

Adair (1928) reported measurements of the osmotic pressure of aqueous hemoglobin (Hb) solutions at a moderate salt concentration (pH ~7.8, 0.1 M KCl, 0.0613 M Na_2HPO_4, 0.0053 M KH_2PO_4) near the pI of Hb (pI = 6.8). The data extend from ~4 to ~340 mg/ml. Hemoglobin concentrations in red blood cells and total cytoplasmic protein concentrations in *E. coli* at high osmolarity approach the upper end of this range. Adair's data were fitted by Ross and Minton

(1977) using McMillan-Mayer theory with the virial expansion for hard spheres (MMHS) but have not been analyzed previously using SPT. Dick (1967) reported an extensive investigation by Adair of the osmotic pressure of Hb solutions (between ~113 and ~509 mg/ml) under conditions similar to those of Adair (1928) (0.1 "ionic strength"; pH 7.43). The extended range of Hb concentrations in this data set is important to provide a more stringent test of the two SPT theories and because total cytoplasmic macromolecular (i.e., total protein and nucleic acid) concentrations in *E. coli* at high osmolarity exceed 340 mg/ml.

Fig. 1 and 2 show our fittings of the two sets of osmotic pressure data. Because uncertainties were not reported, we have assumed that the SD for each osmotic pressure determination is 6% of the measured osmotic pressure, as reported previously by Adair (1925). The MSPT osmotic pressure is calculated using Eq. 10. The BSPT osmotic pressure is calculated from Eqs. 1, 4–6, and 9 using standard numerical algorithms for integration (IMSL).

In the MSPT fittings and in one set of the BSPT fittings, the only adjustable parameter is δ_{Hb}. We find that the best-fitted values of δ_{Hb} from BSPT (0.56 ± 0.01 g/g for Adair's (1928) data and 0.442 ± 0.004 for Adair's data reported by Dick (1967)) are much closer to the range of values of δ_{Hb} obtained by analysis of transport data (e.g., 0.74 g/g, Squire and Himmel (1979); ~0.6 g/g, Kuntz and Kauzmann (1974); ~0.36 g/g, Tanford (1961)) than is the value of δ_{Hb} predicted when MSPT is used (0.03 ± 0.01 g/g for Adair's (1928) data[2] and 0.00 ± 0.01 for Adair's data reported by Dick (1967)). The quality of the BSPT fitting of Adair's data reported by Dick (1967) is substantially better than that of the MSPT fitting, as is demonstrated by the order of magnitude difference in χ_r^2 (see Fig. 2 legend) and by visual inspection of the best-fitted curves (see Fig. 2). Characteristics of the fittings are summarized in Table 1.

In the two-parameter fitting using BSPT, where both the radius of water (r_w) and δ_{Hb} are floated, δ_{Hb} is found to be the same (within 1 SD) as that obtained from the single-parameter fitting (where r_w is fixed at 1.375 Å). Compared with the fixed value of r_w in the single-parameter BSPT fittings, the values of r_w obtained from the two-parameter fittings fall within 1 SD for Adair's (1928) data and within 2 SD for Adair's data reported by Dick (1967). The fitted curve resulting from the two-parameter fitting is superimposable on the fitted curve of the single-parameter fitting (δ_{Hb}) for Adair's (1928) data and almost superimposable on that for Adair's data reported by Dick (1967). For fittings of both data sets, the range of uncertainty in δ_{Hb} calculated in the two-parameter fitting is 10-fold larger than that in the single-parameter fitting. The high correlation between δ_{Hb} and r_w calculated by NONLIN (which yields a correlation coefficient of 0.995 for Adair's (1928) data and 0.99 for Adair's data reported by Dick (1967)) indicates that the errors of the two-parameter fittings are significantly larger than those calculated by NONLIN.

The value of δ_{Hb} from the single-parameter BSPT fitting in Fig. 2 is significantly smaller than that obtained in Fig. 1. This may indicate a real difference resulting from coulombic or other contributions to nonideality not modeled by BSPT, or from some other systematic difference in the data sets. Both values of δ_{Hb} are in the range estimated experimentally from hydrodynamic measurements. For the two-parameter fittings, the errors in both parameters (δ_{Hb} and r_w) are much larger, and the numerical values of both δ_{Hb} and r_w agree within error for the two data sets.

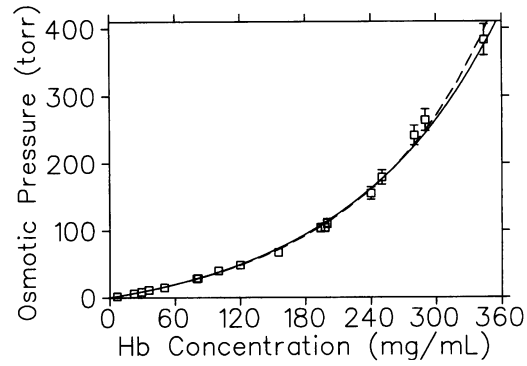

FIGURE 1 Best-fittings to Adair's osmotic pressure data (□) (Adair, 1928). The solid curve is the fitting using MSPT. The dashed curve is the fitting using BSPT with r_w fixed at 1.375 Å. The fitting using BSPT allowing r_w to float is superimposable on the dashed curve. Results of these fittings are summarized in Table 1.

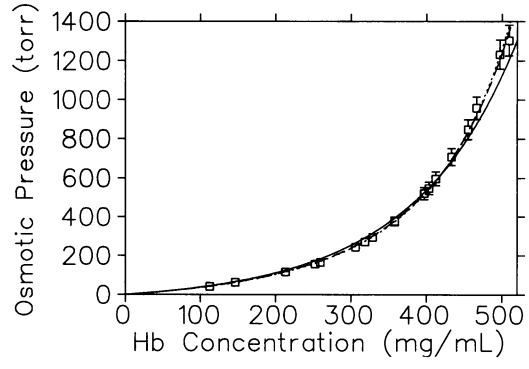

FIGURE 2 Best-fittings to Adair's osmotic pressure data published by Dick (□) (Dick, 1967). The solid curve is the fitting using MSPT ($\chi_r^2 = 1.5$). The long-dashed curve is the fitting using BSPT with r_w fixed at 1.375 Å ($\chi_r^2 = 0.18$). The short-dashed curve is the fitting using BSPT with δ_{Hb} and r_w floated ($\chi_r^2 = 0.14$). Results of these fittings are summarized in Table 1.

[2] In a previous publication, we reported a best-fitted $\delta_{Hb} = 0.045 \pm 0.006$ g/g using MSPT to fit Adair's (1928) data (Cayley et al., 1991). The difference between this value of δ_{Hb} and our currently reported best-fitted value of δ_{Hb} using MSPT arises solely from the methods of assigning errors to the osmotic pressure measurement. Our previous calculation was a conventional unweighted, nonlinear, least-squares fitting based on the assumption of a constant absolute error in each osmotic pressure. However, consideration of earlier work by Adair (1925) and discussion with A. Parsegian (personal communication) led us to conclude that use of a constant percentage error is a more reasonable assumption for the osmotic pressure determinations.

TABLE 1 SPT analyses of thermodynamic data for Hb solutions

Experimental data from:	Protein concentration range (mg/ml)	MSPT δ_{Hb} ($gH_2O \div gHb$)	BSPT* δ_{Hb} ($gH_2O \div gHb$)	BSPT‡ δ_{Hb} ($gH_2O \div gHb$)	BSPT‡ r_w (Å)
Osmotic Pressure§	7–344	0.03 ± 0.01	0.56 ± 0.01	0.6 ± 0.2	1.4 ± 0.3
Osmotic Pressure¶	113–509	0.00 ± 0.01	0.442 ± 0.004	0.50 ± 0.04	1.48 ± 0.07
Sedimentation Equil.‖	5–361	0.014 ± 0.003	0.537 ± 0.005	0.33 ± 0.05	1.03 ± 0.09
Solubility**	195–301	−0.01 ± 0.03	0.48 ± 0.05		

*r_w fixed at 1.375 Å, δ_{Hb} floated.
‡Both r_w and δ_{Hb} floated.
§Adair (1928).
¶Data collected by Adair and published by Dick (1967).
‖Ross et al. (1978), data provided by A. Minton.
**Behe and Englander (1978); r_w and δ_{Hb} are too highly correlated to be fitted independently.

SPT-based analysis of sedimentation equilibrium data for COHb

Ross et al. (1978) carefully investigated thermodynamic nonideality of concentrated aqueous solutions of COHb and HbS (COHbS) by sedimentation equilibrium at temperatures of 2, 10, and 20°C (pH ~ 7.0, 0.1 M phosphate buffer). (These data were kindly provided to us by Dr. A. Minton.) At each temperature, the apparent molecular weight of COHbS was determined as a function of HbS concentration up to a concentration of ~350 mg ml^{-1}. These data were fitted by Ross et al. (1978) using MMHS, but have not been analyzed previously using SPT. Because Ross et al. (1978) found that the nonideality of these solutions was not significantly affected by minor variations in protein structure (i.e., Hb versus HbS) or temperature (2–20°C), we combined all six sedimentation equilibrium data sets and fitted them together.

Fig. 3 and Table 1 summarize the results of our fittings of COHb and COHbS sedimentation equilibrium data. In all of the fittings, we float δ_{Hb} and fix M_{Hb} = 64,610 Da. The best-fitted value of δ_{Hb} obtained by analyzing the sedimentation equilibrium data is 0.014 ± 0.003 g/g using MSPT and 0.537 ± 0.005 g/g using BSPT. The MSPT result is within 1 SD of that obtained by MSPT analysis of osmotic pressure data. The single-parameter BSPT fitting is within 1 SD of that obtained from Adair's (1928) osmotic pressure data, but larger (and outside of 2 SD) than that from the data reported by Dick (1967).

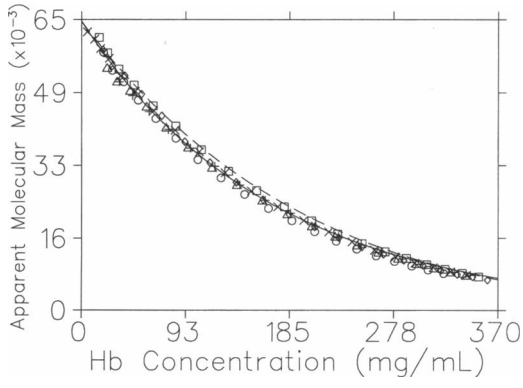

FIGURE 3 Best-fittings to sedimentation equilibrium data (Ross et al., 1978). The solid curve is the fitting using MSPT. (For ease of comparison, the following designations of molecular species are those of Ross et al.) The dashed curve is the fitting using BSPT. Both fittings include all data shown; (□) COHbA at 2°C, (×) COHbA at 10°C, (+) COHbA at 20°C, (◇) COHbS at 2°C, (△) COHbS at 10°C, (○) COHbS at 20°C. Results of these fittings are summarized in Table 1.

For the two-parameter fitting using BSPT, δ_{Hb} is 0.33 ± 0.05 g/g and r_w is 1.03 ± 0.09 Å. This value of δ_{Hb} is less than that reported from all other BSPT fittings. The fitted r_w is also less than that predicted from other two-parameter BSPT fittings and is less than the fixed value (1.375 Å) used in the single-parameter BSPT fittings. However, the correlation coefficient calculated by NONLIN between δ_{Hb} and r_w is 0.991 and, thus, the errors calculated by NONLIN probably are significantly smaller than the true errors. The best-fitted curve from the two-parameter fitting using BSPT is intermediate between those obtained by the MSPT fitting and the one-parameter BSPT fitting.

SPT-based analysis of effects of NHbS protein concentration on HbS solubility

Uncertainties in these important thermodynamic data, which demonstrate a large effect of macromolecular crowding on the solubility of HbS, limit the quantitative treatment of them by SPT. Because our goal for this data set is to compare our analysis with Berg's analysis of Ross and Minton's results (Ross and Minton, 1979; Berg, 1990), we fit the data fitted (visually) by Ross and Minton (1979). These data are plotted in Fig. 4; values of δ_{Hb} from the two SPT analyses are listed in Table 1. The fitted solubility of HbS in the absence of "inert" (NHbS) protein ($c^{ss,0}_{HbS}$) is 206 ± 2 mg/ml, independent of the method (i.e., MSPT or BSPT) used to calculate δ_{Hb}. We find that the fitted amounts of hydration are −0.01 ± 0.03 g/g by MSPT and 0.48 ± 0.05 g/g by BSPT. The δ_{Hb} obtained from fittings using MSPT is the same within error as that predicted by using MSPT to fit both sets of Adair's osmotic pressure data, as well as by using MSPT to fit the sedimentation equilibrium data. The best-fitted value of δ_{Hb} obtained by using BSPT to fit the solubility data is within 1 SD of that predicted by the single- and double-parameter BSPT osmotic pressure fitting of Adair's data published by Dick (1967) and of that predicted by the two parameter BSPT osmotic pressure fitting of Adair's (1928) data, but it does

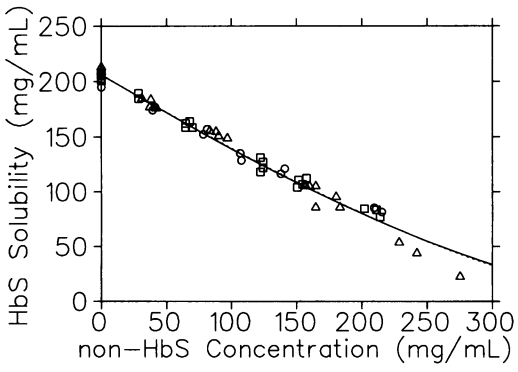

FIGURE 4 Best-fittings to the Behe and Englander (1978) HbS solubility data. The solid curve is the fitting using MSPT. The dashed curve is the fitting using BSPT. Both fittings include all data shown; (△) R-state HbA, (□) BSA, and (○) unhybridized native HbA. (The molecular species designations given here are those of Behe and Englander (1978), for ease of comparison.) Results of these fittings are summarized in Table 1.

differ by slightly more than 1 SD from that predicted by the single-parameter BSPT osmotic pressure fitting of Adair's (1928) data, and from the one- and two-parameter BSPT sedimentation equilibrium fittings. In fitting the solubility data, we find that r_w and δ_{Hb} are too highly correlated to allow simultaneous fitting of r_w as an additional parameter.

Berg also compared MSPT and BSPT as applied to HbS solubility data. His BSPT fitting yielded a radius of Hb of 33 Å. Using this radius, the amount of hydration that we calculated with Eq. 8 (δ_{Hb} = ~0.66 g/g) is higher than the best-fitted values that we obtain using either MSPT or BSPT. This difference in δ_{Hb} is well outside of error, but the discrepancy is difficult to interpret because neither Berg's comparison nor Minton's analysis used a quantitative, nonlinear least-squares fitting of the solubility data. (Both were based on semi-quantitative, visual fittings that did not take into account the protein concentration correction described in Methods.) Nonetheless, the analysis presented here, like that of Berg (1990), leads to the conclusion that BSPT is capable of fitting the solubility data reported by Behe and Englander (1978).

DISCUSSION

Comparison of MSPT and MMHS

From an analysis of Adair's osmotic pressure data (Adair, 1928; cf. Fig. 1) using MMHS (based on McMillan-Mayer theory and the virial expansion with coefficients evaluated using the hard sphere interaction potential), Ross and Minton (1977) reported a fitted volume of Hb that corresponds to $\delta_{Hb} = 0.21 \pm 0.03$ g/g. Similarly, Ross and co-workers (Ross et al., 1978) reported Hb volumes from fittings of sedimentation equilibria derived from MMHS that correspond to $\delta_{Hb} = 0.17 \pm 0.01$ g/g. These estimates of δ_{Hb} are larger than our best-fitted MSPT values; this discrepancy between MSPT and MMHS must be a result of the mathematical approximations inherent in the formulation of these theories, inasmuch as they both are based on a continuum model for solvent and treat the Hb solution as a one-component system.

Comparisons of the pressure predicted by simulations of hard sphere fluids with the hard sphere virial expansion, as well as comparisons of hard sphere simulations of activity coefficients with those calculated by SPT, show that the approximations resulting from the truncated virial expansion of MMHS and those resulting from the derivation of SPT are not the same. Over the range of concentrations of interest here, the truncated virial expansion underestimates by 2–3% the pressure of a hard sphere fluid as compared with molecular dynamics simulations (Ree and Hoover, 1967), whereas the SPT-predicted activity coefficient is 3–4% higher than that predicted using Monte Carlo simulations (Adams, 1974). These differences are consistent with the smaller values of δ_{Hb} predicted here by the MSPT approach as compared with those predicted by MMHS.

The assumption of spherical shape

In applying SPT to a real system containing more than one component, all of the model particles must be assumed to have the same shape, because analytic expressions describing the nonideality due to hard particle interactions in mixtures of particles of different shapes are not available. For the systems analyzed in this paper, we follow the precedent of taking all particles to be spherical. (In their MMHS analysis of Adair's (1928) osmotic pressure data on Hb, Ross and Minton (1977) made a detailed investigation of some alternative simple geometric shapes for Hb and arrived at the conclusion that this protein was at least "quasispherical.") For Hb the axial ratio calculated from the crystal structure as a prolate ellipsoid is ~1.26 (Squire and Himmel, 1979; Kuntz and Kauzmann, 1974). The hard sphere model is probably more appropriate for Hb than for BSA (axial ratio ~3.5; Squire and Himmel (1979)) or water.

Simulations using MC and/or molecular dynamics methods provide an alternative (in principle rigorous) approach to predict the thermodynamic properties of hard particle systems. Simulations could be carried out for mixtures of particles of different shapes, but the relatively high number density of the relatively small solvent molecules could retard equilibration substantially because of insufficient sampling of configuration space (Jackson et al., 1987). The use of SPT to analyze mixtures of spheres of much different sizes (and number densities) is straightforward and computationally much faster than the corresponding simulations. However, for applications of BSPT to analyze the systems and conditions considered in this paper, theoretical tests of SPT predictions by comparisons with simulations incorporating the same model assumptions have not yet been carried out.

Effects of the experimental solution conditions on our fitted values of δ_{Hb}

The small but significant differences between the best-fitted values of δ_{Hb} determined from the osmotic pressure data, the sedimentation equilibrium data, and the solubility data could result from differences in the pH and salt/buffer

concentrations. However, Adair (1928) showed that the osmotic pressure of a solution containing ~200 mg/ml Hb is the same within error at pH 6.8 and 7.9. Thus, any differences in charge (pI = 6.8) in Hb at pH 6.8 and 7.9 do not significantly affect nonideality at 200 mg/ml. At higher concentrations, the charge on a protein could have a more significant effect on its nonideality. This effect may account (at least in part) for the differences in the best-fitted values of δ_{Hb} obtained in our analyses of different data sets. Analyses of sedimentation equilibrium data of Hb and HbS by us and others (Ross et al., 1978) show no significant effect of temperature on the best-fitted hard particle radius. The small dependences of these data on pH and (to an even lesser extent) on T are consistent with the expectation that a hard particle model can provide an accurate description of nonideality in concentrated protein solutions, at least when the net charge on the protein is small.

Physical interpretation of δ_{Hb}

The results presented in this paper demonstrate that diverse types of equilibrium measurements can be fitted with a single value of the parameter δ_{Hb} as defined in Eq. 8. This finding supports the applicability of the hard sphere model to solutions of Hb, but does not necessarily guarantee a simple physical interpretation of the best-fitted hard sphere radius. The magnitude of δ_{Hb} could be affected either by deficiencies in the hard sphere model as an accurate basis for predicting measurable properties of a concentrated solution of real macromolecules or by deficiencies in SPT as a predictor of excluded volume nonideality in model hard sphere fluids. Evidence that the latter concern may be significant, at least for MSPT calculations, is implied by the MC results reported by Adams (1974).

A radius as large as the hydrodynamic radius may appear inappropriate as the hard sphere radius needed to describe repulsions between proteins at the molecular level (for the purpose of SPT calculations of thermodynamic properties). Minton proposed that the relevant hard particle volume should be greater than the partial specific volume but substantially less than the hydrodynamic volume (Minton, 1980). An implication of this proposal is that there is no large short range repulsion between macromolecules at interparticle separations of twice the hydrodynamic radius. However, recent osmotic stress experiments on collagen triple helices indicate a large short range repulsive force that is independent of [NaCl] and that extends over a distance approximately equal to twice the hydrodynamic helical radius (cf. Fig. 2 in Leikin et al., 1994). Such a large, short range repulsive force could be well approximated by a hard core repulsion.

Because the BSPT analyses do yield reasonable values for δ_{Hb} and yield better fittings (than those obtained from MSPT) of the Hb osmotic pressure data at Hb concentrations above 350 mg/ml, we conclude that crowding effects from water (in the BSPT formulation) can be described usefully using a two-state model that classifies water as either hydration or bulk. The bulk water molecules are modeled as small hard spheres, and the water of hydration is assumed to increase the effective hard sphere radius of the macromolecule. An analogous treatment of the water of hydration is standard in the analysis of transport data to obtain information about the size and shape of globular macromolecules (Tanford, 1961). However, we know of no previous two-state treatment of bulk water and hydration water for the calculation of the excluded volume contributions to thermodynamic nonideality of concentrated macromolecular solutions.

Summary of the applicability of MSPT and BSPT in crowding calculations

Below ~350 mg/ml, both MSPT and BSPT (when suitably parameterized) yield accurate fittings of the thermodynamic properties considered here (albeit with different macromolecular radii). When the range of macromolecular concentrations extends above ~350 mg/ml, BSPT provides more accurate fittings of the osmotic pressure data than does MSPT. In summary, BSPT can predict accurately all of the three types of thermodynamic properties with similar hard sphere radii of the hydrated Hb particle (corresponding to $\delta \cong 0.5$ $gH_2O/gprotein$). MSPT accurately predicts the thermodynamic properties of Hb solutions with a hard sphere radius nearly equal to that of unhydrated Hb (corresponding to $\delta \cong 0.0$ $gH_2O/gprotein$), provided the macromolecular concentration does not exceed ~350 mg/ml. Accordingly, if MSPT is used to estimate macromolecular activity coefficients in solutions where the concentration of Hb does not exceed 350 mg/ml, then the unhydrated radius of Hb (i.e., $\delta_{Hb} = 0$) should be used. To generalize from this result, we predict that if a hydrated macromolecular radius is used with MSPT (e.g., Zimmerman and Trach, 1991), then the thermodynamic effects of crowding will be greatly overestimated.

Deviations from SPT predictions of nonideality: possible role of coulombic effects

It is clear from this and previous analyses that thermodynamic data in Hb solutions obtained near the isoelectric point at moderate salt concentrations can be fitted successfully by excluded volume theory neglecting coulombic contributions to nonideality. Minton and Edelhoch (1982) found that light scattering data as a function of BSA concentration (up to ~90 mg/ml) at different pH values could be described quantitatively by assuming a hard sphere model. Although these data could be well fitted by the hard sphere model, many of the best-fitted effective specific volumes of BSA were larger than those found for any other protein system analyzed using hard sphere models. The effective hard sphere radius was found to increase as the pH increases from the isoelectric point of BSA; this effect was attributed to increased coulombic repulsions (BSA has a net charge of ~ -20 at a pH of ~7.6 and ~0 at a pH of ~4.4).

For the BSA data, effects of increases in protein charge can be parameterized successfully (at least over a limited

concentration range) by increasing the effective hard sphere radius. However, Vérétout et al. (1989) found that their osmotic pressure data for α-crystallin (up to ~390 mg/ml) could not be fitted using an approach equivalent to MMHS with any reasonable choice of hard sphere radius for the protein. The results of their fitting procedures show clear *qualitative* disagreement between experimental data and the best-fitted theoretical curves. Vérétout et al. noted that under the conditions of their experiment the charge on α-crystallin is great enough (-46 ± 12, as inferred by Siezen and Owen (1983) from electrophoretic measurements (Niyogi and Koenig, 1962)) to indicate that coulombic interactions cannot be neglected. The thermodynamic consequences of these interactions in α-crystallin solution are much larger than would be expected for Hb, which has at most a small charge under the conditions where the data fitted in the present paper were acquired.

Our analysis of the osmotic pressure data reported for α-crystallin by Vérétout et al. is shown in Fig. 5, which demonstrates that even at the highest salt concentration investigated by them neither MSPT nor BSPT provides an adequate fitting for any reasonable value of δ (or the molecular weight). (At any lower salt concentration, coulombic contributions to nonideality are expected to be even more substantial.) The inadequacy of both MMHS and SPT (in either form) as means of accounting for thermodynamic data for α-crystallin solutions indicates that hard sphere radii cannot always be adjusted to parameterize the contribution of coulombic interactions to nonideality, and that predictions based on hard sphere models can be qualitatively inconsistent with experimental observations at high concentrations of relatively highly charged macromolecules. The failure of SPT in either form to fit data to which neither should be applicable tends to support the physical significance of the parameters obtained here from data that can be fitted by SPT. The inapplicability of SPT to solutions of α-crystallin does not necessarily preclude the relevance of a two-state model for water in solutions of highly charged macromolecules, but does imply that for such systems another method of allowing for coulombic interactions in addition to hard sphere interactions will be needed to predict the concentration dependence of the macromolecular activity coefficient.

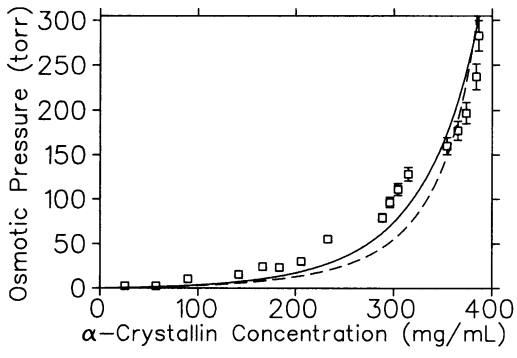

FIGURE 5 Best-fittings to osmotic pressure data of (□) α-crystallin in high salt (Vérétout et al., 1989). The solid curve is the fitting using MSPT with δ as the only floated parameter. The long-dashed curve is the fitting using BSPT with δ as the only floated parameter.

CONCLUSIONS

In this paper, we have demonstrated that BSPT can be used to fit a variety of thermodynamic data on Hb (or closely related globular proteins) with best-fitted values of δ_{Hb} that are comparable with that obtained from hydrodynamic measurements. In our applications of BSPT, a constant pressure constraint was used to determine the concentration of bulk water in the macromolecular solution, instead of the approximate analytic expression derived by Berg, which is less accurate toward the high end of the experimental range of Hb concentrations. The results presented in this paper indicate that BSPT is more accurate than MSPT as a predictor of the contribution of macromolecular crowding to thermodynamic properties of Hb solutions when the range of Hb concentrations exceeds 350 mg/ml. In a subsequent report, we will compare the applicability of MSPT and BSPT to the analysis of excluded volume nonideality in concentrated solutions of a flexibly coiling polymer.

Although Berg included water as hard spheres in his calculation of the solute activity coefficient, he did not use a two-state classification of water to interpret the enhancement of the macromolecular radius (due to hydration) as distinct from the bulk hard sphere water. We interpret the increase in the BSPT-predicted Hb radius (relative to that predicted from the partial specific volume of unhydrated Hb) not only as a hard sphere measure of the extent of hydration of Hb, but also as indicative of the potential applicability of a simple, two-state model for water (hydration and bulk) in a macromolecular solution. On the basis of the thermodynamic data, we conclude that this two-state model of water suffices to characterize the thermodynamic effects due to crowding in a macromolecular solution, at least when the charge density on the macromolecules is sufficiently low that coulombic contributions to nonideality are inconsequential.

We thank Allen Minton for a copy of the original data from Ross et al. (1978) for COHb and for discussions of SPT, Walter Englander for a detailed copy of Fig. 2 from Behe and Englander (1978), Adrian Parsegian for helpful discussions of osmotic pressure measurements, Jeff Bond for enlightening discussions on statistical mechanics and osmotic pressure, and Steve Zimmerman and the reviewers for their thoughtful comments on the manuscript. We thank Sheila Aiello for help in preparation of the manuscript.

This work was supported by National Institutes of Health grant GM47022. H. J. Guttman gratefully acknowledges support from a Molecular Biophysics graduate training grant (NIH GM08293).

APPENDIX

The osmotic pressure, Π, is defined as the difference in the mechanical pressures acting on two solutions in dialysis equilibrium separated by a membrane that is impermeable to at least one of the components. When (as in Adair's experiments on hemoglobin) one or more types of low molecular weight solutes as well as water are diffusible across the membrane, Π generally is called a "Donnan" osmotic pressure. If the membrane is impermeable only to solute component "2," Π can be expressed as a function of the activity coefficient, γ_2, of this nondiffusible solute (here assumed to be uncharged, for the sake of simpler expressions). In

accordance with the following conventional expression the chemical potential, μ_2, of component 2 is expressed in terms of its standard-state chemical potential $\mu_2^\circ(T, P)$, a function only of temperature and pressure, and its activity, $\gamma_2 c_2$

$$\mu_2 = \mu_2^\circ(T, P) + RT \ln \gamma_2 c_2. \quad \text{(A1)}$$

In this equation γ_2 and c_2 are understood to be dimensionless functions whose numerical magnitudes are determined by the units chosen to specify c_2 (such as grams of solute 2 per ml of solution.)

The derivation of a general relationship between Π and γ_2 can be initiated by considering an isothermal change in the composition of a multicomponent solution that is not maintained at constant pressure. For this situation, the Gibbs-Duhem equation is

$$VdP = \sum_i n_i d\mu_i, \quad \text{(A2)}$$

where the summation runs over all components in the system, including the solvent. In the context of a dialysis equilibrium, if solute "2" is the only nondiffusible component in the solution of interest, and provided that the change in the composition of this solution is made in such a way that the chemical potentials of all the diffusible components (including solvent) do not change, then from (A2) the following equation describes changes in P and in μ_2 that are caused by changes in c_2

$$M_2\left(\frac{\partial P}{\partial c_2}\right)_{T,\{\mu_{i\neq 2}\}} = \left(\frac{\partial \mu_2}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}}. \quad \text{(A3)}$$

Introducing (A1) into (A3)

$$M_2\left(\frac{\partial P}{\partial c_2}\right)_{T,\{\mu_{i\neq 2}\}} = \left(\frac{\partial \mu_2^\circ}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}} + RT\left(1 + \left(\frac{\partial \ln \gamma_2}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}}\right). \quad \text{(A4)}$$

Under the specified constraints (all $\mu_{i\neq 2}$ held constant), the indicated partial derivative of μ_2° does not vanish because both μ_2° and c_2 change with the variable pressure. A useful alternative form of the first term on the right-hand side of Eq. A4 is obtained by applying the chain rule and Euler reciprocity (which relates the pressure derivative of μ_2° to $M_2\bar{v}_2^\circ$)

$$\left(\frac{\partial \mu_2^\circ}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}} = \left(\frac{\partial \mu_2^\circ}{\partial P}\right)_{T,\{\mu_{i\neq 2}\}}\left(\frac{\partial P}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}} \quad \text{(A5a)}$$

$$= c_2 \bar{v}_2^\circ M_2\left(\frac{\partial P}{\partial c_2}\right)_{T,\{\mu_{i\neq 2}\}}. \quad \text{(A5b)}$$

Introducing Eq. A5b into Eq. A4

$$\frac{M_2}{RT}\left(\frac{\partial P}{\partial c_2}\right)_{T,\{\mu_{i\neq 2}\}} = (1 - c_2\bar{v}_2^\circ)^{-1}\left(1 + \left(\frac{\partial \ln \gamma_2}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}}\right). \quad \text{(A6)}$$

To express the osmotic pressure, Π, resulting from the isothermal addition of the nondiffusible solute 2 to a solution where the chemical potentials of all diffusible components are the same in the final as in the initial state, (A6) is integrated as follows (by noting $\bar{v}_2^\circ$ is not a function of c_2)

$$\frac{\Pi M_2}{RT} = \int_0^{c_2} (1 - c_2\bar{v}_2^\circ)^{-1}\left(1 + \left(\frac{\partial \ln \gamma_2}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}}\right) dc_2 \quad \text{(A7a)}$$

$$= -\frac{1}{\bar{v}_2^\circ}\ln(1 - c_2\bar{v}_2^\circ) + \int_0^{c_2} (1 - c_2\bar{v}_2^\circ)^{-1}\left(\frac{\partial \ln \gamma_2}{\partial \ln c_2}\right)_{T,\{\mu_{i\neq 2}\}} dc_2 \quad \text{(A7b)}$$

$$= -\frac{1}{\bar{v}_2^\circ}\ln(1 - c_2\bar{v}_2^\circ) + \int_0^{c_2} \frac{c_2}{(1 - c_2\bar{v}_2^\circ)}\, d\ln \gamma_2 \quad \text{(A7c)}$$

$$= -\frac{1}{\bar{v}_2^\circ}\ln(1 - c_2\bar{v}_2^\circ) + \frac{c_2 \ln \gamma_2}{1 - c_2\bar{v}_2^\circ} - \int_0^{c_2} \frac{\ln \gamma_2}{(1 - c_2\bar{v}_2^\circ)^2} dc_2 \quad \text{(A7d)}$$

The invariance of the set of chemical potentials $\{\mu_{i\neq 2}\}$ is ensured experimentally when the composition (as well as T and P) of the solution *not* containing component 2 is the same in the initial and final states (e.g., before and after an addition of component 2 to the solution on the other side of the dialysis membrane). Over the range of concentrations investigated by Adair (1928), the composition of the dialyzing solution was reported to have been essentially constant. When Ross and Minton (1977) applied MMHS to their analysis of Adair's data, they also in effect assumed that μ_{Hb} is the only chemical potential to vary in response to changes in c_{Hb}.

In our applications of (A7d) to arrive at theoretical predictions of Π, we do not rely upon SPT to evaluate $\bar{v}_{Hb}^\circ$, because the underlying model contains no information about the short range attractive forces that cause the volume of the real system, a condensed liquid, to be much less than that of the analogous ideal gas mixture. (Although $\bar{v}_i = RT/P$ for any component i in an ideal gas mixture, the corresponding $\bar{v}_i^\circ = 0$, because for an ideal gas μ_i° is a function only of temperature.) Instead of assuming the "ideal gas" value, we set $\bar{v}_{Hb}^\circ$ equal to the experimental value inferred from the measurements reported by Adair and Adair (1947). This assumption implies that the interaction-dependent contributions to μ_{Hb} can be separated into two additive terms. The first, contributing to $\mu_{Hb}^\circ(T, P)$, depends exclusively on pressure at a given temperature and yields when differentiated with respect to P the correct (experimental) value of $\bar{v}_2^\circ$. Only the second term, $RT \ln \gamma_{Hb}$, is taken to be directly comparable with the predictions of one or the other of the two versions of SPT used in this paper to analyze experimental data.

An equation equivalent to (A7d) was used, in conjunction with a virial expansion analysis of osmotic pressure data (Adair, 1928), to analyze sedimentation equilibrium data on Hb (Ross and Minton, 1977). This analysis yielded the same effective hard sphere volume of Hb that was obtained from a virial expansion analysis of the osmotic pressure data (which did not require (A7d) in any form). A subsequent virial expansion analysis (reported by Eaton and Hofrichter (1990)) of the same set of sedimentation equilibrium data (based on an equation analogous to (A7d), but from which the term $c_2\bar{v}_2^\circ$ was omitted) yielded a value for the hard sphere volume of Hb that is lower (by ~15%) than that obtained from the previous virial expansion analysis of Adair's osmotic pressure data. This discrepancy has no direct bearing on any of the analyses reported in the present paper, which are not predicated on the virial expansion for Π and do not use SPT to evaluate virial coefficients.

In summary, the predictions of MSPT and of BSPT are introduced into the thermodynamic expression for Π (Eq. 9 above) in the following ways. The explicit expression for Π given by Eq. 10 is derived by analytic integration of the integral in Eq. 9 using the formula for $\ln \gamma^{MSPT}$ obtained from Eqs. 1 and 3. The BSPT prediction for Π was obtained by numerical evaluation of the integral in Eq. 9, where $\ln \gamma_{Hb}^{BSPT}$, as defined in Eq. 5, is evaluated by determining the implicit dependence of ν_w on ν_{Hb} from the constant P^{BSPT} criterion described in connection with Eq. 7. This approach to the analysis of Π appears to be most closely analogous to that introduced by Berg (1990) in his analysis of HbS solubility data. Alternative adaptations of SPT to predict Π for systems where the solvent is modeled explicitly are under investigation in this laboratory.

REFERENCES

Adair, G. S. 1925. A critical study of the direct method of measuring the osmotic pressure of hemoglobin. *Proc. R. Soc. Lond.* 108:627–637.

Adair, G. S. 1928. A theory of partial osmotic pressures and membrane equilibria, with special reference to the application of Dalton's Law to hemoglobin solutions in the presence of salts. *Proc. R. Soc. Lond. Ser. A.* 120:573–603.

Adair, G. S., and M. E. Adair. 1947. The density increments of proteins. *Proc. R. Soc. Lond. Ser. A.* 190:341–356.

Adams, D. J. 1974. Chemical potential of hard-sphere fluids by Monte Carlo methods. *Mol. Phys.* 28:1241–1252.

Behe, M. J., and S. W. Englander. 1978. Sickle hemoglobin gelation reaction order and critical nucleus size. *Biophys. J.* 23:129–145.

Berg, O. G. 1990. The influence of macromolecular crowding on thermodynamic activity: solubility and dimerization constants for spherical and dumbbell-shaped molecules in a hard-sphere mixture. *Biopolymers.* 30: 1027–1037.

Cantor, C. R., and P. R. Schimmel. 1980. Biophysical Chemistry II. Techniques for the Study of Biological Structure-Function. W. H. Freeman & Co. 584–586.

Cayley, S., B. A. Lewis, H. J. Guttman, and M. T. Record, Jr. 1991. Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity: implications for protein-DNA interactions *in vivo*. *J. Mol. Biol.* 222:281–300.

Dick, D. A. T. 1967. An approach to the molecular structure of the living cell by water flux studies. *In* Physical Bases of Circulatory Transport. E. B. Reeve and A. G. Guyton, editors. W. B. Saunders Co., Philadelphia. 217–234.

Eaton, W. A., and J. Hofrichter. 1990. Sickle cell hemoglobin polymerization. *In* Advances in Protein Chemistry, Vol. 40. C. B. Anfinsin, J. T. Edsall, F. M. Richards, and D. S. Eisenberg, editors. Academic Press, New York. 263–265.

Fulton, A. B. 1982. How crowded is the cytoplasm? *Cell.* 30:345–347.

Guttman, H. J. 1994. Assessing the physical chemical effects of the intracellular environment on molecular interactions in the cytoplasm of *Escherichia coli* K-12 using statistical thermodynamics and quadrupolar NMR spectroscopy. Ph.D. Thesis. University of Wisconsin-Madison. 225 pp.

Hill, T. L. 1960. An Introduction to Statistical Thermodynamics. Dover Publications, Inc., New York. 508 pp.

Jackson, G., J. S. Rowlinson, and F. van Swol. 1987. Computer simulations of mixtures of hard spheres. *J. Phys. Chem.* 91:4907–4912.

Johnson, M. L., and S. G. Frasier. 1985. Nonlinear least-squares analysis. *Methods Enzymol.* 117:301–342.

Kruger, S., S.-H. Chen, J. Hofrichter, and R. Nossal. 1990. Small angle neutron scattering studies of HbA in concentrated solutions. *Biophys. J.* 58:745–757.

Kuntz, I. D., Jr., and W. Kauzmann. 1974. Hydration of proteins and polypeptides. *Adv. Protein Chem.* 28:239–345.

Leikin, S., D. C. Rau, and V. A. Parsegian. 1994. Direct measurements of forces between self-assembled proteins: temperature-dependent exponential forces between collagen triple helices. *Proc. Natl. Acad. Sci. USA.* 91:276–280.

Lebowitz, J. L., E. Helfand, and E. Praestgaard. 1965. Scaled particle theory of fluid mixtures. *J. Chem. Phys.* 43:774–779.

McMillan, W. L., and J. E. Mayer. 1945. The statistical thermodynamics of multicomponent systems. *J. Chem. Phys.* 13:276–305.

Minton, A. P. 1980. Thermodynamic nonideality and the dependence of partition coefficients upon solute concentration in exclusion chromatography. Application to self-associating and non-self-associating solutes, application to hemoglobin. *Biophys. Chem.* 12:271–277.

Minton, A. P. 1981. Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers.* 20:2093–2120.

Minton, A. P. 1983. The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences. *Mol. Cell Biochem.* 55:119–140.

Minton, A. P., and H. Edelhoch. 1982. Light scattering of bovine serum albumin solutions: extension of the hard particle model to allow for electrostatic repulsion. *Biopolymers.* 21:451–458.

Niyogi, S. K., and V. L. Koenig. 1962. Physico-chemical properties of α-crystallin from ox lens. *Biochim. Biophys. Acta.* 69:283–295.

Pierotti, R. A. 1965. Aqueous solutions of nonpolar gases. *J. Phys. Chem.* 69:281–288.

Ree, F. H., and W. G. Hoover. 1967. Seventh virial coefficients for hard spheres and hard disks. *J. Chem. Phys.* 46:4181–4197.

Reiss, H., H. L. Frisch, and J. L. Lebowitz. 1959. Statistical mechanics of rigid spheres. *J. Chem. Phys.* 31:369–380.

Ross, P. D., R. W. Briehl, and A. P. Minton. 1978. Temperature dependence of non-ideality in concentrated solutions of hemoglobin. *Biopolymers.* 17:2285–2288.

Ross, P. D., and A. P. Minton. 1977. Analysis of non-ideal behavior in concentrated hemoglobin solutions. *J. Mol. Biol.* 112:437–452.

Ross, P. D., and A. P. Minton. 1979. The effect of non-aggregating proteins upon the gelation of sickle cell hemoglobin: model calculations and data analysis. *Biochem. Biophys. Res. Commun.* 88:1308–1314.

Siezen, R. J., and E. A. Owen. 1983. Interactions of lens proteins: self-association and mixed-association studies of bovine α-crystallin and γ-crystallin. *Biophys. Chem.* 18:181*a*. (Abstr.)

Squire, P. G., and M. E. Himmel. 1979. Hydrodynamics and protein hydration. *Arch. Biochem. Biophys.* 196:165–177.

Straume, M., S. G. Frasier-Cadoret, and M. L. Johnson. 1991. Least-squares analysis of fluorescence data. *In* Topics in Fluorescence Spectroscopy. J. R. Lakowicz, editor. Plenum Press, New York. 177–240.

Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, New York. 710 pp.

Vérétout, F., M. Delaye, and A. Tardieu. 1989. Molecular basis of eye lens transparency. Osmotic pressure and X-ray analysis of α-crystallin solutions. *J. Mol. Biol.* 205:713–728.

Zimmerman, S. B., and S. O. Trach. 1991. Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*. *J. Mol. Biol.* 222:599–620.