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Direct allowance for the effects of thermodynamic nonideality in the quantitative characterization of protein self-association by osmometry

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ABSTRACT

A procedure is described for the direct analysis of osmotic pressure data for reversibly dimerizing proteins that makes allowance for effects of thermodynamic nonideality on the statistical–mechanical basis of the potential-of-mean-force between molecules. Detailed consideration is also given to calculation of the magnitudes of the required virial coefficients. After illustration of the approach with analysis of simulated osmotic pressure data, the method is used to obtain dimerization constants from published osmotic pressure data for soybean proteinase inhibitor, hemoglobin and α-chymotrypsin.

1. Introduction

For an extended period commencing four decades ago, the potential of osmometry for the characterization of protein interactions was explored only on the basis of empirical procedures for taking into account the effects of thermodynamic nonideality [1–6], a practice common to all physicochemical studies of interacting systems during that period. Since then considerable progress has been made in the analogous tasks of quantifying protein self-association through the application of sedimentation equilibrium [7–10] and static light scattering [11–13] techniques. In particular, a major advance has been the replacement of the empirical relationships used initially for the activity coefficients of monomer and oligomer species [1–6,8] by expressions emanating from considerations of thermodynamic nonideality on the statistical–mechanical basis of the potential-of-mean-force between molecules [14–17].

Direct analysis was employed in a much later osmotic pressure study of α-chymotrypsin [18], but no specific account was taken of the reversible dimerization undergone by this protein. Instead, the results were analyzed in terms of the standard expression for osmotic pressure (Π),

$$\Pi / (RTc) = 1/M + B_2' c + B_3' c^2 + \ldots \quad (1)$$

Here the B_i' are the virial coefficients expressed in terms of the expansion on the protein weight concentration scale c [units typically g/L], M is the molar mass, R is the universal gas constant and T is the absolute temperature. Consequent to this approach, the only conclusion to be drawn was that the pH-dependence of the ordinate intercepts and negative values of B_2' signified nonconformity with the osmotic behavior of a nonassociating solute system. Biochemists rightly realize that such physicochemical investigations should yield a more rewarding quantitative description of solute self-association.

A treatment of nonideal protein self-association with greater appeal to biochemists has been employed recently [19] for direct analysis of published osmotic pressure results [20] for immunoglobulin G. However, this approach based on a scaled-particle-theory model of protein self-association [21] is also empirical for characterization of charged systems in that it necessitates an assumption that the consequences of electrostatic repulsion can be accommodated adequately by increasing the effective size of the uncharged sphere to which scaled-particle theory applies. Unfortunately, demonstrations of the adequacy of this approach to account for thermodynamic nonideality in noninteracting protein systems bearing net charge [22–24] do not necessarily establish its complete validity in relation to all analyses of results reflecting nonideal self-association of charge-bearing solutes. In this communication we allow for the effects of charge in the direct analysis of osmotic pressure data reflecting solute self-association by basing electrostatic effects on the often-used statistical–mechanical model of the potential-of-mean-force between charged macromolecules [14–17]. The method is first illustrated with simulated membrane osmometry data based on the reversible dimerization of α-chymotrypsin under slightly acidic conditions (pH 4, I_M 0.05); and then applied to published osmotic pressure results for hemoglobin [2] and soybean proteinase inhibitor [25] as well as α-chymotrypsin [18].

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2. Theory

2.1. Allowance for effects of thermodynamic nonideality

For a noninteracting single solute with molar mass M, the osmotic pressure Π, defined under constraints of constant temperature and solvent chemical potential, can be written as a virial expansion in molar solute concentration, $C = c/M$. Thus

$$\Pi/(RT) = C + B_2C^2 + B_3C^3 + \ldots \quad (2)$$

where the virial coefficients are related to the molar activity coefficient γ by the expression [15,16]

$$\gamma = \exp\left[2B_2C + (3/2)B_3C^2 + \ldots\right] \quad (3)$$

The activity coefficient arises when, because of thermodynamic nonideality, the molar activity z of the solute is needed in place of the concentration C in the familiar expression for the solute chemical potential

$$\mu = \mu^\circ + RT\ln z = \mu^\circ + RT\ln(\gamma C) \quad (4)$$

which applies to a solution under "osmotic conditions" of constant temperature and chemical potential of solvent. By definition $z = \gamma C$ and it is obvious that $\gamma \to 1$ as $C \to 0$ in the ideal limit, and that nonideality can be thought of as adding terms to the ideal chemical potential. A consequence of this interrelationship between γ and the virial coefficients is that B_2 and B_3 find simple statistical–mechanical interpretation in terms of the physical interactions between pairs and triplets of solute molecules [14–16]. At first sight the use of Eq. (2) to describe the dependence of osmotic pressure upon concentration of a charged protein solute may seem to imply neglect of the contribution to P that arises from the Donnan redistribution of electrolyte ions to maintain constant solvent chemical potential and electroneutrality on both sides of the semipermeable membrane [26–29]. However, the demonstrated equivalence [30,31] of the McMillan-Mayer treatment of electrostatic repulsion between charged non-diffusible molecules [14] and the classical approach means that considerations of the potential-of-mean-force between two charged molecules [14–16] does incorporate the consequences of the Donnan effect, provided the osmotic membrane is permeable to the simple electrolyte. In this case no account need be taken of the quasi-osmotic force of attraction between protein molecules resulting from the inability of small molecules to penetrate the space between two macromolecules in imminent contact [32,33]. In any case, this contribution to the potential-of-mean-force is considered to be insignificant for electrolyte concentrations below 1 M [34].

On the grounds that a solute undergoing self-association can be regarded thermodynamically as a single solute in that specification of the total concentration defines the composition, Hill and Chen [35] have established that an expression analogous to Eq. (2) can still be used to describe the osmotic pressure by regarding the self-association as another form of nonideality. Upon adopting this viewpoint we adapt Eqs. (2) and (3) so that they are now written in terms of $\overline{C}$, the total base-molar concentration of solute (still total weight concentration divided by monomer molecular weight); and then the activity coefficient of monomer γ_1 is given by the relationship

$$\gamma_1 = \exp\left[2B_2\overline{C} + (3/2)B_3\overline{C}^2 + \ldots\right] \quad (5)$$

and the polynomial coefficients B_i pertain to the corresponding series expansion of osmotic pressure in terms of $\overline{C}$, namely

$$\Pi/(RT) = \overline{C} + B_2\overline{C}^2 + B_3\overline{C}^3 + \ldots \quad (6)$$

For a self-associating solute, typified in the present study by a solute undergoing reversible dimerization, these virial coefficients include contributions from terms reflecting chemical interaction as well as those reflecting the physical interactions between solute clusters that were mentioned above, specifically,

$$B_2 = -K_2 + B_{11} \quad (7)$$

$$B_3 = 4K_2^2 - 2K_2(4B_{11} - B_{12}) + B_{111} \quad (8)$$

In these expressions the association constant for dimerization represents the appropriate ratio of molar activities

$$K_2 = exp\left(-\frac{\mu_2^0 - 2\mu_1^0}{RT}\right) = \frac{z_2}{z_1^2} = \frac{\gamma_2}{\gamma_1^2}\frac{C_2}{C_1^2} \quad (9)$$

which reduces to the familiar ratio of concentrations C_2/C_1^2 in the ideal limit. The quantities B_{11} and B_{111} are the 2nd and 3rd virial coefficients that arise from the non-associative forces of interaction between pairs and triplets of monomers respectively, whereas B_{12} is the corresponding parameter for physical interaction between a monomer and a dimer species.

An obvious requirement of the above approach is a need for rapid convergence of the series in Eq. (6), a constraint which limits its application to studies of extremely weak self-association. Indeed, for the association constants that are likely to be encountered with most dimerizations ($K_2 > 10^3\ \mathrm{M}^{-1}$) the series diverges. However, as noted elsewhere [9,10], this problem can be overcome by expressing the osmotic pressure as the corresponding power series in monomer activity, z_1,

$$\Pi/(RT) = z_1 + (K_2 - B_{11})z_1^2 + \left(2B_{11}^2 - B_{111}/2 - K_2B_{12}\right)z_1^3 + \ldots \quad (10)$$

Eliminating the problem of series divergence by this means has, of course, been effected at the expense of the independent experimental variable $\overline{C}$, which has the related form [9,10]

$$\overline{C} = z_1 + 2(K_2 - B_{11})z_1^2 + 3\left(2B_{11}^2 - B_{111}/2 - K_2B_{12}\right)z_1^3 + \ldots \quad (11)$$

It might be thought that iterative incorporation of the solution to this cubic expression for z_1 into Eq. (10) would afford a means of evaluating the dimerization constant provided that values can be assigned to the various virial coefficients: K_2 then remains the only parameter of unknown magnitude. Such a procedure is unlikely to circumvent numerically the divergence implicit in Eq. (6). However, we have had success by eliminating the cubic terms from Eqs. (10) and (11) by combining them in the form

$$Y = 3\Pi/(RT) - \overline{C} = 2z_1 + (K_2 - B_{11})z_1^2 \quad (12)$$

the solution of this quadratic for the thermodynamic activity of monomer being, in terms of the transformed experimental variable Y,

$$z_1 = \frac{-2 + 2\sqrt{1 + (K_2 - B_{11})Y}}{2(K_2 - B_{11})} \quad (13)$$

Nonlinear regression analysis of the osmotic pressure data according to Eq. (10) with z_1 defined by Eq. (13) then yields the dimerization

constant (K_2) as the only remaining parameter to be evaluated from the curve-fitting.

Alternatively, we could have adopted a widely accepted viewpoint that Eqs. (10) and (11) truncated at the quadratic terms should suffice to describe the thermodynamic nonideality of proteins below 10 g/L. On that basis the thermodynamic activity of monomer would be given by the following solution of the truncated Eq. (11),

$$z_1 = \frac{-1 + \sqrt{1 + 8(K_2 - B_{11})\overline{C}}}{4(K_2 - B_{11})} \tag{14}$$

whereupon nonlinear curve-fitting of the osmotic pressure data according to the expression

$$\Pi / (RT) = z_1 + (K_2 - B_{11})z_1^2 \tag{15}$$

with z_1 now defined by Eq. (14) would yield the dimerization constant as the only parameter of unknown magnitude. However, the dangers of such truncation of Eqs. (10) and (11) are illustrated later by analyses of simulated data for a monomer–dimer system.

An obvious prerequisite for application of the suggested analysis to experimental osmotic pressure data is the availability of suitable estimates for the two second virial coefficients B_{11} and B_{12} as well as for the third virial coefficient for self-interaction, B_{111}. Procedures for calculating those estimates now follow.

2.2. Evaluation of second virial coefficients

For two identical spherical molecules of radius R_i the electrical repulsion contribution to the second virial coefficient B_{ii}^{elec} is [14]

$$B_{ii}^{\mathrm{elec}} = -2\pi L \int_{2R_i}^{\infty} f_{ii}(r) r^2 dr \tag{16}$$

where $f_{ii}(r)$, the Mayer f-function, is given by

$$f_{ii}(r) = \exp\left[-u_{ii}(r) / (kT)\right] - 1 \tag{17}$$

In this expression $u_{ii}(r)$ specifies the potential energy of the two molecules as a function of the center-to-center separation r, and k the Boltzmann constant. Avogadro's number L is included in Eq. (16) to convert the second virial coefficient from a molecular to a molar basis. For spherical molecules with radius R_i the energy function $u_{ii}(r)$ is described in terms of the Debye–Hückel inverse screening length κ, the dielectric constant of the solvent medium ε, electronic charge e and the net charge Z_i of the molecules by

$$u_{ii}(r) = \begin{cases} \infty & r < 2R_i \\ \dfrac{Z_i^2 e^2 \exp[-\kappa(r - 2R_i)]}{\varepsilon(1 + \kappa R_i)^2 r} & r \geq 2R_i \end{cases} \tag{18}$$

An approximate analytical solution for the evaluation of B_{ii}^{elec} is facilitated by expanding the exponential term in Eq. (17) as

$$f_{ii}(r) = -u_{ii}(r) / (kT) + \left[u_{ii}(r) / (kT)\right]^2 / 2 - \ldots \tag{19}$$

whereupon the integration of Eq. (16) gives

$$B_{ii}^{\mathrm{elec}} = \frac{Z_i^2(1 + 2\kappa R_i)}{4I_M(1 + \kappa R_i)^2} + \frac{Z_i^4\left(1000\kappa^3\right)}{128\pi L I_M^2 \varepsilon(1 + \kappa R_i)^4} + \ldots \tag{20}$$

in which the magnitude in cm^{-1} of the inverse screening length κ at 293 K may be calculated from the magnitude in mol/L of the ionic strength I_{M} as $3.27 \times 10^7 \sqrt{I_{\mathrm{M}}}$; and in which the factor of 1000 in the numerator of the second term converts κ^3 from cm^{-3} and maintains L/mol as the units of B_{ii}^{elec}. The overall second virial coefficient for self-interaction is then obtained by adding the hard-sphere contribution of $16\pi L R_i^3/3$ to the electrical repulsion contribution.

The reliability of the value of B_{ii} that incorporates the calculation of B_{ii}^{elec} from Eq. (20) depends upon the extent of conformity with the assumption $u_{ii}(r)/(kT) << 1$ that is inherent in Eq. (19), the expanded form of $f_{ii}(r)$. As shown later, this requirement precludes the use of Eq. (20) for the calculation of second virial coefficients for highly charged proteins in media of low ionic strength. For those systems numerical integration must be used to solve the combination of Eqs. (16)–(18), a practice adopted previously in a sedimentation equilibrium study of α-chymotrypsin under acidic conditions (pH 4.0) and an ionic strength of 0.05 M [10].

Adaptation of the above approach to calculate the electrical repulsion contribution to the second virial coefficient for different species (i and j) requires substitution of the expressions

$$B_{ii}^{elec} = -4\pi L \int_{R_i + R_j}^{\infty} f_{ij}(r) r^2 dr \tag{21}$$

$$f_{ij}(r) = \exp\left[-u_{ij}(r) / (kT)\right] - 1 \tag{22}$$

$$u_{ii}(r) = \begin{cases} \infty & r < R_i + R_j \\ \dfrac{Z_i Z_j e^2 \exp\left[-\kappa\left(r - R_i - R_j\right)\right]}{\varepsilon(1 + \kappa R_i)\left(1 + \kappa R_j\right) r} & r \geq R_i + R_j \end{cases} \tag{23}$$

for Eqs. (16)–(18). Incorporation of these changes then leads to

$$B_{ii}^{\mathrm{elec}} = \frac{Z_i Z_j\left(1 + \kappa R_i + \kappa R_j\right)}{2I_M(1 + \kappa R_i)\left(1 + \kappa R_j\right)} - \frac{Z_i^2 Z_j^2\left(1000\kappa^3\right)}{64\pi L I_M^2(1 + \kappa R_i)^2\left(1 + \kappa R_j\right)^2} + \ldots \tag{24}$$

as the electrostatic contribution to the second virial coefficient (L/mol) for interaction between dissimilar species. This contribution is then supplemented by addition of the term $4\pi L(R_I + R_J)^3/3$ to obtain the overall second virial coefficient describing the excluded volume interaction between dissimilar species.

2.2.1. Evaluation of the third virial coefficient for self-interaction

As noted previously [7,9,10], the remaining virial coefficient required for the present interpretation of osmotic pressure data for a reversibly dimerizing protein system, B_{111}^{elec}, can only be obtained by numerical integration of the expression

$$B_{111}^{\mathrm{elec}} = -\left(8\pi^2 L^2 / 3\right) \int_0^{\infty} r_{ij} f\left(r_{ij}\right) dr_{ij} \int_0^{\infty} r_{jk} f\left(r_{jk}\right) dr_{jk} \int_{|r_{ij} - r_{jk}|}^{r_{ij} + r_{jk}} r_{ik} f(r_{ik}) dr_{ik} \tag{25}$$

where the "Mayer f-function" continues to be given by Eq. (21) but with r_{ij} now the center-to-center distance between two of three identical molecules (i, j, k), and $u_{ij}(r)$ given by Eq. (17) because of the identity of i and j molecules. As before, the calculation of the third virial coefficient for self-interaction of monomer, B_{111}, requires the addition of a contribution, $160\pi^2 L^2 R_1^6/9$, to cover the hard-sphere interaction of three spherical particles with radius R_1.

3. Practical considerations

Before embarking upon application of the above theoretical expressions to simulated and experimental osmotic pressure results there are several practical aspects that require comment.

3.1. Choice of concentration scale

The present expression of concentrations and virial coefficients in molar terms does, of course, deviate from experimental practice in osmotic pressure studies, where custom dictates the use of weight-based concentrations in Eq. (1). For a noninteracting solute the ordinate intercept of the concentration dependence of $\Pi/(RTc)$ then yields the solute molar mass M, whereas the slope of the limiting tangent (as $c \rightarrow 0$) defines the magnitude of a second virial coefficient B_2' in units of L mol g^{-2} (usually expressed as mL mol g^{-2}). Curvature of the dependence can also be used to estimate a third virial coefficient B_3' in units of L^2 mol g^{-3}. In view of the clearly defined relationships between virial coefficients expressed on the two bases $B_2' = B_2/M^2$ and $B_3' = B_3/M^3$, the use of Eq. (1) has the advantage of providing a thermodynamic description of thermodynamic nonideality without prior knowledge of the solute molar mass.

From the viewpoint of determining the equilibrium constant governing solute dimerization the results of osmotic pressure studies are most readily analyzed according to Eq. (10). For such systems there is no useful transform akin to Eq. (1) because the virial expansion is in terms of the thermodynamic activity of monomer (z_1, a parameter of unknown magnitude) rather than the independent variable (the constituent solute concentration $\overline{C}$ or its weight concentration counterpart $\overline{c} = M_1\overline{C}$). The only viable course of action for the conversion of Eqs. (10) and (11) to a weight-concentration basis would entail their expression as

$$M_1\Pi/(RT) = M_1 z_1 + \left[(X_2/2) - B_{11}^*\right](M_1 z_1)^2 + \left[2\left(B_{11}^*\right)^2 - \left(B_{111}^* + X_2 B_{12}^*\right)/2\right](M_1 z_1)^3 + \ldots \quad (26a)$$

$$\overline{c} = M_1 z_1 + \left(X_2 - 2B_{11}^*\right)(M_1 z_1)^2 + 3\left[2\left(B_{11}^*\right)^2 - \left(B_{111}^* + X_2 B_{12}^*\right)/2\right](M_1 z_1)^3 + \ldots \quad (26b)$$

where $X_2 = 2K_2/M_1$ is the weight-based dimerization constant (L/g), $B_{11}^* = B_{11}/M_1$, $B_{12}^* = B_{12}/M_1$ and $B_{111}^* = B_{111}/M_1^2$. However, no advantage is gained by these conversions because knowledge of M_1 is a prerequisite for assigning magnitudes to the various virial coefficients, which can only be calculated on a molecular (and hence molar) basis. In studies of solute dimerization it is therefore simpler to accept at the outset that molecules interact on a mole-for-mole basis, whereupon molarity becomes the logical concentration scale for the quantitative characterization of molecular events.

3.2. Adequacy of the analytical expressions for second virial coefficients

In most experimental studies thus far reported the second virial coefficients have been calculated on the basis of estimates of B_{ii}^{elec} and B_{ii}^{elec} obtained from the first terms of Eqs. (20) and (24) respectively. The need for extension of the series was recognized by disparities between such estimates of B_{ii} and B_{ij} and values obtained by numerical integration of Eqs. (16) and (21) in a study of the dimerization of α-chymotrypsin at low ionic strength [10]; and although that need has been addressed briefly [17,36], the adequacy of the extended expression has not been tested. That situation is now remedied.

To illustrate the effects of protein charge (valence) and ionic strength on the magnitude of the second virial coefficient for self-interaction, Table 1 summarizes values of B_{ii} that have been calculated for spherical species with solvated radii of 2.44 and 3.50 nm, the respective Stokes radii of α-chymotrypsin and serum albumin. In the absence of net charge ($Z_i = 0$) the sole contributor to the second virial coefficient is the volume mutually excluded by two identical hard spheres, a quantity which is independent of supporting electrolyte concentration in the absence of any ionic-strength-dependent protein conformational changes. Introduction of net charge Z_i, taken to be uniformly distributed over the sphere surface, leads to an increase in the exclusion volume because of electrostatic repulsion between like-charged molecules. As is evident from Table 1 the extent of this increase in B_{ii} can be offset to some extent by increasing the concentration of supporting electrolyte in the solvent medium.

Of major interest in the present context is the comparison between values of the second virial coefficient calculated with B_{ii}^{elec} defined by Eq. (20) and those based only on the first term of that expression (the numbers in parentheses in Table 1). For low values of net charge ($Z_i \leq 4$) the inclusion of the extra term in B_{ii}^{elec} has little effect on the magnitude of the calculated second virial coefficient at all ionic strengths examined. However, an ever-increasing disparity between the two estimates occurs with increased protein valence, this effect being more pronounced for the smaller protein at the lowest ionic strength examined (0.05 M). Although the value that includes the contribution from the final term of Eq. (20) is undoubtedly the better estimate, its reliability comes into question with increasing size of the disparity because of the potential for truncation at the second term of Eq. (20) also being premature. In that regard the second virial coefficient for the α-chymotrypsin system with $Z_i = 10$ and $I_M = 0.05$ M has been determined by numerical integration as 364 L/mol [10], which shows that the value of B_{ii} based on Eq. (20) is an underestimate whereas that based on its truncated form is an overestimate. Eq. (20) must therefore be regarded as just the first two terms of a more extended series. For low values of net charge there is rapid convergence of the series; but a stage is inevitably

Table 1
Effect of truncating the expression for B_{ii}^{elec} on the magnitude of the second virial coefficient (B_{ii}) for proteins (radius R_i) as a function of net charge (Z_i) and molar ionic strength of the medium (I_M).

I_M	Second virial coefficient B_{ii} (L/mol)[a]							
	$Z_i = 0$	$Z_i = 2$	$Z_i = 4$	$Z_i = 6$	$Z_i = 8$	$Z_i = 10$	$Z_i = 12$	$Z_i = 15$
α-chymotrypsin ($R_i = 2.44$ nm)								
0.05	147[b]	158 (158)	191 (194)	239 (253)	291 (335)	334 (441)	348 (511)	265 (810)
0.10	147	151 (151)	165 (166)	187 (190)	212 (224)	239 (268)	260 (322)	270 (421)
0.15	147	149 (149)	158 (158)	171 (172)	187 (192)	205 (218)	222 (258)	240 (307)
0.20	147	148 (148)	154 (154)	163 (164)	175 (178)	188 (195)	201 (217)	219 (256)
Serum albumin ($R_i = 3.50$ nm)								
0.05	432	442 (442)	470 (471)	514 (519)	570 (587)	634 (674)	697 (780)	772 (976)
0.10	432	436 (436)	448 (448)	466 (467)	490 (494)	519 (529)	551 (571)	599 (650)
0.15	432	435 (435)	441 (441)	452 (453)	466 (468)	484 (488)	504 (513)	536 (556)
0.20	432	434 (434)	438 (438)	446 (446)	456 (456)	468 (470)	482 (486)	505 (517)

[a] Numbers in parentheses denote values calculated with B_{ii}^{elec} truncated at the first term.
[b] Hard sphere contribution ($16\pi L R_i^3/3$).

reached where further increase in Z_i leads to divergence of the series. Consequently, little or no confidence can be placed on the reliability of the final two entries for the α-chymotrypsin system with $I_M = 0.05$ M because of slow convergence of the series in Eq. (20): in view of the relatively large contribution from the second term of the final entry (−542 cf 663 L/mol from the first term) the listed value undoubtedly underestimates the second virial coefficient by a considerable margin. As noted earlier, numerical integration should be used to evaluate the second virial coefficient in such circumstances.

4. Results and discussion

Before using the suggested approach to extract dimerization constants from osmotic pressure results it was deemed desirable to first illustrate the method by analyzing a data set for which the magnitudes of all parameters were known.

4.1. Analysis of simulated osmotic pressure data

Simulation has been used to generate a $[\bar{C}, \Pi/(RT)]$ data set for α-chymotrypsin ($M_1 = 25$ kDa) under slightly acid conditions (pH 4.0, I_M 0.05) for which the parameters $B_{11} = 364$ L mol^{-1}, $B_{12} = 1140$ L mol^{-1}, $B_{111} = 36{,}100$ L^2 mol^{-2} and $K_2 = 1570$ M^{-1} had been determined in a previous sedimentation equilibrium study of the enzyme [10]. Specifically, Eqs. (10) and (11) were used to calculate values of $\Pi/(RT)$ and $\bar{C}$ respectively for assigned values of monomer activity z_1 at 25 μM intervals in the range of 25–300 μM to yield a data set in which the base-molar protein concentration $\bar{C}$ ranged between 26 and 392 μM ($0.61 \le \bar{c} \le 9.80$ g/L). Values of $\Pi/(RT)$ were then modified by incorporating a random error with a standard deviation of 0.2 μM, which corresponds to 0.5 Pa (or 0.05 mm H_2O) in osmotic pressure Π. These corrected values of $\Pi/(RT)$ were then combined with the corresponding $\bar{C}$ to calculate the values of Y [Eq. (12)] to be used in the solution of Eq. (13) for z_1.

Analysis of the simulated $[\bar{C}, Y, \Pi/(RT)]$ data for α-chymotrypsin (pH 4.0, I_M 0.05) is summarized in Fig. 1A, where the solid line corresponds to the best-fit description obtained by nonlinear least-squares curve-fitting to Eqs. (10) and (13) by means of SCIENTIST software (Micromath Scientific Software Inc., Salt Lake City, UT). As required for a situation in which the same expressions are used for simulation and analysis of data, the returned estimate (±2SD) of 1550 (±60) M^{-1} is in excellent agreement with the input dimerization constant (K_2) of 1570 M^{-1}. On the other hand, a much lower estimate, 710 (±80) M^{-1} is obtained by the analysis based on Eqs. (14) and (15), which reflect the truncation of Eqs. (10) and (11) at the quadratic terms in monomer activity. That the consequent best-fit description (- - -, Fig. 1A) exhibits systematic deviation from the fitted data is established more clearly by presenting the results in conventional osmotic pressure format (Fig. 1B). Analyses via Eqs. (14) and (15) are thus likely to be of limited validity.

4.2. Analysis of osmotic pressure results for soybean proteinase inhibitor

An early study of protein self-association by osmometry [25] entailed characterization of the reversible dimerization of soybean proteinase inhibitor on the basis of thermodynamic ideality, a simplifying assumption made because of the relatively small size of the protein ($M_1 = 8$ kDa) and the low concentration range (below 2.5 g/L) examined. Although the physicochemical characterization of protein interactions under conditions approaching thermodynamic ideality is prevalent, the inability to quantify the extent to which assumed thermodynamically ideal behavior has affected the resultant magnitude of K_2 must always be viewed as a cause for some concern. The current aim is to address this concern by subjecting the experimental data to analysis according to Eqs. (14) and (15), which at least takes some account of thermodynamic nonideality.

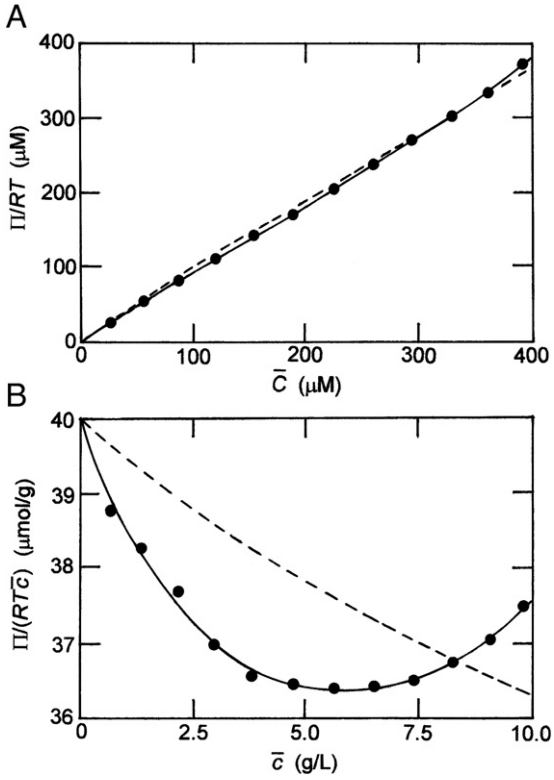

Fig. 1. Illustration of the present approach for extracting the dimerization constant from osmotic pressure data reflecting effects of thermodynamic nonideality. (A) Analysis of simulated data for α-chymotrypsin (pH 4.0, I_M 0.05): the solid line denotes the best-fit description ($K_2 = 1550$ M^{-1}) obtained by nonlinear least-squares curve-fitting to Eqs. (9) and (12), whereas the broken line refers to the corresponding description ($K_2 = 710$ M^{-1}) obtained by analysis in terms of Eqs. (13) and (14), which neglect third-order effects. (B) Replot of the same information in conventional osmotic pressure format.

For that purpose we examine the experimental concentration dependence of number-average molecular mass, the reciprocal of $\Pi/(RT\bar{C})$, for the proteinase inhibitor in 0.01 M potassium phosphate (pH 7) supplemented with 0.1 M KCl.

The advantage of adopting the simplified analysis is its requirement for only one virial coefficient, B_{11}, the calculation of which needs careful consideration because of a relatively high net charge ($Z_1 = -4.6$) on such a small protein ($R_1 = 1.3$ nm [25]). In that regard the value of 46 L/mol obtained on the basis of Eq. (20) for B_{11}^{elec} is only 5 L/mol smaller than that obtained by neglecting the final term. Consequently, the former should suffice as a satisfactory estimate (within 5%) of B_{11}.

Experimental results deduced from Fig. 1 of Harry and Steiner [25] are summarized in Table 2, together with the corresponding best-fit estimates of $\Pi/(RT)$ and $\Pi/(RT\bar{C})$ emanating from nonlinear least-squares curve-fitting of the data to Eqs. (14) and (15)). The resulting estimate (±2 SD) of 3500 (±200) M^{-1} for K_2, which is unchanged by setting $B_{11} = 0$, encompasses the reported dimerization constant of 3600 M^{-1} obtained on the basis of thermodynamic ideality. In that regard the current analysis affords a more explicit means of defining the study of an interaction under conditions approaching thermodynamic ideality as a situation in which $K_2 >> B_{11}$, whereupon the coefficient of the quadratic term in the series expansion of $\Pi/(RT)$ as a function of monomer activity ($K_2 - B_{11}$) approximates the dimerization constant. Instead of citing the low solute concentration range as the reason for assuming ideality, it is probably more rigorous to justify the assumption on the basis of a sufficiently large dimerization constant to render negligible the difference between K_2 and $K_2 - B_{11}$. After all, the fact that the interaction could be studied over a low solute

Table 2
Analysis of osmotic pressure data on the reversible dimerization of soybean proteinase inhibitor.

$\bar{c}$ (g/L)	$\overline{M}_n$ (kDa)	$10^5\overline{C}$ (M)	$10^5\Pi/(RT)$ (M)	$10^5\Pi/(RT\bar{c})$ mol/g	$10^5\Pi/(RT)_{bf}$[a]	$10^5\Pi/(RT\bar{c})_{bf}$[a]
0.28	8.7	3.50	3.22	11.50	3.21	11.46
0.59	9.3	7.38	6.32	10.71	6.38	10.81
0.88	9.6	11.0	9.16	10.41	9.16	10.41
1.16	10.0	14.5	11.6	10.00	11.7	10.09
1.48	10.0	18.5	14.8	10.00	14.6	9.86
2.08	10.5	26.0	19.8	9.52	19.8	9.52
2.36	10.7	29.5	22.0	9.32	22.1	9.36

[a] Best-fit value based on the value of 3500 M^{-1} obtained by nonlinear least-squares curve-fitting of the $[\bar{C}, \Pi/(RT)]$ data to Eqs. (12) and (13).

concentration range is the consequence, not the cause, of K_2 being so large.

4.3. Osmotic pressure studies of hemoglobin dissociation

In another early osmotic pressure study of protein self-association Guidotti [2] introduced the concept of direct analysis to characterize a monomer–dimer equilibrium, the $2\alpha\beta \leftrightarrows \alpha_2\beta_2$ interconversion undergone by human hemoglobin in various liganded states. In present terminology the concentration dependence of osmotic pressure was written as

$$\frac{\Pi}{RT\bar{c}} = \frac{1}{2M_1}\left[1 + \frac{-1 + \sqrt{1 + 4X_2\bar{c}}}{2X_2\bar{c}}\right] + B'\bar{c} \qquad (27)$$

where B' was an empirically defined second virial coefficient akin to that used initially by Adams and Fujita [37] in the context of sedimentation equilibrium studies before extension to osmometry [1,4,6]. Unfortunately, the problem with such formulation of nonideality is that B', actually a concentration-dependent variable [38,39], is regarded as a constant. The effect of this approximation on the value of the dimerization constant is examined for carbonmonoxyhemoglobin (CO-hemoglobin) in 0.2 M NaCl, and oxyhemoglobin in 0.4 M $MgCl_2$, both at pH 7. Because hemoglobin is essentially isoelectric ($Z_1 = Z_2 = 0$) the virial coefficients are described adequately by the term for hard-sphere interaction, whereupon $B_{11} = 16\pi L R_1^3/3 = 154$ L mol^{-1}, $B_{12} = 4\pi L(R_1 + R_2)^3/3 = 445$ L mol^{-1}, and $B_{111} = 160\pi^2 L^2 R_1^6/9 = 148{,}000$ L^2 mol^{-2} with R_1 taken as 2.48 nm on the grounds that the effective solvated radius R_2 of the $\alpha_2\beta_2$ form of hemoglobin is 3.13 nm [40,41].

Osmotic pressure results taken from Fig. 2 of the Guidotti article [2] for CO-hemoglobin in 0.2 M NaCl are presented in Fig. 2, together with the dependence corresponding to their best-fit description obtained by nonlinear least-squares analysis of the untransformed $[\Pi/(RT), \bar{C}]$ data according to Eqs. (10) and (13). That dependence (——), corresponding to a K_2 of 140,000 (±20,000) M^{-1}, clearly provides a much better description of the results (●) than the dependence calculated for a dimerization constant (K_2) of 200,000 M^{-1}, the value inferred by Guidotti [2] from curve-fitting to Eq. (27) with $B' = 6.1 \times 10^5$ mL mol g^{-2}. However, that estimate of an empirically defined second virial coefficient, deduced from the seemingly linear concentration dependence of $\Pi/(RT\bar{c})$ over a higher concentration range (Fig. 1 of [2]), actually refers to a nonassociating solute with a molecular mass of 56.4 kDa and a B_{ii} of 194 L mol^{-1}. The consequent over-correction for the effect of thermodynamic nonideality is responsible for the overestimation of K_2.

Corresponding results taken from Fig. 3 of the Guidotti article [2] for oxyhemoglobin in 0.4 M $MgCl_2$ are also presented (○) in Fig. 2, where the solid line again denotes the best-fit description of untransformed osmotic pressure data in terms of Eqs. (10) and (13). For this system there is an even greater discrepancy between the

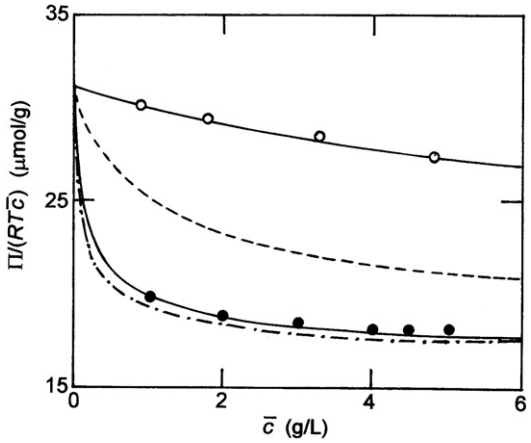

Fig. 2. Best-fit descriptions of osmotic pressure data [2] for CO-hemoglobin in 0.2 M NaCl (●) and oxyhemoglobin in 0.4 M $MgCl_2$ (○) obtained by nonlinear least-squares curve-fitting of the untransformed data to Eqs. (9) and (12), which yielded dimerization constants of 140,000 M^{-1} and 1600 M^{-1} for the respective systems. Broken lines (–·–·– and - - -) are the corresponding plots calculated on the basis of the K_2 values of 200,000 M^{-1} and 17,000 M^{-1} inferred by Guidotti [2] for the CO-hemoglobin and oxyhemoglobin systems respectively.

returned K_2 of 1600 (±200) M^{-1} and that of 17,000 M^{-1} reported by Guidotti [2], which provides a poor description of the experimental results when more realistic account is taken of thermodynamic nonideality (- - -, Fig. 2). On this occasion the overestimation of the dimerization constant reflects not only the use of an inappropriate magnitude for B' but also a greater deviation from its assumed constancy because of the weaker extent of oxyhemoglobin dimerization in 0.4 M $MgCl_2$.

4.4. Experimental results for chymotrypsin

We conclude these considerations of published osmotic pressure data for self-associating proteins with an examination of results for α-

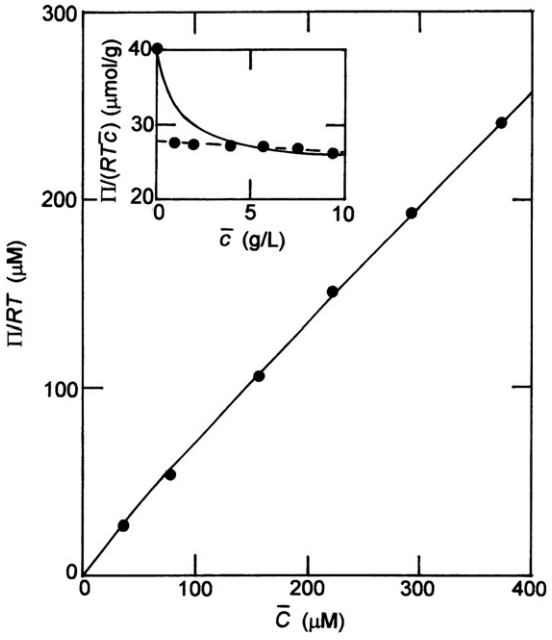

Fig. 3. Evaluation of the dimerization constant (14,500 M^{-1}) from published osmotic pressure data [18] for α-chymotrypsin in 0.1 M phosphate buffer (pH 6.0) by the nonlinear least-squares analysis in terms of Eqs. (9) and (12). The inset, a replot of the same information in traditional osmotic pressure format also indicates the best-fit thermodynamic description (- - -) reported in the original publication [18].

chymotrypsin studied over a wide range of pH and ionic strength [18]. Unfortunately, the absence of information on the binding of sulfate and phosphate by α-chymotrypsin precludes reliable estimation of protein net charge for most of the situations investigated, which invariably used one of those ions in the supporting electrolyte medium. As an indication of the need for caution in any calculations of net charge based on pH-titration data, the isoelectric point of α-chymotrypsin shifts from pH 8.1 in univalent salts [42] to pH 6.2 in phosphate buffer [43]. It therefore seems reasonable to assume that the set of results reported in Table 2 of [18] for α-chymotrypsin in 0.1 M sodium phosphate, pH 6.0, I_M 0.12, refers to essentially uncharged enzyme, whereupon hard-sphere interactions should dominate the magnitudes of the various virial coefficients. On the basis of spherical geometry and a monomer radius of 2.44 nm (as in Table 1), B_{11} is taken as 147 L mol^{-1}, B_{12} as 422 L mol^{-1}, and B_{111} as 13,400 L^2 mol^{-2}.

The outcome of nonlinear least-squares curve-fitting of those results to Eqs. (10) and (13)) is summarized in the main section of Fig. 3, where the solid line corresponds to the best-fit description, $K_2 = 14{,}500\ (\pm 2000)$ M^{-1}. Although this dimerization constant is lower than the estimate of 26,000 M^{-1} (2.07 L/g) deduced over a comparable concentration range for α-chymotrypsin in phosphate buffer, pH 6.2, I_M 0.2 [43], the disparity is consistent with an inverse dependence of K_2 upon ionic strength such as that observed under slightly acid conditions [44]. The relatively large uncertainty (±2 SD) of the present best-fit dimerization constant reflects disparate data and a sparsity of results in the crucial low-concentration data range, a factor evident from the inset in Fig. 3, which presents the results in traditional osmotic pressure format. Also shown is their best-fit description (- - -) reported in the original publication [18], a seemingly valid thermodynamic analysis except for the fact that an ordinate intercept of $1/M_1$ should be regarded as an obligatory feature of which account should be taken in any such description of a system undergoing reversible dimerization.

5. Concluding remarks

This investigation into the characterization of nonideal protein self-association by osmometry has served several roles. First, we develop a procedure that allows for the effects of thermodynamic nonideality on the statistical–mechanical basis of the potential-of-mean-force between molecules. Secondly, further insight into the evaluation of the virial coefficients required for application of the procedure is provided by consideration of (a) the adequacy of approximate analytical expressions for their determination and (b) the numerical integration procedures to be followed in the event of their inadequacy. Thirdly, the feasibility of the direct analysis has been illustrated by its application to simulated osmotic pressure data for α-chymotrypsin under weakly acidic conditions (pH 4, I_M 0.05). Finally, the method has been used to determine dimerization constants from published osmotic pressure data for soybean proteinase inhibitor, hemoglobin and α-chymotrypsin, thereby revealing shortcomings in some of the earlier analyses employed to characterize nonideal protein self-association by osmometry.

Whether the present analysis of osmotic pressure data reflecting protein self-association represents an advance on the scaled-particle-theory approach used by Minton and coworkers [19–24] depends, of course, upon the relative extents of solute compliance with the hard-particle models upon which the two procedures are predicated. In that regard the current method would certainly apply to self-associating systems that conform reasonably well with description as impenetrable spheres with net charge spread evenly over their surfaces. Such description of proteins is clearly an over-simplification, but its adoption for at least two proteins has been vindicated by the conformity of measured second virial coefficients for ovalbumin and lysozyme with statistical–mechanical interpretation of experimentally determined values for the Stokes radius and net charge [45,46]. Although further support for the likely generality of these isolated findings would clearly be desirable, the difficulties associated with reliable estimation of protein net charge [47] seemingly preclude the prospect of any widespread testing of the extent to which the current model accounts for thermodynamic nonideality of proteins.

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