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Biophysical Chemistry 93 (2001) 231–240

Biophysical Chemistry

www.elsevier.com/locate/bpc

Analysis of thermodynamic non-ideality in terms of protein solvation

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Received 18 January 2001; received in revised form 25 April 2001; accepted 26 April 2001

Abstract

The effects of thermodynamic non-ideality on the forms of sedimentation equilibrium distributions for several isoelectric proteins have been analysed on the statistical–mechanical basis of excluded volume to obtain an estimate of the extent of protein solvation. Values of the effective solvation parameter δ are reported for ellipsoidal as well as spherical models of the proteins, taken to be rigid, impenetrable macromolecular structures. The dependence of the effective solvated radius upon protein molecular mass exhibits reasonable agreement with the relationship calculated for a model in which the unsolvated protein molecule is surrounded by a 0.52-nm solvation shell. Although the observation that this shell thickness corresponds to a double layer of water molecules may be of questionable relevance to mechanistic interpretation of protein hydration, it augurs well for the assignment of magnitudes to the second virial coefficients of putative complexes in the quantitative characterization of protein–protein interactions under conditions where effects of thermodynamic non-ideality cannot justifiably be neglected.

Keywords: Protein solvation; Sedimentation equilibrium; Thermodynamic non-ideality; Virial coefficients

1. Introduction

In the past 20 years there has been a growing interest in quantitative interpretation of the effect of thermodynamic non-ideality on protein self-association [1–4] and interaction between dissimilar proteins [5,6]. This has necessitated the assignment of a solvated volume (or radius for a spherical protein) to each interacting species so that the effects of thermodynamic non-ideality could be assessed on the statistical–mechanical basis of excluded volume [6–8]. Although statistical–mechanical interpretation of the second virial

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0301-4622/01/$ - see front matter
PII: S0301-4622(01)00223-X

coefficient for a stable non-associating solute [9] provides an unequivocal estimate of the required radius, the paucity of reliable estimates of second virial coefficients has usually necessitated substitution of the Stokes radius for the effective thermodynamic radius of a solvated protein.

Because its magnitude emerged from the concentration dependence of the reciprocal of the apparent molecular mass in sedimentation equilibrium studies [10], the second virial coefficient has been reported for many proteins. However, apart from the report of its magnitude as the slope of the concentration dependence used to evaluate the molecular mass of the protein, little attention has been paid to its molecular significance. Indeed, there have been relatively few studies in which the magnitude of the second virial coefficient has been subjected to statistical–mechanical interpretation [11–15].

This investigation explores the possibility that measurement of the second virial coefficient by sedimentation equilibrium [14,15] may provide a convenient means of assessing the effective solvated volume of a globular protein; and hence provide information on the extent of protein solvation. Being mindful of the concerns expressed by Ogston [16,17] about the meaning of macromolecular hydration, we trust that we are not introducing yet another sense in which the term protein solvation is used. However, the results of experiments thus far seemingly indicate that the effective thermodynamic radius being measured closely resembles its hydrodynamic counterpart; and that it is therefore providing information on protein solvation as defined by Oncley [18] and by Kauzmann in a footnote to an ultracentrifugal study by Schachman and Lauffer [19]. By 'protein solvation' we mean solvent that is associated with the protein. Although such solvent association (water plus small electrolyte in the case of aqueous systems) is a dynamic process in that there is a continual exchange of free solvent with associated solvent, the effective time-averaged volume of a protein is larger than its anhydrous volume insofar as hydrodynamic and thermodynamic properties are concerned.

2. Theoretical basis of the approach

For 30 years the nature of the thermodynamic activity applied to sedimentation equilibrium experiments remained unresolved. However, it has now been demonstrated [15,20,21] that the parameter governing the solute distribution is the molar thermodynamic activity, z_A, defined under conditions of constant temperature (T) and chemical potential of solvent (μ_s). Consequently, sedimentation equilibrium distributions for single protein species may be used to determine osmotic virial coefficients, which are amenable to interpretation on the statistical–mechanical basis of excluded volume.

2.1. Osmotic virial coefficients and thermodynamic activity

The osmotic pressure, Π, of a solution of single solute species A may be expressed as the following series in molar solute concentration C_A.

$$[\Pi/(RT)]_{T,\mu_s} = -(\mu_s - \mu_s^\circ)/(RTV_s) = C_A + B_{AA}C_A^{2+} + \ldots \quad (1)$$

where B_{AA} is the second virial coefficient describing non-associative interaction between molecules of the single species A [9,20]: R is the universal gas constant, T the absolute temperature, μ_s° the standard state chemical potential of solvent and V_s its molar volume. This expression for the chemical potential of solvent as a function of solute concentration now needs to be related to the corresponding relationship for the chemical potential of solute as a function of concentration, namely,

$$(\mu_A)_{T,\mu_s} = (\mu_A^\circ)_{T,\mu_s} + RT\ln z_A = (\mu_A^\circ)_{T,\mu_s} + RT\ln(\gamma_A C_A) \quad (2)$$

where $(\mu_A^\circ)_{T,\mu_s}$ is the standard state chemical potential of solute and $\gamma_A = z_A/C_A$ is the molar activity coefficient.

On the basis of the Gibbs–Duhem equation

($\sum \mu_i dn_i = 0$) Hill [22] has established that

$$(\partial \Pi / \partial C_A)_{T,\mu_s} = C_A (\partial \mu_A / \partial C_A)_{T,\mu_s} \quad (3)$$

Differentiation of Eqs. (1) and (2) with respect to C_A to obtain the required partial derivatives then leads to the conclusion [22] that

$$\ln \gamma_A = 2B_{AA}C_A + \ldots \quad (4)$$

The expression relating the molar activity to the molar concentration of solute is therefore

$$z_A = C_A \exp[2B_{AA}C_A + \ldots] \quad (5)$$

Measurement of the molar activity of a protein as a function of its molar concentration thus offers a potential means for evaluating the magnitude of the second virial coefficient.

2.2. Measurement of the second virial coefficient by sedimentation equilibrium

The application of Eq. (5) for the determination of second virial coefficients requires measurements in which the molar activity and molar concentrations are the dependent and independent variables, respectively. In sedimentation equilibrium of a single solute species at constant temperature (T) and angular velocity (ω) the molar activity, $z_A(r)$, at radial distance r is related to that, $z_A(r_F)$, at a selected reference radial distance r_F by the expression [3]

$$z_A(r) = z_A(r_F)\psi_A(r) \quad (6a)$$

$$\psi_A(r) = \exp\left[M_A(1 - \bar{v}_A \rho_s)\omega^2 (r^2 - r_F^2)/(2RT)\right] \quad (6b)$$

where M_A is the molecular mass of the protein with partial specific volume $\bar{v}_A$; and where ρ_s is the solvent density. The dependent variable, $z_A(r)$, has thus been expressed in terms of a constant thermodynamic activity, $z_A(r_F)$, and a dependent variable, $\psi_A(r)$. Combination of Eqs. (4), (6a) and (6b) then yields the relationship [15]

$$\psi_A(r) = [C_A(r)/z_A(r_F)]\exp[2B_{AA}C_A(r) + \ldots] \quad (7)$$

which, in principle, allows second virial coefficients to be obtained by non-linear curve-fitting of [$\psi_A(r)$, $C_A(r)$] data to this expression.

Alternatively, from the viewpoint that $\psi_A(r)$ may also be regarded as an acceptable transform of the independent variable r, Eq. (5) can be replaced by the corresponding expression for molar concentration as a function of molar activity, namely [7]

$$C_A(r) = z_A(r)\exp[-2B_{AA}z_A(r) + \ldots] \quad (8a)$$

$$= z_A(r) - 2B_{AA}z_A(r)^2 + \ldots \quad (8b)$$

Substitution of Eq. (6b) for $z_A(r)$ then gives rise to the expression

$$C_A(r) = z_A(r_F)\psi_A(r) - 2B_{AA}[z_A(r_F)\psi_A(r)]^2 + \ldots \quad (9)$$

The constant parameter $z_A(r_F)$ and the second virial coefficient may thus be evaluated as the two curve-fitting parameters to emerge from non-linear regression analysis of the dependence of $C_A(r)$ upon $\psi_A(r)$ in accordance with Eq. (9).

2.3. Statistical–mechanical interpretation of the second virial coefficient

Consideration of thermodynamic non-ideality on the statistical–mechanical basis of excluded volume [9] gives rise to the expression [1]

$$B_{AA} = 16\pi N R_A^3/3 + \frac{Z_A^2(1 + 2\kappa R_A)}{(4I)(1 + \kappa R_A)^2} + \ldots \quad (10)$$

for the second virial coefficient of a rigid, impenetrable spherical solute with radius R_A and net charge Z_A distributed uniformly over its surface. Avogadro's number (N) is included to express B_{AA} on a molar rather than a molecular basis. The inverse screening length, κ, is calculated as $3.27 \times 10^7 \sqrt{I}$ (cm^{-1}) from the molar ionic strength

I (mol/l). Although Eq. (10) affords, in principle, the prospect of evaluating R_A from the magnitude of B_{AA} obtained under any conditions, the current unavailability of methodology for determining the net charge Z_A has necessitated restriction of this investigation to sedimentation equilibrium studies of each protein at its isoelectric point ($Z_A = 0$) so that $2B_{AA}$ becomes the co-volume, $4\pi N(2R_A)^3/3 = 8V_h$ where V_h is the solvated (hydrated) volume of the protein molecule.

For uncharged, impenetrable ellipsoids of revolution with semi-major and semi-minor axes a and b, respectively, the co-volume (twice the second virial coefficient) is [23]

$$2B_{AA} = V_h\left[2 + \frac{3}{2}\left\{1 + \frac{\sin^{-1}\varepsilon}{\varepsilon(1-\varepsilon^2)^{1/2}}\right\} \times \left\{1 + \frac{(1-\varepsilon^2)}{2\varepsilon}\ln\frac{(1+\varepsilon)}{(1-\varepsilon)}\right\}\right] \quad (11)$$

where $\varepsilon = (1 - b^2/a^2)^{1/2}$. V_h, the solvated volume of the molecule, is $4\pi Nab^2/3$ for a prolate ellipsoid and $4\pi Na^2b/3$ for an oblate ellipsoid of revolution. Extraction of the solvated solute dimensions is facilitated by the availability of COVOL [24], a software program that also allows rationalisation of B_{AA} in terms of a triaxial ellipsoid, for which all three semi-axes can differ and for which the requisite co-volume expression is also available [25].

Evaluation of a magnitude for B_{AA} allows calculation of a molar solvated volume (V_h, ml/mol) for an assigned regular shape of the protein molecule. For a spherical solute $2B_{AA} = 8V_h$, whereas for an ellipsoid of revolution the corresponding relationship is evident from Eq. (11). The ratio $2B_{AA}/V_h$, termed the reduced co-volume, u_{red}, in COVOL [24], has a minimum value of 8 (the value for a spherical solute). A magnitude for this reduced co-volume parameter is obtained by applying COVOL to the semiaxial ratios a/b, b/c for the triaxial ellipsoid that are deduced by ELLIPSE [26] for the protein molecule from the atomic structural co-ordinates [27] listed in the X-ray crystallographic database. On the grounds that the difference between V_h and the unsolvated volume, $V_u = M_A\bar{v}_A$, represents the increase in volume arising from solvation, the hydration parameter δ is taken as

$$d = (V_h - V_u)\rho_s/M_A \quad (12)$$

where the molecular mass and solvent density are introduced to accord the extent of solvation its usual dimensions (g/g).

3. Experimental

Bovine pancreatic ribonuclease A, bovine erythrocyte carbonic anhydrase, ovalbumin (chicken egg), bovine serum albumin, rabbit muscle creatine phosphokinase and rabbit muscle aldolase were commercial preparations obtained from either Sigma–Aldrich or ICN Biochemicals. Solutions (200 μl, 20 mg/ml) of the various proteins in the appropriate buffer were subjected to zonal chromatography at 0.2 ml/min on a 600 × 7.8 mm Biosep-SEC-3000 column (Phenomenex, Tor-

Table 1
Experimental details for sedimentation equilibrium studies of various proteins under isoelectric conditions

Protein	M_A (kDa)	Isoelectric conditions	Reference	Rotor speed (rev./min)
Ribonuclease A	13.7	Ethanolamine-chloride, pH 9.5, I 0.2	[29]	45 K
Carbonic anhydrase	28.9	Acetate-phosphate, pH 5.3, I 0.1	[30]	32 K
Ovalbumin	44.0	Acetate-chloride, pH 4.6, I 0.1	[31,32]	23 K
Bovine serum albumin	66.0	Acetate-chloride, pH 4.6, I 0.1	[33]	21 K
Creatine phosphokinase	81.0	Phosphate, pH 6.08, I 0.1	[34]	21 K
Aldolase	160	Phosphate, pH 6.05, I 0.1	[35]	13 K

rance, CA), pre-equilibrated with the same buffer. This exclusion chromatography step served not only to remove any contaminating material with markedly different molecular mass but also to provide a solution in dialysis equilibrium with the buffer to be used in the ultracentrifuge studies. The resulting protein solutions (approx. 2 mg/ml) were then subjected to sedimentation at 20°C in a Beckman XL-I analytical ultracentrifuge operating at a rotor speed commensurate with generation of equilibrium distributions of the meniscus-depletion type [28]. The rotor speed for each protein is listed in Table 1, which also details the isoelectric conditions used and the source of that information.

The resulting sedimentation equilibrium distributions, recorded by the Rayleigh interference optical system, were corrected for baseline variation on the basis of a minor linear radial dependence of the absolute fringe displacement. These distributions in terms of Rayleigh fringe displacement, J(r), were then converted to the corresponding weight-concentration distributions by assuming a common value for the refractive index increment of proteins — a frequently used approximation in ultracentrifuge studies. Specifically, the weight-concentration of protein at each radial distance, $c_A(r)$, was then calculated as $c_A(r) = J(r)/3.33$ [36] and converted to a molar concentration $C_A(r)$, on the basis of the molecular mass listed in Table 1. Non-linear regression analysis of the $[C_A(r), r]$ distribution according to Eq. (7) and/or Eq. (9) with $r_F = 7.05$ cm was then used to obtain $z_A(r_F)$ and B_{AA} as the two curve-fitting parameters. In that regard the value of $\psi_A(r)$ was based on the buoyant molecular mass, $M_A(1 - \bar{v}_A\rho_s)$, obtained from absorption optical records (A_{280}) of sedimentation equilibrium experiments with lower protein concentrations and lower angular velocities — a protocol introduced to obviate the assignment of magnitudes to the protein partial specific volume and the buffer density for the determination of $\psi_A(r)$.

4. Results

Procedural aspects of the evaluation of the solvation parameter are illustrated by the analysis of sedimentation equilibrium results obtained for isoelectric carbonic anhydrase (Fig. 1). The left-hand panel (Fig. 1a) presents the results (●) of a low-speed (15 000 rev./min) sedimentation equilibrium experiment [37] in which the enzyme concentration ranged between 0.03 and 0.22 mg/ml across the distribution. Also shown is the best-fit description (—) in terms of Eqs. (6a) and (6b) with $z_A(r)$ and $z_A(r_F)$ replaced by the respective absorbances at 280 nm [$r_F = 7.0000$ cm, $A(r_F) = 0.201$]. Such treatment of the sedimentation equilibrium distribution yielded a buoyant molecular mass of 7420 (±80) Da, which on combination with a partial specific volume of 0.735 ml/g signifies a molecular mass of 28.5 (±0.3) kDa — a value comparing favourably with that of 28.9 kDa deduced from the amino acid sequence. The right-hand panel (Fig. 1b) presents the Rayleigh interference record of a meniscus-depleted sedimentation equilibrium distribution (obtained at 32 000 rev./min) in which the concentration range sufficed for determination of the second virial coefficient. Analysis of those results in terms of Eqs. (7) and (9) with $r_F = 6.9998$ cm are summarised in Fig. 2a,b, respectively. Whereas a reference thermodynamic activity, $z_A(r_F)$ and co-volume, $2B_{AA}$, of 5.36 (±0.01) μM and 326 (±18) l/mol, respectively, emanate from Fig. 2a, the corresponding estimates (±2 S.D.) from Fig. 2b are 5.35 (±0.01) μM and 293 (±12) l/mol. Either analysis clearly suffices; and for illustrative purposes we shall proceed with further analyses on the basis of co-volumes ($2B_{AA}$) determined by the latter procedure [Eq. (9)]. To complete the evaluation of δ we note that the X-ray structure of carbonic anhydrase can be represented as a triaxial ellipsoid with dimensions 55 × 42 × 39 Å [38], or, with reasonable approximation, as a prolate ellipsoid with $a/b = 1.36$ ($b/a = 0.736$). On the grounds that u_{red} [the term within square parentheses in Eq. (11)] is 8.201, $V_h = 35.6$ l/mol, which exceeds the anhydrous volume, ($M_A\bar{v}_A$ = 21.2 l/mol) by 14.4 l/mol. A solvation parameter, δ of 0.50 (±0.05) g/g is then obtained from Eq. (12).

This analysis of the sedimentation equilibrium experiment presented in Fig. 2 is summarised in

Table 2, together with that of a duplicate experiment on carbonic anhydrase and a range of corresponding studies on other isoelectric proteins. In that regard it should be noted that thermodynamic rigor concludes at column 6 — the report of the second virial coefficient ($2B_{AA}$ to be precise). Thereafter the analysis on the statistical-mechanical basis of excluded volume is clearly model-dependent and hence devoid of thermodynamic rigor. Nevertheless, as emphasised by Eisenberg [45], such departure from thermodynamic rigor is justified provided that it leads to greater appreciation of events at the molecular level.

Interpretation of the difference between $2B_{AA}/u_{red}$ and $M_A\bar{v}_A$ in terms of Eq. (12) leads to the values of the solvation parameter δ reported in columns 7 and 8 for the respective spherical and ellipsoidal models of the proteins. Inasmuch as u_{red} is minimal ($u_{red} = 8$) for a spherical solute, the values of the solvation parameter for this model are greater than those calculated for an ellipsoidal model of the molecule. The final column lists R_A, the effective thermodynamic radius of the solvated protein on the basis of its depiction as a rigid, impenetrable sphere — the thermodynamic parameter that finds hydrodynamic equivalence in the Stokes radius.

In a departure from the customary convention of expressing protein solvation as a fixed amount of solvent per gram of protein, we examine solvation in the light that most (if not all) of the solvation is external to the protein [13,19] — a time-averaged solvation layer (averaged over the dynamic interchange between free and associated solvent). From the viewpoint of predicting the magnitude of R_A from the anhydrous molar volume ($V_u = M_A\bar{v}_A$), the question at issue is the number of layers of solvent that comprise the putative shell. We note that the difference between the effective solvated thermodynamic radius (R_A) deduced from the co-volume of the solute ($2B_{AA}/8$), and the anhydrous radius (R_u) determined from $M_A\bar{v}_A$ is approximately 0.5 nm; and therefore explore the possibility that the effective solvated thermodynamic radius (R_A) may

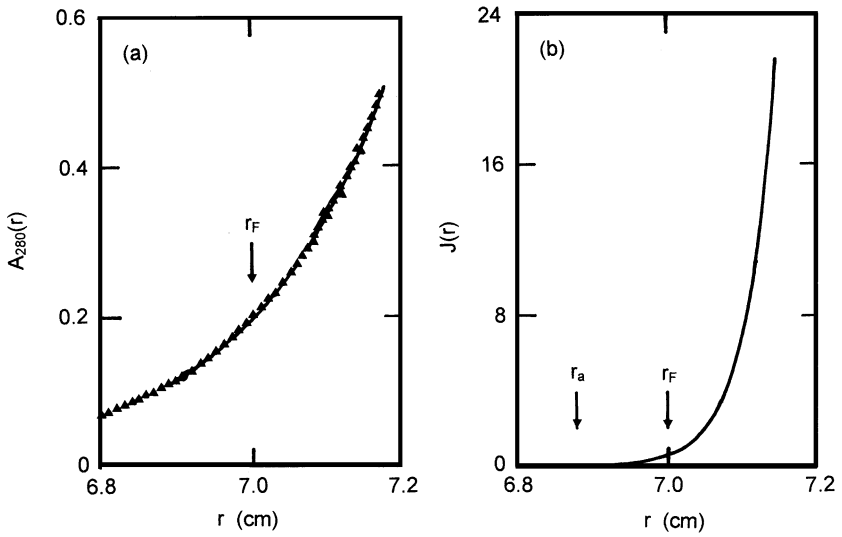

Fig. 1. Sedimentation equilibrium distributions used in the evaluation of the second virial coefficient for isoelectric carbonic anhydrase (pH 5.3, I 0.1). (a) Low-speed (15 000 rev./min) sedimentation equilibrium distribution used to determine the magnitude of the buoyant molecular mass, $M_A(1 - \bar{v}_A\rho_s)$, required for the calculation of the psi function. (b) Rayleigh interferometric record of the high-speed (32 000 rev./min) sedimentation equilibrium distribution used to evaluate the second virial coefficient.

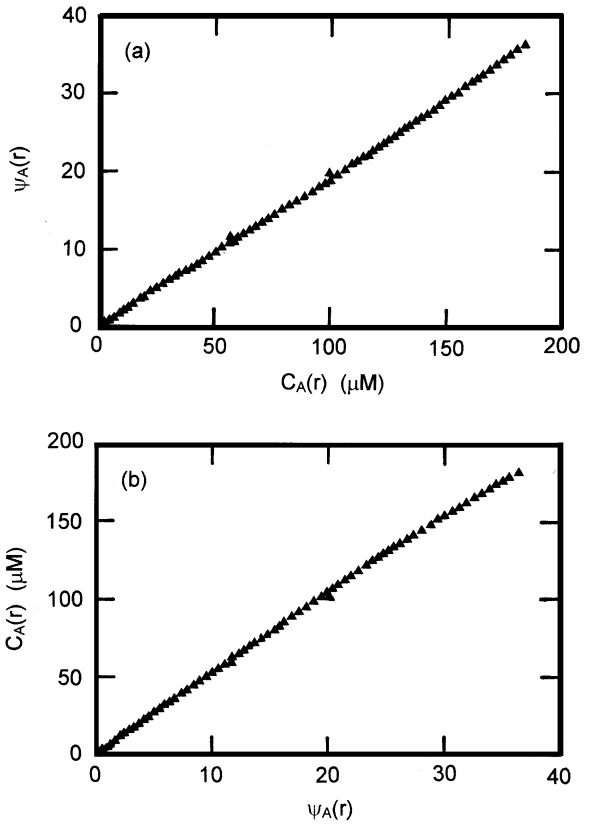

Fig. 2. Determination of the second virial coefficient for isoelectric carbonic anhydrase by sedimentation equilibrium. (a) Plot of results from Fig. 1b in accordance with Eq. (7). (b) Corresponding analysis in terms of Eq. (9).

be predicted with reasonable precision by regarding the solvated molecule as its unsolvated counterpart surrounded by a halo of solvent corresponding to two water molecules.

The dependence of the experimental estimate of the effective solvated radius (R_A) upon protein molecular mass is summarised in Fig. 3a, together with the theoretical relationship (—) for proteins with $\bar{v}_A = 0.735$ ml/g and a 0.56-nm solvent shell (equivalent to two water molecules with a radius of 0.14 nm). Solid symbols denote effective thermodynamic radii determined in this study, whereas open symbols refer to estimates of R_A from reported second virial coefficients for proteins. From the viewpoint of determining the magnitude of the second virial coefficient, B_{AA}, via R_A for a protein with known M_A, the agreement between experimental and predicted solvated radii seems to suffice — an important consideration in allowance for effects of thermodynamic non-ideality on the characterization of reversible association [3,6,8,46]. Extension of this comparison to co-volumes measured for a wider range of proteins is dependent upon refinement of methods for net charge determination in order to eliminate the current restrictive requirement that the sedimentation equilibrium study be conducted under isoelectric conditions.

Unfortunately, the situation is not so clear from the viewpoint of understanding what is meant by the extent of protein solvation. The present magnitudes of the solvation parameter (Table 2) are in general larger than the value of 0.3 g/g assigned to δ by Oncley [18] on the basis of hydrodynamic parameters, and supported to some extent by the NMR studies of Kuntz [47,48]. In that regard we note that the concept of a 'time-averaged' solvation shell with finite average thickness is inconsistent with a common value of δ, which is instead predicted to decrease with increasing size of the protein (Fig. 3b). Our decision to restrict such considerations to spherical geometry for the model of the rigid, impenetrable protein reflects avoidance of a dilemma encountered in applying the same approach to ellipsoidal models. The assignment of a solvation shell with fixed thickness necessitates account to be taken of the consequent changes in the axial ratios a/b and b/c from those pertinent to anhydrous protein. Alternatively, consideration of solvation to be without effect on the axial ratios implies a solvation shell of varying thickness. Only for spherical geometry does this dilemma disappear.

From the viewpoint of excluded volume, protein solvation may be taken into account satisfactorily by considering the unsolvated protein to be surrounded by a shell approximately two water molecules thick — a concept that is clearly reminiscent of the solvation model in which the protein is surrounded by a shell of structured water [49–51]. However, although consistent with such a proposition, R_A is merely an effective

Table 2
Second virial coefficients and extents of protein solvation determined by sedimentation equilibrium

Protein	M_A (kDa)	$\bar{v}_A$ (ml/g)	Ellipsoidal shape[a]	u_{red}	$2B_{AA}$[b] (l/mol)	δ (g/g)		R_A (nm)
						Sphere	Ellipse	
Carbonic anhydrase	28.9	0.735	Prolate [38]	8.201	292 (±14)	0.53	0.50	2.43
					314 (±14)	0.62	0.59	2.50
Ribonuclease A	13.7	0.703	Triaxial [39]	8.692	204 (±14)	1.16	1.01	2.17
Ovalbumin	44.0	0.735	Prolate [40]	8.891	480 (±30)	0.61	0.47	2.88
					440 (±10)[c]	0.50	0.38	2.80
					500[d]	0.69	0.53	2.92
Bovine serum albumin	66.0	0.734	Oblate [41]	10.262	640 (±27)	0.48	0.21	3.17
Creatine phosphokinase	81.0	0.743	Prolate [42]	8.506	691 (±72)	0.39	0.26	3.25
Aldolase	160	0.742	Oblate [43,44]	8.222	1570 (±30)	0.48	0.45	4.27

[a] Reference to crystal structure denoted in square parentheses.
[b] Uncertainties quoted as (±2 S.D.)
[c] Value reported in Wills et al. [15].
[d] Value reported in Jeffrey et al. [11].

thermodynamic radius; and hence not amenable to detailed mechanistic interpretation.

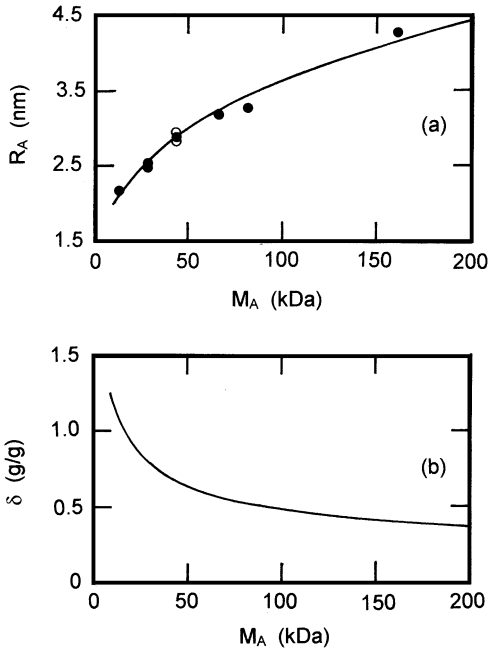

Fig. 3. (a) Comparison of the experimental dependence of the effective solvated radius, R_A, upon protein molecular mass (●) with the theoretical relationship (—) predicted on the basis of the anhydrous molecular volume and a 0.52-nm solvation shell. (b) Corresponding dependence of the predicted magnitude for the solvation parameter δ.

5. Concluding remarks

This investigation has re-emphasised the validity and simplicity of using sedimentation equilibrium for the evaluation of second virial coefficients describing the non-associative self-interaction of proteins [11,14,15]. By restriction of the study to sedimentation equilibrium studies of isoelectric proteins, the second virial coefficient so determined is related directly to the co-volume, which is amenable to statistical–mechanical interpretation without any need for considering the contribution stemming from charge-charge repulsion.

Statistical–mechanical interpretation of the magnitudes of second virial coefficients in terms of the size of the solvated protein has led to a magnitude of the solvation parameter that is larger than the usually assumed value of 0.3 g/g for δ. Inasmuch as the present results are seemingly consistent with interpretation in terms of a model in which the unsolvated protein is surrounded by a solvation shell with a thickness equivalent to two water molecules (Fig. 3), the concept of an invariant value for δ becomes open to question. Although the present estimates of solvated protein radii are thermodynamically (statistical–mechanically) based, their magnitudes

follow reasonably closely their hydrodynamic counterpart (the Stokes radius). The characterization of thermodynamic non-ideality arising from non-associative self-interactions of proteins may therefore provide a readier means of appraising the extent of protein solvation.

Acknowledgements

Aspects of this investigation performed at the University of Queensland were funded in part by the Australian Research Council. The support of research at the National Centre for Macromolecular Hydrodynamics by the United Kingdom Biotechnology and Biomolecular Sciences Research Council as well as the Engineering and Physical Sciences Research Council is also gratefully acknowledged.

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