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Allowance for thermodynamic nonideality and Donnan effects in binding studies

Activity coefficients of charged ligands in the presence of albumin

Keith E. Shearwin and Donald J. Winzor

Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia

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A combination of ultrafiltration with either equilibrium dialysis or frontal gel chromatography has been used to evaluate the effects of thermodynamic nonideality in mixtures of bovine serum albumin and charged ligands. Studies with methyl orange, chlorpromazine and chromate as ligand all demonstrated inadequacy of the Donnan effect for description of the difference between the concentrations of free ligand in a mixture and the protein-free phase with which it is in dialysis equilibrium. On the basis of a quantitative relationship derived for the situation in which Donnan and thermodynamic nonideality effects both operate, values of the second virial coefficient for albumin and ligand have been determined. For albumin and either methyl orange or chlorpromazine the magnitude of this second virial coefficient has been rationalized on the statistical-mechanical basis of excluded volume. For the albumin-chromate system, however, the thermodynamic nonideality was manifested as a negative deviation from Raoult's Law, in keeping with the classical behaviour of electrolyte ions. From the viewpoint of the characterization of ligand binding a unique feature of the ultrafiltration/gel chromatography and ultrafiltration/equilibrium dialysis methods is their ability to define not only the binding function but also the activity coefficient of ligand for a given acceptor-ligand mixture. Consequently, irrespective of whether the ligand is charged or uncharged, the intrinsic binding constant that is determined is the thermodynamic parameter instead of the apparent value that is obtained from methods based on assumed thermodynamic ideality.

1. Introduction

The possible consequences of thermodynamic nonideality arising from the space-filling effects of inert solutes were first highlighted by the theoretical demonstration [1], in response to an experimental observation [2], that inclusion of a solute such as poly(ethylene glycol) could displace a self-association equilibrium from the point of non-detectability to a situation in which polymer is essentially the sole species present. Subsequent physicochemical studies have confirmed that expression of activity coefficients in terms of the statistical-mechanical concept of excluded volume can provide a quantitative description of the effects of thermodynamic nonideality on several self-associating enzymes [3–5]. Moreover, thermodynamic nonideality due to the presence of inert space-filling solutes has been used as a probe for the detection of enzyme isomerization, be it substrate-induced [6–9] or pre-existing [10,11].

Although such space-filling effects of non-participating solutes on interactions entailing gross conformational and/or volume changes could well be substantial in the highly concentrated physiological environment, there are also likely to be wider-ranging consequences. For example, the resultant changes in the thermodynamic activities of

Correspondence address: D.J. Winzor, Department of Biochemistry, University of Queensland, St. Lucia, Qld. 4067, Australia.

ions and hence in the effective ionic strength of the local physiological environment [12] will clearly alter the magnitude of the thermodynamic association constant for any interaction with an electrostatic component. These indirect effects of macromolecular crowding and Donnan redistribution whereby the thermodynamic constant is changed could well be far more dramatic than the entropically driven displacement achievable by a minor change in volume and/or asymmetry during the reactant-to-product transformation.

In the absence of specific chemical interaction between macromolecular species and a small solute, the effects of high concentrations of the former on the thermodynamic activity of the small solute may be measured experimentally either by equilibrium dialysis [13,14] or by frontal gel chromatography [15,16]. However, the measurement of the thermodynamic activity coefficient of small solutes has only been attempted for systems where nonideality is restricted either to excluded-volume effects [10,16] or to the Donnan redistribution of ions [13,14]. The present investigation not only considers the problem of estimating the extent of thermodynamic nonideality under conditions wherein both effects operate, but also illustrates experimental procedures for measuring the activity coefficient of a charged ligand under such circumstances. Firstly, a combination of equilibrium dialysis and ultrafiltration [17,18] is used to measure interaction coefficients for methyl orange and chlorpromazine in albumin solutions supplemented with these charged ligands. Secondly, the possibility of substituting frontal gel chromatography [16] for the equilibrium dialysis step is explored in a study with albumin as the macromolecular solute imparting nonideality to the distribution of chromate ion.

2. Experimental

2.1. Measurement of ligand binding by equilibrium dialysis and ultrafiltration

Solutions of bovine serum albumin (10 ml, 10–70 mg/ml) were prepared by dissolving salt-free protein (fraction V, from Sigma) in either Tris-chloride buffer, pH 7.4, I 0.15, or acetate-chloride buffer, pH 5.5, I 0.15. Protein concentrations were determined spectrophotometrically on the basis of an absorption coefficient ($A^{1\%}_{1cm}$) of 6.6 at 280 nm [19] and a molecular weight of 66 000 [20]. The albumin solutions in Tris-chloride buffer were then dialyzed for 24 h (4×500 ml) at 25°C against buffer supplemented with 20 μM methyl orange (BDH Chemicals). Those in acetate-chloride were dialyzed vs buffer containing 150 μM chlorpromazine (Sigma), precautions being taken to minimize exposure to light. Sacs were weighed before and after dialysis in order to monitor any variation in volume and hence change in albumin concentration from the original value.

Each dialyzed protein solution (7 ml) was then placed in an Amicon 8MC ultrafiltration assembly fitted with a YM10 membrane that had been pre-equilibrated with aliquots of the same solution to eliminate effects of ligand adsorption [21]. A pressure of approx. 14 psi (lb/inch^2) was applied to enable the collection of ultrafiltrate (1 ml) in pre-weighed tubes so that the change in volume and hence protein concentration of the mixture could be assessed [22]: the mean of the initial and final concentrations was taken as that ($\bar{C}^{\alpha}_{A}$) to which the concentration of ligand in the ultrafiltrate (C^{α}_{S}) referred. The latter concentration and also its counterpart in the final diffusate from equilibrium dialysis (C^{β}_{S}) were measured spectrophotometrically on the basis of molar absorption coefficients of 26 800 $M^{-1}\,cm^{-1}$ at 465 nm for methyl orange [23] and 4400 $M^{-1}\,cm^{-1}$ at 300 nm for chlorpromazine [24].

2.2. Studies of the albumin-chromate system by gel chromatography and ultrafiltration

In studies of the effect of albumin concentration upon the activity of chromate ion, mixtures containing potassium chromate (0.25 mM) and albumin (16–25 mg/ml) in 0.15 I Tris-chloride buffers pH 7.4, were subjected to the above ultrafiltration procedure to determine C^{α}_{S}, and to frontal gel chromatography on a column (2.2×9 cm) of Sephadex G-25 to determine C^{β}_{S} [16,26]. A molar absorption coefficient of 4000 $M^{-1}\,cm^{-1}$ at 384 nm was used to monitor chromate concentra-

tion, this wavelength being selected because it represents an isosbestic point for the chromate-albumin system.

3. Results and discussion

Under thermodynamically ideal conditions ligand binding is readily quantified by techniques such as equilibrium dialysis [23,25] and frontal gel chromatography [26], in which the concentration of ligand in the acceptor-free phase is a direct measure of its equilibrium concentration in the acceptor-ligand mixture. Furthermore, in the absence of ligand binding these two techniques make possible the quantification of nonideality, since the composition of the acceptor-free phase now reflects the thermodynamic activity of ligand in the mixture [14,16]. This situation also prevails in the event that ligand binding is being studied under thermodynamically nonideal conditions, but inability to relate the thermodynamic activity of ligand to its equilibrium concentration in the mixture precludes the use of such data for characterizing either the ligand-binding phenomenon or the thermodynamic nonideality. The problem of characterizing both phenomena is first solved by coupling equilibrium dialysis with ultrafiltration of the resulting acceptor-ligand mixture [17,18]. Analysis of the ultrafiltrate yields the concentration of free ligand required for binding studies [18], whereas information on thermodynamic nonideality comes from comparison of the ligand concentrations in the ultrafiltrate (C_S^α) and the final diffusate of the equilibrium dialysis step (C_S^β). Indeed, the ratio C_S^β/C_S^α defines the activity coefficient of ligand in the acceptor-ligand mixture. On previous occasions advantage was taken of this ratio of ligand concentrations to evaluate the mean net charge (valence) of proteins [17,18]. Such interpretation of the results in terms of the Donnan redistribution of ions does, of course, presume the absence of significant space-filling effects, an assumption that is now addressed in a study of the interaction between bovine serum albumin and methyl orange.

3.1. Interaction of methyl orange with bovine serum albumin

In a previous investigation of this interaction in Tris-chloride buffer, pH 7.4 [18], the emphasis was on evaluation of the net acceptor charge; and accordingly the ionic strength ($I = 0.047$) was relatively low to enhance the accuracy with which the Donnan redistribution of ions could be measured. To facilitate the detection of superimposed thermodynamic nonideality due to excluded-volume effects the earlier protocol has been amended by (i) increasing the ionic strength ($I = 0.15$) to diminish the Donnan contribution to the nonidentity of C_S^α and C_S^β, and (ii) extending the study to much higher albumin concentrations.

Results of the present investigation are summarized in fig 1a, where the experimental points

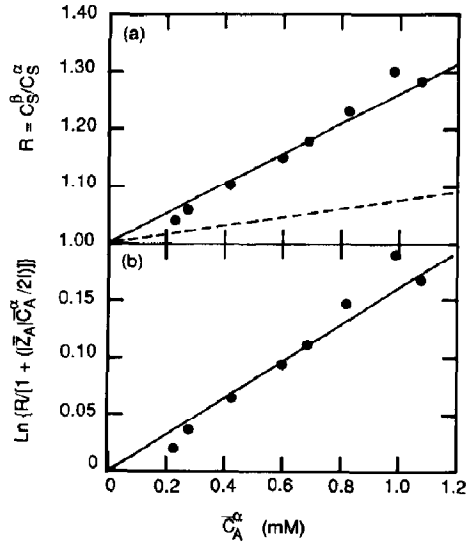

Fig. 1. Distribution of free ligand between the protein-free and protein-containing phases in equilibrium dialysis of bovine serum albumin against methyl orange (pH 7.4, I 0.15). (a) Effect of albumin concentration ($\bar{C}_A^\alpha$) on the ratio of the diffusate ligand concentration (C_S^β) to that of free methyl orange in the mixture (C_S^α) obtained by ultrafiltration of the dialyzed mixture. (b) Plot of same results in accordance with eq. 4c to obtain the second virial coefficient for albumin and methyl orange. In each case the solid line joins the origin to the mean of the experimental points, whereas the broken line in (a) is the relationship predicted on the basis of Donnan effects (eq. 1).

denote the ratio of free ligand concentrations in the two phases, $R = C_S^\beta / C_S^\alpha$, for mixtures in which the net negative charge ($\bar{Z}_A$) on albumin has been increased from -22 [18] to -23 by ligand binding. In that regard the value of unity obtained for the binding function, ν, is in accordance with earlier characterizations [18,22,27–29] of the interaction (15 sites with an association constant of 3300 M^{-1}) and conservation of charge [18]. At first sight, the approximately linear dependence of C_S^β / C_S^α upon total albumin concentration ($\bar{C}_A^\alpha$) is seemingly consistent with the quantitative expression for Donnan redistribution of univalent ions [18], namely,

$$R = C_S^\beta / C_S^\alpha = 1 + \left(| \bar{Z}_A | \bar{C}_A^\alpha / 2I \right); \qquad (1)$$

but the theoretical relationship for such dependence (broken line in fig. 1) is clearly a poor description of the experimental results. An attempt is therefore made to reconcile the disparity between experimental points and the theoretical dependence predicted by Donnan considerations in terms of excluded-volume effects.

On the statistical-mechanical basis of excluded-volume the activity coefficient of ligand (y_S^α) in the presence of acceptor, A, is given [3] by

$$y_S^\alpha = \exp\left[\alpha_{S,S} C_S^\alpha + \sum_1^{15} \left(\alpha_{S,AS_i} C_{AS_i}^\alpha \right) \right] \qquad (2)$$

provided that consideration is restricted to second virial coefficients. Furthermore, for small ligands the various second virial coefficients for the interaction of ligand with acceptor species will be of similar magnitude ($\alpha_{S,AS_i} \cong \alpha_{S,A}$) [29], whereupon the use of a ligand concentration (20 μM) that is considerably smaller than that of albumin (0.2–1.1 mM) allows simplification of this expression to

$$y_S^\alpha \cong \exp\left(\alpha_{S,A} \bar{C}_A^\alpha \right). \qquad (3)$$

If thermodynamic ideality of the protein-free phase (diffusate) in equilibrium dialysis is assumed, eq. 3 describes the ratio of ligand concentrations in the diffusate and protein-containing phases (C_S^β / C_S^α) under conditions where excluded-volume effects alone prevail, whereas eq. 1 is the corresponding relationship for Donnan redistribution of ligand ions in the absence of excluded-volume effects. The results of fig. 1a are therefore considered in terms of the relationship

$$R = C_S^\beta / C_S^\alpha = \left[1 + \left(| \bar{Z}_A | \bar{C}_A^\alpha / 2I \right) \right] \exp\left(\alpha_{S,A} \bar{C}_A^\alpha \right). \qquad (4a)$$

Introduction of the approximations $\ln[1 + (|\bar{Z}_A| \bar{C}_A^\alpha / 2I)] \cong |\bar{Z}_A| \bar{C}_A^\alpha / 2I$ and $\ln(C_S^\beta / C_S^\alpha) \cong (C_S^\beta / C_S^\alpha) - 1$ into the logarithmic form of eq. 4a leads to the expression

$$C_S^\beta / C_S^\alpha \cong 1 + \left[\alpha_{S,A} + \left(| \bar{Z}_A | / 2I \right) \right] \bar{C}_A^\alpha \qquad (4b)$$

which shows that the operation of an excluded-volume effect in addition to the Donnan redistribution of ligand ions should still result in the observed linear dependence of C_S^β / C_S^α upon total protein concentration, $\bar{C}_A^\alpha$ (fig. 1a).

Although eq. 4b offers, in principle, a method of evaluating $\alpha_{S,A}$ from the difference between the slopes of the solid and broken lines in fig. 1a, a more accurate estimate of the second virial coefficient is obtained by analysis in terms of the following logarithmic form of eq. 4a.

$$\ln\left\{ R / \left[1 + \left(| \bar{Z}_A | \bar{C}_A^\alpha / 2I \right) \right] \right\} = \alpha_{S,A} \bar{C}_A^\alpha \qquad (4c)$$

In accordance with prediction, the dependence of the left-hand side of eq. 4c upon total protein concentration is described adequately by a linear relationship passing through the origin (fig. 1b), a value of 160 ($\pm$19) l/mol for the second virial coefficient ($\alpha_{S,A}$) being obtained from the slope of the line joining the origin to the mean of the experimental points. On the statistical-mechanical basis of excluded-volume this second virial coefficient is related to molecular parameters by the expression [3]

$$\alpha_{S,A} = U_{S,A} - M_A \bar{v}_A + \frac{\bar{Z}_A Z_S (1 + \kappa r_A + \kappa r_S)}{2I (1 + \kappa r_A)(1 + \kappa r_S)} \qquad (5)$$

where the covolume, $U_{S,A}$, is given by $4\pi N (r_A + r_S)^3 / 3$ for spherical acceptor and ligand molecules with radii r_A and r_S, respectively. The second term on the right-hand side of eq. 5 denotes the molar volume of anhydrous acceptor, and the final term describes charge-charge interactions in terms of the respective valences (Z) and radii of acceptor

and ligand: for albumin $M_A = 66000$, $\bar{v}_A = 0.735$ ml/g and $r_A = 3.51$ nm [20], and the product $\bar{Z}_A Z_S$ is +23, since acceptor and ligand both bear net negative charge. The inverse screening length, κ, has been taken as $3.27 \times 10^7 \sqrt{I}$, the value for 1:1 electrolytes. An iterative procedure using an initial value of $\bar{Z}_A Z_S / 2I$ for the charge-charge term yields a covolume, $U_{S,A}$ of 149 (±19) l/mol and a consequent effective thermodynamic radius of 0.38 (±0.16) nm for methyl orange. This value of r_S essentially duplicates the effective thermodynamic radius of 0.34 nm obtained [5] for sucrose, a small solute with similar molecular weight (M_r 342 cf. 355). We therefore conclude that the results presented in fig. 1a for the methyl orange-bovine serum albumin system find quantitative explanation in terms of the combined operation of excluded-volume and Donnan effects.

3.2. *Interaction of bovine serum albumin with chlorpromazine*

Having considered a situation in which the ligand and acceptor are both anionic, we now focus attention on the interaction of a cationic ligand, chlorpromazine, with albumin under conditions (pH 5.5, I 0.15) where the protein still bears net negative charge. On the basis of sedimentation equilibrium [30] and gel chromatographic [31] studies under comparable conditions, there is essentially no micellization of chlorpromazine at the concentration (150 μM) used in this investigation: the ligand may therefore be assigned a valence (Z_S) of +1. At this pH a valence (Z_A) of −10 for albumin is deduced from application of Gorin theory [32] to the electrophoretic mobility of -3.2×10^{-5} cm^2 s^{-1} V^{-1} reported by Alberty [33], and also from analysis of the pH titration curve after allowance for chloride binding [34].

The dependence of C_S^β / C_S^α upon total albumin concentration ($\bar{C}_A^\alpha$) is again described adequately by a linear relationship passing through the origin (fig. 2a). Furthermore, as with methyl orange, there is poor correlation between the observed dependence and the relationship predicted solely on the basis of Donnan considerations (eq. 1) and a mean valence ($\bar{Z}_A$) of −9 for the albumin

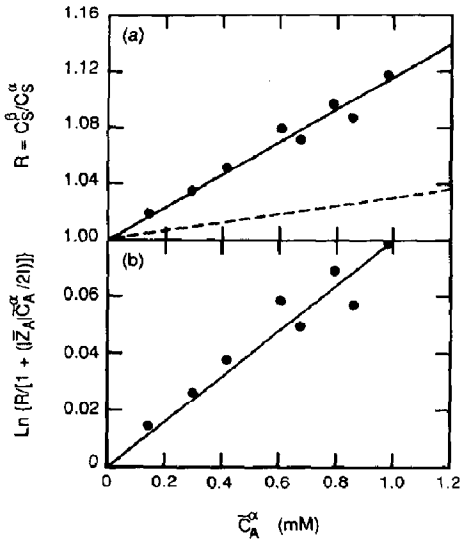

Fig. 2. Distribution of free ligand between the protein-free and protein-containing phases in equilibrium dialysis of bovine serum albumin against chlorpromazine (pH 5.5, I 0.15). (a) Comparison of the protein concentration dependence of the ratio of chlorpromazine concentrations in the diffusate (C_S^β) and protein-containing (C_S^α) phases (●) with the relationship predicted (eq. 1) for Donnan effects alone (------). (b) Replot of results in accordance with eq. 4c to obtain the second virial coefficient ($\alpha_{S,A}$) for albumin and chlorpromazine.

(broken line in fig. 2). The latter is based on the above valence of −10 for unliganded albumin and essential saturation of the single binding site, the present observation that $\nu = 0.9$–1.0 being in substantial agreement with prediction based on the intrinsic association constant of 10^5 M^{-1} reported [31] for the binding of monomeric chlorpromazine to human serum albumin under the same conditions.

The analysis of the results for chlorpromazine and albumin in terms of eq. 4c is summarized in fig. 2b, where the slope of the line signifies a second virial coefficient ($\alpha_{S,A}$) of 80 (±9) l/mol. Although this value is only half of the corresponding second virial coefficient for albumin and methyl orange, it signifies an essentially identical covolume because the charge-charge term in the expression relating $\alpha_{S,A}$ to covolume (eq. 5) has opposite sign for the two systems. Indeed, the resulting covolume of 147 (±9) l/mol for albumin and chlorpromazine corresponds to an effective

thermodynamic radius of the drug, 0.38 (±0.08) nm, that is indistinguishable experimentally from that for methyl orange and sucrose: the molecular weight (328) is also comparable with those of the other two small solutes.

Three important points are noted in relation to these studies of the interactions of albumin with methyl orange (fig. 1) and chlorpromazine (fig. 2). (i) The ratio C_S^β/C_S^α is greater than unity irrespective of the relative signs of the net charges on acceptor and ligand (figs 1a and 2a), this being a consequence of the failure of ultrafiltration to include a counterionic contribution to the concentration of free ligand. (ii) The sign of the acceptor valence in relation to that of ligand does affect the magnitude of the second virial coefficient ($\alpha_{S,A}$) because of the dependence of the sign of the charge-charge term upon the product of the acceptor and ligand valences (eq. 5). (iii) The fact that there is a covolume contribution to the nonideality of these charged ligands must be attributed to their non-charged portions, since electrolyte ions exhibit negative deviations from Raoult's Law. This aspect is considered further in section 3.3, where the effect of albumin on the thermodynamic activity of chromate ion is examined.

3.3. Effect of albumin on the activity of chromate ion

In anticipation that the distribution of chromate ion would reflect the dialysis behaviour of a non-binding bivalent electrolyte ion, a mixture of potassium chromate (0.25 mM) and bovine serum albumin (21.1 mg/ml) in 0.15 I Tris-chloride buffer, pH 7.4, was subjected to frontal gel chromatography on Sephadex G-25 for determination of the ratio C_S^β/C_S^α [15,16]. However, the resulting trailing elution profile (fig. 3) is clearly at variance with the Donnan requirement that the concentration of non-counterion be less in the mixture than in the protein-free phase ($C_S^\alpha < C_S^\beta$). The consequent inference that chromate must be binding to albumin was confirmed by subjecting an aliquot of the same albumin-chromate mixture to ultrafiltration in order to determine C_S^α, the concentration of free chromate in the mixture, and hence the binding function ν. This value of C_S^α, indicated by the dotted line in fig. 3, clearly meets the Donnan requirement that $C_S^\beta > C_S^\alpha$; and also sig-

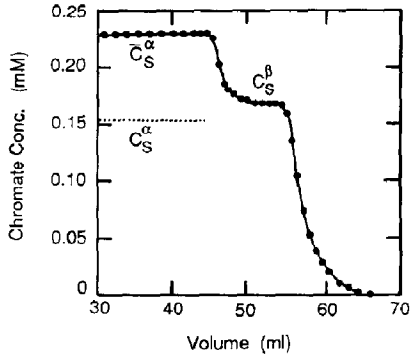

Fig. 3. Trailing elution profile obtained for chromate ion in frontal gel chromatography of a mixture of bovine serum albumin (21.1 mg/ml) and potassium chromate (0.25 mM) in Tris-chloride buffer (pH 7.4, I 0.15) on a column (2.2×9 cm) of Sephadex G-25: (·····) concentration of free chromate ion in the mixture (C_S^α), as determined by ultrafiltration.

Table 1
Determination of the second virial coefficient for chromate (S) and bovine serum albumin (A) by a combination of frontal gel chromatography and ultrafiltration

$\bar{C}_A^\alpha$ (mM)	$\bar{C}_S^\alpha$ (mM)	C_S^β [a] (mM)	C_S^α [b] (mM)	ν	$\alpha_{S,A}$ [c] (l/mol)	$U_{S,A}$ [d] (l/mol)
0.320	0.250	0.168	0.153	0.23	137	35
0.243	0.250	0.201	0.189	0.25	115	13
0.372	0.250	0.176	0.162	0.24	88	−14

[a] Concentration in the protein-free phase of the trailing elution profile [15,16].
[b] Concentration of chromate in the ultrafiltrate [17,18].
[c] Obtained by application of eq. 6.
[d] Apparent value obtained from eq. 5: for excluded-volume effects to be operating this value must exceed 109 l/mol, the hydrated molar volume of albumin.

nifies a value of 0.25 for ν. It should be noted that the combination of frontal gel chromatography and ultrafiltration provides a more rapid and more convenient alternative to equilibrium dialysis and ultrafiltration for characterizing the interacton of a charged ligand with an acceptor under thermodynamically nonideal conditions.

Interpretation of the results from fig. 3 in terms of the counterpart of eq. 4c for a bivalent ligand, namely

$$\ln\left\{R/\left[1+\left(|\bar{Z}_A|\bar{C}_A^{\alpha}/2I\right)\right]^2\right\}=\alpha_{S,A}\bar{C}_A^{\alpha}, \qquad (6)$$

is summarized in the top row of table 1, which indicates a positive value (137 l/mol) for the second virial coefficient, $\alpha_{S,A}$. However, its substitution into eq. 5 with $\bar{Z}_A=-22.5$ and $Z_S=-2$ yields a calculated covolume (35 l/mol) that is smaller than the hydrated molar volume of albumin (109 l/mol) – an outcome that is verified by results for two other albumin-chromate mixtures reported in table 1. The physically unacceptable inference that chromate ion has a negative radius signifies failure of the thermodynamic nonideality of this electrolyte ion to be describable by the excluded-volume concept. However, the value of $\alpha_{S,A}$ retains validity from the viewpoint of defining the relative thermodynamic activities of ligand in the mixture and protein-free phases (eq. 3). For the characterization of ligand binding this ratio of concentrations is also regarded as the activity coefficient of ligand in the mixture due to definition of the association constant in terms of the concentration of free ligand ion (C_S^{α}) instead of the mean ionic activity ($a_{\pm}$) of the electrolyte system. Since mean activity coefficients of electrolytes in solutions with physiological ionic strength differ markedly from unity [35,36], the equilibrium constant obtained by the present procedures would not, strictly speaking, be the true thermodynamic value, but rather the apparent thermodynamic constant extrapolated to zero acceptor concentration.

3.4. Thermodynamic characterization of ligand binding

Thus far the primary concern of this investigation has been the molecular interpretation of the second virial coefficient ($\alpha_{S,A}$) obtained by the equilibrium dialysis/ultrafiltration or the gel chromatography/ultrafiltration procedure. An additional application of these procedures is their use for thermodynamic characterization of relatively weak ligand-binding phenomena. Since the high acceptor concentrations required for such characterization are inevitably going to impart thermodynamic nonideality to the binding data, some comment on the use of the present approach for evaluating the thermodynamic binding constant seems appropriate.

On the basis of reasonable assumptions that charge is conserved on succesive ligand attachment to acceptor [18] and that such ligand addition is essentially without effect on acceptor radius ($r_{AS_i}\cong r_A$), the binding function for the interaction of ligand with p equivalent and independent sites on acceptor is given [29] by

$$\nu=\frac{pK_A\left(y_A^{\alpha}y_S^{\alpha}/y_{AS}^{\alpha}\right)C_S^{\alpha}}{1+K_A\left(y_A^{\alpha}y_S^{\alpha}/y_{AS}^{\alpha}\right)C_S^{\alpha}} \qquad (7)$$

where K_A is the intrinsic association constant for the acceptor-ligand interaction. For evaluation of the activity coefficient ratio, eq. 10 of ref. 29 is more conveniently expressed in terms of $\bar{C}_A^{\alpha}$ rather than C_A^{α} and C_{AS}^{α}, the result being

$$\left(y_A^{\alpha}y_S^{\alpha}/y_{AS}^{\alpha}\right)=\exp\left[\alpha_{S,A}\bar{C}_A^{\alpha}-\frac{Z_S(1+\kappa r_A+\kappa r_S)\left[\bar{Z}_A\bar{C}_A^{\alpha}+Z_SC_S^{\alpha}\right]}{2I(1+\kappa r_A)(1+\kappa r_S)}\right] \qquad (8)$$

an expression that simplifies to eq. 11 of ref. 29, as required, in the event that $Z_S=0$. In eq. 8 y_S^{α} is considered to be given by eq. 3 rather than the complete expression (eq. 2), the reason for omission of the self-interaction term ($\alpha_{S,S}C_S^{\alpha}$) being that the measurement of $\alpha_{S,A}$ from C_S^{β}/C_S^{α} entails the implicit assumption that the concentration of ligand in the protein-free (β) phase of equilibrium dialysis or the equivalent gel chromatography step defines its thermodynamic activity in the mixture (α-phase). For consistency, thermodynamic nonideality due to self-interaction of ligand should

also be disregarded in the acceptor-containing phase. As noted previously [29], this activity coefficient ratio will usually be of insignificant magnitude for observable concentration dependence of ν, provided that its calculation entails the use of C_S^{α} (not C_S^{β}). In that context the present equilibrium dialysis/ultrafiltration and frontal gel chromatography/ultrafiltration procedures not only yield the appropriate value of ν (i.e., $[\bar{C}_S^{\alpha} - C_S^{\alpha}]/\bar{C}_A^{\alpha}$), but also have the potential to provide the values of $\alpha_{S,A}$ and Z_A (or $\bar{Z}_A$) required for assessing the magnitude of the activity coefficient ratio.

4. Concluding remarks

This investigation has served to illustrate the use of ultrafiltration in conjunction with either equilibrium dialysis or frontal gel chromatography to study the binding of a charged ligand to an acceptor under conditions where the equilibrium distribution of ligand between protein-containing and protein-free phases is governed by thermodynamic nonideality as well as the Donnan effect. For that purpose expressions have been derived which enable the ratio of free ligand concentrations in the two phases to be used for evaluation of the second virial coefficient ($\alpha_{S,A}$) reflecting nonideality of the ligand due to the presence of acceptor. From experiments with albumin as acceptor and either methyl orange or chlorpromazine as ligand, it appears that this second virial coefficient may be rationalized in terms of excluded-volume effects. However, analogous interpretation of $\alpha_{S,A}$ for chromate and albumin leads to the untenable conclusion that chromate ion has a negative radius, thereby signifying that the thermodynamic nonideality of chromate, like that of other electrolyte ions, is manifested as negative deviations from Raoult's Law rather than the positive deviations associated with space-filling (excluded-volume) effects. Finally, brief consideration has been given to the use of either combination of procedures for the thermodynamic characterization of ligand binding. A unique feature of these two methods is their ability to define not only the magnitude of the binding function (ν) but also the activity coefficient of ligand. Irrespective of whether the ligand is charged or uncharged, the intrinsic association constant that is determined is the thermodynamic parameter instead of the apparent value that is obtained from other methods based on assumed thermodynamic ideality.

Another problem that may be addressed by the application of these procedures is the effect of a high protein/polyelectrolyte concentration on the ionic strength in the immediate vicinity. Although procedures such as equilibrium dialysis [13,14] and frontal gel chromatography [15,16] could be used to evaluate the Donnan redistribution of an ion that did not interact with the macromolecular components, the requirement for inertness of the ion may well not be met at the high macromolecular concentrations encountered in many physiological environments. The combination of either of these procedures with ultrafiltration quantifies not only the extent of any such binding of the ion but also the ratio of its concentrations in the macromolecule-containing and macromolecule-free phases. Substitution of this ratio into the appropriate Donnan expression (eq. 1 for a univalent ion) then yields the effective ionic strength of the macromolecular environment provided that a value may be assigned to Z_A.

It is hoped that this demonstration of two procedures for investigating the binding and Donnan redistribution of charged ligands/ions may prompt further studies of thermodynamic nonideality in concentrated protein/polyelectrolyte solutions, an understanding of which is clearly essential for quantitative rationalization of interactions in the cellular environment.

Acknowledgement

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