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Limitations of the ultracentrifugal approach for measuring the effective net charge of a macroion

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Abstract

Limitations have been detected in a recently published method for macroion valence determination by an ultracentrifugal procedure for quantifying the Donnan distribution of small ions in macroion solutions dialyzed against buffer supplemented with chromate as an indicator ion. The limitation reflects an implicit assumption that sedimentation velocity affords an unequivocal means of separating effects of chromate binding from those reflecting the Donnan redistribution of small ions. Although the assumed absence of significant Donnan redistribution of small ions across the sedimenting macroion boundary seemingly holds for some systems, this approximation is demonstrably invalid for others. Despite preliminary signs of promise, the ultracentrifugal procedure does not afford a simple, readily applied solution to the problem of unequivocal macroion valence determination.
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During recent years, there have been relatively few reports of net charge (valence) determinations for proteins. This situation reflects, to some extent, a widely held belief that such measurements have been rendered redundant by the availability of sequence data that allow accurate calculation of net charge. As highlighted in a recent review [1], the limitations of that approach to determining the effective net charge were recognized long before the advent of the databases and computer programs that render those theoretical calculations possible. Interest in the problem of experimental charge determination has been rekindled by the requirement of a magnitude for the valence in the assessment of effects of thermodynamic nonideality on protein interactions [2–5], a phenomenon of great relevance to the crowded physiological environment due to its importance in concentrated solutions [6–10].

The most direct approach to unequivocal determination of the net charge (valence) of a macroion entails assessment of the Donnan distribution of ions in equilibrium dialysis by measuring the difference between the concentrations of small ions in the solution and diffusate phases [11]. Ultrafiltration has also been used to ascertain the concentration of noncounterions at dialysis equilibrium [12,13]. A recent publication [14] described an additional method in which chromate is included in the buffer to facilitate assessment of the Donnan redistribution of dialyzable ions by a sedimentation velocity procedure.

Inclusion of the indicator ion introduces a potential for erroneous valence estimation unless allowance can be made for any chromate binding by the macroion [14]. Indeed, for the system used to illustrate the procedure, the concentration of chromate in the dialyzed serum albumin solution exceeded that in the diffusate despite the anionic character of the protein under the conditions of the investigation (pH 8.0). However, by using an XL-I ultracentrifuge as the double-beam spectrophotometer for comparing the absorbance (at 375 nm) of the dialyzed solution with that of the diffusate, the contribution arising from chromate binding could be identified by

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increasing the rotor speed from 3000 to 60,000 rpm. The resultant sedimentation velocity pattern signified chromate comigration with the albumin as well as a depleted chromate concentration in the protein-free region of the absorbance scan. This depleted chromate concentration (taken as C_L^{α}, the concentration of free chromate in the dialyzed solution) was then combined with the chromate concentration in the diffusate (C_L^{β}) to obtain the net charge (z_P) of the protein through the generalized form of the Donnan expression [15]

$$(C_L^{\alpha}/C_L^{\beta})^{1/z_L} = 1 - z_P C_P^{\alpha}/(2I), \quad (1)$$

where C_L^{α} denotes the molar concentration of the protein solution in dialysis equilibrium with diffusate with ionic strength I and z_L is the valence (−2) of the indicator (chromate) ion.

Although the use of Eq. (1) is open to criticism due to its inherent assumption that the ratio of mean activity coefficients ($\gamma_{\pm}$) in the solution and diffusate phases is unity, there is experimental support for the validity of that assumption in experiments with varied C_L^{α} but constant ionic strength of the diffusate [16]. Inasmuch as the effects of macroion nonideality have not been taken into account, the apparent magnitudes of z_P obtained from Eq. (1) for a series of C_P^{α} should, strictly speaking, be extrapolated to zero protein concentration to obtain a more accurate estimate of the protein valence. However, the precision of measurements of C_L^{α} at lower protein concentrations does not suffice to warrant such extrapolation.

Unfortunately, the conclusion [14] that the ultracentrifugal approach should provide an unequivocal method for determining macroion valence requires modification in the light of further experience with dextran sulfate and bovine serum albumin as the charged macromolecular species. This article reports that additional evidence.

Materials and methods

In the first series of experiments, a solution of dextran sulfate (Pharmacia) was prepared by dissolving the polysaccharide directly in phosphate buffer (0.02 M NaH_2PO_4–0.02 M Na_2HPO_4), pH 6.8, I 0.08, supplemented with 0.2 mM K_2CrO_4 to provide an indicator ion. After dialysis against the same buffer to effect the Donnan redistribution of ions, the difference between chromate concentrations in the polysaccharide and diffusate solutions was monitored by means of the absorption optical system of a Beckman XL-I ultracentrifuge. As in the previous investigation [14], wavelengths of 375 and 500 nm were used for this purpose, with the scan at the latter wavelength being taken as a baseline ($\Delta A_{375} = 0$). Initial scans were recorded at 3000 rpm, after which the rotor speed was increased to 60,000 rpm to create a polysaccharide-free region in subsequent scans. Migration of the sedimenting macroion boundary was monitored by means of the Rayleigh interference optical system.

The same protocol [14] was followed to determine the chromate ion distribution in experiments on bovine serum albumin (Sigma Fraction V) dialyzed against chromate-supplemented sodium phosphate buffer (pH 6.8, I 0.04) as well as on serum albumin and dextran sulfate solutions in which the phosphate was replaced by Tris–chloride buffer (pH 8.0, I 0.05).

Results and discussion

Because of the higher charge of dextran sulfate (two $-OSO_3^-$ per monosaccharide residue), the difference in chromate concentration between the solution and diffusate phases is larger, and hence more easily quantified, than in the previous attempt to measure protein valence [14]. Scans at 375 and 500 nm obtained by centrifuging a solution of dextran sulfate (10 mg/ml) dialyzed against chromate-supplemented 0.08 I phosphate buffer (diffusate in the reference sector) at 3000 rpm are presented in Fig. 1A. In keeping with the anionic nature of dextran sulfate, the negative increment in A_{375} signifies that chromate is a noncounterion. Furthermore, interpretation of that absorbance increment in terms of a standard

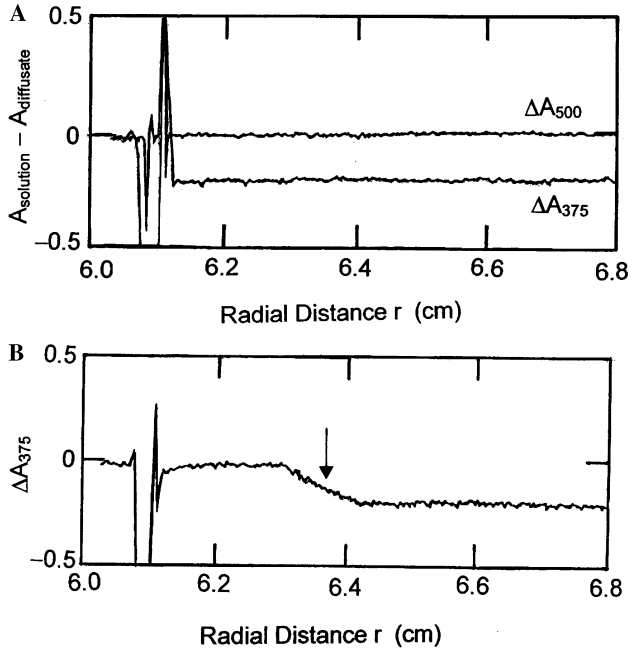

Fig. 1. Ultracentrifuge scans comparing the chromate ion concentration in the diffusate with that in a solution of dextran sulfate (10 mg/ml) dialyzed against 0.08 I phosphate buffer (pH 6.8) supplemented with 0.2 mM K_2CrO_4. (A) Scans at 375 and 500 nm (baseline) obtained at 3000 rpm. (B) Scan at 375 nm after an increase in rotor speed to effect migration of the macroion. The arrow denotes the position of the dextran sulfate boundary (detected by the interference optical system of the XL-I ultracentrifuge.

curve (as in [14]) gives an estimate of 0.16 mM for C_L^{α} (cf. 0.20 mM for (C_L^{β}); using Eq. (1), this gives an effective net charge (z_P) of −0.56 per monosaccharide residue ($C_P^{\alpha} = 0.033$ M on a monosaccharide basis). This estimate of z_P is lower than the theoretical value of −2, a reflection of charge screening (shielding) in highly charged macroion solutions [16–24].

Inherent in the above calculation is the presumed absence of chromate binding. Although the negative increment in A_{375} is certainly consistent with such an assumption, that observation alone does not constitute proof of the absence of chromate binding; it could merely signify predominance of the Donnan effect over any such chromate binding. As in the previous study [14], the rotor speed was increased to 60,000 rpm in the belief that the polysaccharide-free region of a sedimentation velocity scan would provide unequivocal information on C_L^{α} and, hence, on the Donnan distribution of chromate ion. However, that expectation on the basis of the previous experience with bovine serum albumin [14] was not realized (Fig. 1B). Instead, the identity of chromate concentrations in the diffusate and polysaccharide-free region of the scan ($\Delta A_{375} = 0$) signified reestablishment of the Donnan distribution of small ions across the sedimenting boundary of dextran sulfate that coincided with the decrease in ΔA_{375} in Fig. 1B. In retrospect, this observation offers visible proof of the assertion [24] that the refractive index difference in the macroion-free region of a Rayleigh sedimentation velocity pattern for undialyzed heparin in 0.15 M NaCl should be amenable to quantitative interpretation in terms of the Donnan distribution of supporting electrolyte across the migrating polysaccharide boundary.

Because of the disparity between the form of Fig. 1B and its published counterpart for serum albumin (Fig. 2B in [14]), a sedimentation velocity experiment was performed on bovine serum albumin dialyzed against 0.04 *I* sodium phosphate (pH 6.8) supplemented with 0.2 mM chromate. In that regard, the relatively high protein concentration (15 mg/ml) and lower ionic strength were used to enhance the magnitude of ΔA_{375} and, hence, the certainty of the observation. From Fig. 2A in the current article, it is evident that the Donnan equilibrium has also been reestablished across the sedimenting albumin boundary ($\Delta A_{375} = 0$ in the protein-free region).

Verification of the earlier finding [14] is afforded by Fig. 2B, which presents the sedimentation velocity scan at 375 nm for bovine serum albumin (15 mg/ml) in 0.05 *I* Tris–chloride (pH 8.0) supplemented with 0.2 mM chromate—the conditions used previously. The reported existence of a depleted chromate concentration in the protein-free region [14] is clearly confirmed. Therefore, we conclude that the difference between the forms of the sedimentation velocity scans in Figs. 2A and B reflects a more rapid reestablishment of the Donnan distribution of

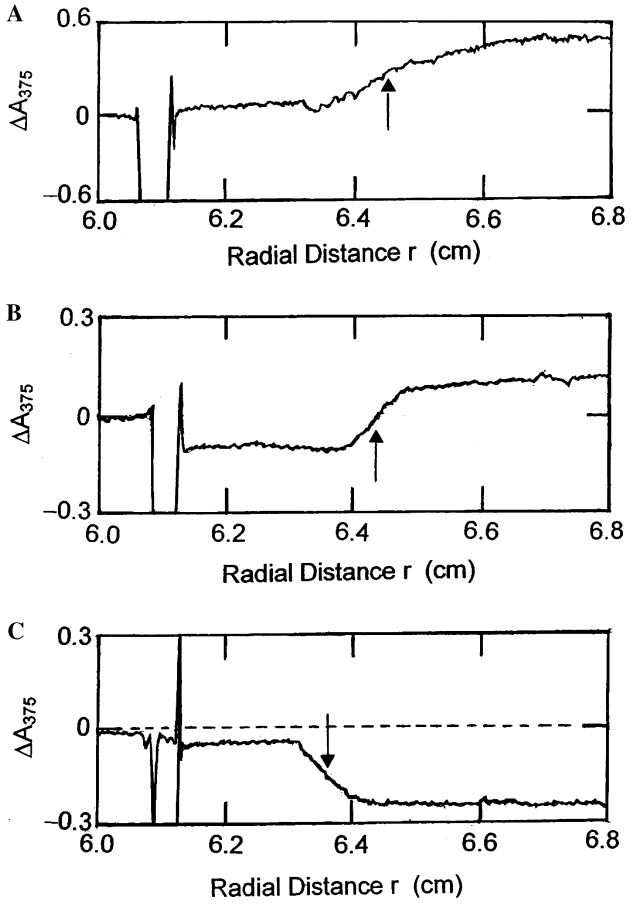

Fig. 2. Absorbance scans (at 375 nm) obtained in sedimentation velocity experiments (60,000 rpm) on macroion solutions in dialysis equilibrium with buffers containing 0.2 mM chromate as indicator ion. (A) Bovine serum albumin (15 mg/ml) in phosphate buffer, pH 6.8, *I* 0.04. (B) Bovine serum albumin (15 mg/ml) in Tris–chloride buffer, pH 8.0, *I* 0.05. (C) Dextran sulfate (10 mg/ml) in Tris–chloride, pH 8.0, *I* 0.05. Arrows denote the positions of the migrating macroion boundary.

small ions across the migrating macroion boundary when Na^+ (rather than $Tris^+$) is the counterion. Support for that contention comes from Fig. 2C, which presents the sedimentation velocity scan at 375 nm from an experiment with dextran sulfate (10 mg/ml) in the Tris–chloride environment. Although some redistribution of chromate ion has occurred across this more slowly migrating macroion boundary at $s_{20} = 1.8$ S (cf. 4.2 S for albumin), the small but significant extent of chromate depletion ($\Delta A_{375} = -0.04$) in the polysaccharide-free region indicates incomplete attainment of the Donnan equilibrium between the solution and macroion-free phases.

In retrospect, it is evident that this procedure for valence determination [14] entails an implicit assumption that ultracentrifugation ensures the generation of the macroion-free phase with sufficient rapidity to preclude any significant Donnan redistribution of small ions across the migrating boundary. Although that assumption/approximation seemingly holds in sedimentation velocity experiments on bovine serum albumin in

Tris–chloride buffer (Fig. 2B; see also Fig. 2B in [14]), it certainly does not apply to the same protein in sodium phosphate buffer (Fig. 2A in the current article). Even in instances where ΔA_{375} is nonzero in the macroion-free region, there must inevitably be uncertainty about the validity of using this region to obtain C_L^{α} and, hence, of quantifying the Donnan distribution of chromate at dialysis equilibrium. Thus, no additional evidence with unequivocal relevance to the determination of macroion valence is afforded by the sedimentation velocity experiment. As demonstrated above, the results obtained at 3000 rpm may be analyzed in accordance with Eq. (1) by assuming the absence of chromate binding. However, the ultracentrifuge then serves little useful purpose inasmuch as the same information could have been obtained by comparing the absorbances of the diffusate and dialyzed macroion solution in a conventional spectrophotometer.

Concluding remarks

The most important outcome of this investigation is the detection of limitations imposed by an enforced assumption inherent in the ultracentrifugal procedure for valence determination whereby sedimentation velocity is used to identify and quantify any contribution of chromate binding to the difference between concentrations of the indicator ion (chromate) in diffusate and macroion solutions at dialysis equilibrium [14]. Whereas the assumed absence of significant Donnan redistribution of small ions across the migrating polymer boundary is seemingly valid for bovine serum albumin under some conditions (Fig. 2B), this approximation does not hold under other conditions (Fig. 2A). Such disparity between findings for different counterions ($Tris^+$ and Na^+) also exposes a corresponding limitation in the Braswell procedure for valence estimation [24] that relies on the validity of the other extreme assumption—establishment of the Donnan equilibrium across the migrating boundary in a sedimentation velocity experiment on undialyzed macroion solution. Despite initial signs of promise [14,24], neither sedimentation velocity procedure provides an unequivocal solution to the problem of macroion valence determination. Although the net charge of a macroion is a fundamental parameter, the measurement of its magnitude continues to be troublesome [1]. The current problem of distinguishing between binding and Donnan distribution of the indicator ion could well be overcome by substituting ultrafiltration ([12,13]) for ultracentrifugation to effect more rapid separation of the macroion-free phase. Alternatively, it should be feasible to adapt a gel chromatographic procedure that has solved the analogous problem of obtaining binding data for macroion–metal ion interactions that include a correction for the Donnan distribution of metal ion [25].

Acknowledgments

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