

Determination of protein charge by capillary zone electrophoresis

Donald J. Winzor,^{a,*} Susan Jones,^b and Stephen E. Harding^c

^a Department of Biochemistry, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Qld. 4072, Australia

^b Department of Biochemistry, University of Sussex, Falmer, Brighton BN1 9RH, UK

^c National Center for Macromolecular Hydrodynamics, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, UK

Received 7 April 2004

Available online 24 June 2004

Abstract

The feasibility of employing classical electrophoresis theory to determine the net charge (valence) of proteins by capillary zone electrophoresis is illustrated in this paper. An outline of a procedure to facilitate the interpretation of mobility measurements is demonstrated by its application to a published mobility measurement for Staphylococcal nuclease at pH 8.9 that had been obtained by capillary zone electrophoresis. The significantly higher valence of +7.5 (cf. 5.6 from the same series of measurements) that has been reported on the basis of a “charge ladder” approach for charge determination signifies the likelihood that the latter generic approach may be prone to error arising from nonconformity of the experimental system with an inherent assumption that chemical modification or mutation of amino acid residues has no effect on the overall three-dimensional size and shape of the protein.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Capillary zone electrophoresis; Mobility–charge relationship; Protein charge; Staphylococcal nuclease

A recent review paper on experimental determination of the net charge (valence) of proteins [1] has again drawn attention to the potential of electrophoretic mobility measurements for this purpose. Despite the demise of moving boundary electrophoresis, the classical procedure for mobility determination [2], the same information can still be obtained by either gel electrophoresis [3] or capillary [4] electrophoresis. Of these two procedures, the latter can afford a direct measure of electrophoretic mobility provided that effects of electroosmosis are suppressed [5,6].

Quantitative interpretation of protein and peptide mobilities obtained by capillary zone electrophoresis to this point has been based on generic relationships for their dependence on net charge [4]. This approach has seemingly allowed the net charge (valence) of a protein to be determined solely on the basis of mobility differences stemming from alteration of the protein charge either by mutation [6] or by chemical modification [7]. The major goal of this study is to examine more closely

the reliability of valence estimates obtained by this “charge ladder” method. That endeavor entails the interpretation of mobility data in terms of classical electrophoresis theory [8,9], with which very few users of current electrophoresis protocols are familiar. Therefore, it has been deemed advisable to address specifically the manner in which those classical expressions may be applied to measurements of electrophoretic mobility—the second goal of this study.

Theoretical considerations

Classical interpretation of the electrophoretic mobility of a protein, u_A , on its valence, z_A , is based [8,9] on the expression

$$u_A = \frac{z_A e f(\kappa r_A)(1 + \kappa r_A)}{6\pi\eta_b r_A [1 + \kappa(r_A + r_b)]} \quad (1)$$

for a spherical protein with radius r_A . In Eq. (1), e denotes electronic charge (1.602×10^{-19} C), κ is the inverse screening length encountered in Debye–Hückel theory [10], and $f(\kappa r_A)$ the Henry function [11]. The inclusion of r_b , the average radius of a buffer ion, allows for the fact

* Corresponding author. Fax: +61-7-3365-4699.

E-mail address: d.winzor@mailbox.uq.edu.au (D.J. Winzor).

that $(r_A + r_b)$ is the distance of closest approach by buffer ions to the center of the spherical protein with radius r_A . η_b denotes the dynamic viscosity of the buffer medium.

For a prolate ellipsoid possessing the same volume as a sphere with radius r_A , the expression for mobility requires modification to the form

$$u_A/\psi = \frac{z_A e f(\kappa r_A)(1 + \kappa r_A)}{6\pi\eta_b r_A [1 + \kappa(r_A + r_b)]}, \quad (2)$$

where the correction factor ψ is given by the relationship

$$\psi = \frac{6[B + \ln\{(b + R_b)/b\}](1 + \kappa R_p + \kappa R_b)}{[2/(3S)]^{1/3}(1 + S)F'(\kappa b)f(\kappa R_p)(1 + \kappa R_b)}, \quad (3a)$$

where B represents the following ratio of Bessel functions $\mathbf{K}_0(\kappa b + \kappa R_b)$ and $\mathbf{K}_1(\kappa b + \kappa R_b)$

$$B = \frac{\mathbf{K}_0(\kappa b + \kappa R_b)}{\{\kappa b + \kappa R_b\}\mathbf{K}_1(\kappa b + \kappa R_b)} \quad (3b)$$

and S is the asymmetry factor [$S = a/b$]. In Eq. (3a), $F'(\kappa b)$ is a parameter that takes into account the random orientation of the prolate ellipsoid.

Experimental aspects of charge determination by electrophoresis

Measurement of mobility by capillary zone electrophoresis

In capillary electrophoresis, the migration of a protein is quantified by means of a retention time (t_A), that is, the time taken for the protein zone to migrate the fixed distance (x) from the point of sample application to the position in the capillary that is being monitored by the detector. However, this retention time may be converted to an electrophoretic mobility by the relationship

$$u_A = [(x/t_A) - (x/t_{EO})]/E, \quad (4)$$

where inclusion of the term in retention time for an uncharged marker such as mesityl oxide (t_{EO}) allows correction for the contribution of electroosmotic flow to the velocity of protein migration. In Eq. (4), E , the electric field strength, is taken as the applied potential gradient divided by the total length of the capillary across which the constant voltage is applied. Because the reliability of measurements of u_A by capillary zone electrophoresis relies heavily on the accuracy of the correction for electroosmotic flow, it is preferable to suppress the extent of the phenomenon by coating the inner wall of the capillary with a noncrosslinked polyacrylamide layer [5,6].

Calculation of the inverse screening length

The first stage in the application of Eq. (1) or Eq. (2) entails calculation of κ , the inverse screening length, which has traditionally been defined [10] as

$$\kappa = [8\pi N_A e^2 I / (1000 D k_B T)]^{1/2}, \quad (5a)$$

where N_A is Avogadro's number, e is the electron charge, D is the dielectric constant of the medium, T is the absolute temperature, and k_B is the Boltzmann constant. However, at that time, the unit of charge was the statcoulomb (3.336×10^{-10} C) and the Boltzmann constant was 1.381×10^{-16} erg K⁻¹ (rather than 1.381×10^{-23} J K⁻¹). In terms of SI units, the expression for inverse screening length needs to be written as

$$\kappa = [8\pi N_A (2.998 \times 10^9 e)^2 I / \{1000 D (10^7 k_B) T\}]^{1/2}. \quad (5b)$$

Substitution of values for the various parameters in Eqs. (5a) and (5b) reveals [9] that the magnitude of κ (m⁻¹) at 25 °C (298 K) is related to the molar ionic strength of the medium, I , by the expression

$$\kappa = 3.27 \times 10^9 \sqrt{I}. \quad (6)$$

Furthermore, the inverse screening length is relatively insensitive to temperature in that a change in T from 25 to 0 °C leaves the coefficient in Eq. (6) essentially unchanged [9].

Stokes radius of the protein

From Eq. (1), it is also evident that a magnitude needs to be assigned to the radius of the protein (r_A), a parameter taken as the Stokes radius of the solvated protein under the conditions pertaining to the environment for which u_A has been determined. The Stokes radius, r_A , may be obtained from the sedimentation coefficient and the relationship

$$r_A = M_A(1 - \bar{v}_A \rho_b) / (6\pi N_A \eta_b s_A), \quad (7)$$

where \bar{v}_A is the partial specific volume of the protein with molecular mass M_A and ρ_b is the buffer density. Alternatively, the translational diffusion coefficient, D_A , obtained by dynamic light scattering may be substituted into the expression

$$r_A = RT / (6\pi N_A \eta_b D_A) \quad (8)$$

to obtain the radius of the protein. In Eq. (8), R is the universal gas constant (8.314 J K⁻¹ mol⁻¹). Because the ionic strengths of buffers used in capillary zone electrophoresis are usually very low ($I < 0.02$ M), the density and dynamic viscosity of water (0.998 g cm⁻³ and 0.001 N s m⁻², respectively, at 20 °C) afford acceptable approximations of ρ_b and η_b . Under other circumstances, simple density and viscometry measurements can provide direct estimates of these parameters. The magnitude of \bar{v}_A (typically 0.71–0.74 cm³ g⁻¹) is readily calculated from the amino acid composition of the protein [12,13].

Empirical procedures for determining the protein radius entail calibration of gel chromatographic data (elution volumes) in terms of Stokes radius [14,15] rather than molecular mass [16,17].

Evaluation of the Henry function

The Henry function is defined [11] as

$$f(\kappa r_A) = 1 + \frac{(\kappa r_A)^2}{16} + \frac{5(\kappa r_A)^3}{48} + \frac{(\kappa r_A)^4}{96} + \frac{(\kappa r_A)^5}{96} - \exp(\kappa r_A) \left[\frac{(\kappa r_A)^4}{8} - \frac{(\kappa r_A)^6}{96} \right] + \int_0^{\kappa r_A} [\exp(-\beta^2)/\beta] d\beta, \quad (9)$$

an expression that allows the calculation of $f(\kappa r_A)$ from the dimensionless product of the inverse screening length κ (nm⁻¹) and protein radius r_A (nm). Fortunately, that rather daunting task can be sidestepped by using the approximate relationship

$$f(\kappa r_A) = 1 + \frac{0.5}{1 + \exp[d\{1 + \log(\kappa r_A)\}]}, \quad (10)$$

with $d = 2.8$ for $\kappa r_A < 10$ and 2.5 for $\kappa r_A > 10$ [3].

Allowance for protein asymmetry

Assignment of a magnitude to the asymmetry factor (axial ratio a/b), S , is the next step in the process of evaluating protein valence from electrophoretic mobility. Because b actually relates to a cylinder with the same volume as the sphere, it needs to be calculated as

$$b = [2/(3S)]^{1/3} r_A. \quad (11)$$

Whereas the axial ratio of a protein was of unknown magnitude when the theory was introduced [9], the availability of X-ray crystallographic or NMR structures for many proteins often allows this parameter to be assigned a realistic magnitude. In that regard, there is an algorithm (ELLIPSE) available for calculating axial ratios from structure coordinates [18,19].

Although the stage is now set for calculation of the charge correction factor ψ (Eqs. (3a) and (3b)), a major obstacle is the need for assignment of values to the random orientation parameter, $F'(\kappa b)$, and the term containing the ratio of Bessel functions (B). To facilitate the calculation of ψ , Figs. 1A and B present the respective dependencies of the random orientation parameter $F'(\kappa b)$ and the Bessel function term on the appropriate dimensionless products κb and $(\kappa b + \kappa r_b)$. The data for these plots have been taken from Tables 8 and 9, respectively, in [9]. Thus, estimates of $F'(\kappa b)$ and $\mathbf{K}_0(\kappa b + \kappa r_b)/[(\kappa b + \kappa r_b)\mathbf{K}_1(\kappa b + \kappa r_b)]$ may be obtained as the ordinate values in Figs. 1A and B that correspond to the previously calculated magnitudes for κb and $(\kappa b + \kappa r_b)$, respectively. An experimenter is then left with the relatively trivial task of employing Eqs. (3a) and (3b) to calculate the magnitude of ψ , which is then substituted into Eq. (2) to determine the protein valence (z_A) as the only parameter of unknown magnitude.

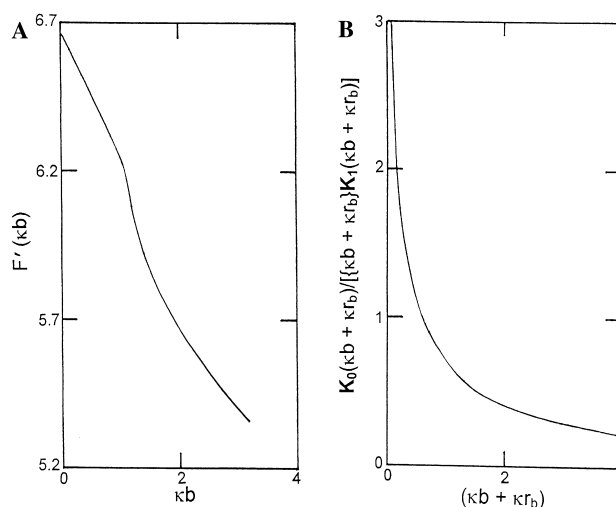


Fig. 1. Graphical aids for assigning magnitudes to (A) the random orientation parameter $F'(\kappa b)$ and (B) the ratio of Bessel functions in the application of Eqs. (3a) and (3b) to evaluate ψ and, hence, the net charge of a protein modeled as a prolate ellipsoid. Data taken from Tables 8 and 9 in [9].

Determination of the valence of Staphylococcal nuclease by capillary electrophoresis

In a study of the pH dependence of the mobility of Staphylococcal nuclease by capillary zone electrophoresis [6], the inner walls of the capillary tubes were coated with noncrosslinked polyacrylamide to eliminate electroosmotic flow and thereby render unequivocal the interpretation of retention times solely in terms of u_A . Attention is focused initially on the electrophoretic mobility of $1.31 \times 10^{-8} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1}$ obtained at pH 8.9 and 25 °C in buffer with ionic strength 0.014 M—conditions under which the enzyme exists in its tightly folded native state and also the conditions for which the valences of the various mutants are listed (Table 1 in [6]). As in [6], η_b has been taken as $0.00089 \text{ N s m}^{-2}$, the dynamic viscosity of water at 25 °C [20].

Assignment of values of $3.87 \times 10^8 \text{ m}^{-1}$ to κ (on the basis that $\kappa = 3.27 \times 10^9 \sqrt{I}$ [9]) and 2.1 nm to r_A (the gel chromatographically deduced Stokes radius of the nuclease [21] as well as the value inferred from its radius of gyration [22]) leads to magnitudes of 0.812 and 0.097 for κr_A and κr_b (where r_b is taken as 0.25 nm [9]), respectively. Substitution of these parameters and the corresponding value of 1.023 for $f(\kappa r_A)$ (Eq. (10)) yields an estimate (via Eq. (1)) of +4.9 for the valence (z_A) of a spherical Staphylococcal nuclease molecule under these conditions (pH 8.9, I 0.014 M, 25 °C). This estimate is a minimal value inasmuch as its determination is based on consideration of the enzyme as a sphere ($\psi = 1$ in Eq. (2)).

Application of the algorithm for calculating axial ratios from structural coordinates [18,19] to the listing

for Staphylococcal nuclease in the SwissProt Data Base signifies that a prolate ellipsoid with axial ratio (a/b) of 1.31 provides a better model of the enzyme. From Eq. (11), the minor semi-axis (b) of the prolate ellipsoid is 1.68 nm, whereas $\kappa b = 0.650$ and $(\kappa b + \kappa r_b) = 0.747$. Reference to Fig. 1 then yields values of 0.650 for $F'(\kappa b)$ (Fig. 1A) and 0.747 for $\mathbf{K}_0(\kappa b + \kappa r_b)/[(\kappa b + \kappa r_b)\mathbf{K}_1(\kappa b + \kappa r_b)]$ (Fig. 1B). On the basis of the consequent correction factor ψ (Eqs. (3a) and (3b)) of 0.88, the estimate of z_A increases to +5.6 for this model of Staphylococcal nuclease.

The net charge (valence) that emanates from consideration of the mobility at pH 8.9 in terms of classical electrophoresis theory [8,9] is smaller than the original estimate [6] of +7.5 that was obtained by the charge ladder method. Furthermore, a similar situation applies to the two estimates of the nuclease valence under other conditions—a factor evident from Table 1, which summarizes results for the compact form of the enzyme (first three entries) as well as for the “swollen” state at pH 4.1 (final entry). Although both estimates of charge are obviously monitoring the same variation in z_P with pH, discrepancies between the two persist for all four conditions. A potential source of error in the current estimates is their reliance on the premise that the three-dimensional shape deduced by X-ray crystallography also applies to the nuclease in solution. On the other hand, the valences determined by the charge ladder approach are obtained on the basis that mutation of an amino acid has no effect on the size and shape of the enzyme. A possible indicator of the weakness of that assumption is the observation of variations in the mobilities of mutants with the same charge. For example, mobilities of $1.28 \times 10^{-8} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1}$ (cf. $1.31 \times 10^{-8} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1}$) were obtained for four of the six mutants with unaltered charge at pH 8.9, and there were also mobility variations among the sets of mutants differing in charge by +1 and +2 from the wild-type nuclease (Table 4 in [6]). Indeed, these discrepancies were attributed [6] to differences in the tertiary fold of the mutants, a situation at variance with the fundamental assumption on which the charge ladder method is based.

An observation that may be construed as signifying overestimation of z_A by the charge ladder method is the consequent calculation (from Eq. (7) and Fig. 8 in [6]) of a Stokes radius of 2.5 nm (rather than 2.1 nm) for the nuclease; at pH 8.9, a charge of +5.3 spread uniformly over a sphere with a radius of 2.1 nm would infer the same surface charge density as would that for the larger sphere ($r_A = 2.5 \text{ nm}$) with $z_A = +7.5$.

Concluding remarks

This study has illustrated the feasibility of employing classical electrophoresis theory [8,9] to determine the net charge (valence) of proteins by capillary zone electrophoresis, an endeavor that requires specification of the size as well as the shape of the protein. In that regard, it is seemingly less acceptable than the charge ladder approach to valence determination by capillary zone electrophoresis [6,7], which requires no such information. However, the above comparison signifies the possibility that values obtained by the charge ladder approach may be prone to error arising from failure of the experimental system to comply rigorously with the inherent assumption that chemical modification [7] or mutation [6] of amino acid residues has no effect on the overall three-dimensional size and shape of the protein.

From the biological viewpoint, the use of capillary electrophoresis for protein valence determination is currently of limited utility due to buffer selection on the basis of resolving power rather than physiological considerations. Thus, protein mobilities tend to be measured in buffers with very low ionic strength ($I < 0.03 \text{ M}$), whereupon the relevance of the valence estimate to the state of the protein in the chloride-rich biological environment ($I \approx 0.15 \text{ M}$) is uncertain. For a purified protein, there is in principle no impediment to the conduct of capillary electrophoresis under the latter conditions despite the slower migration and greater spreading of the zone at higher ionic strength. Indeed, the corresponding adaptation of polyacrylamide gel electrophoresis has already been demonstrated [23].

Table 1

Comparison of valence estimates for Staphylococcal nuclease obtained by the current approach and the “charge ladder” approach

pH	I (M)	$10^8 u_P$ ($\text{m}^2 \text{ V}^{-1} \text{ s}^{-1}$)	r_A (nm)	ψ	Protein valence z_P	
					From Eq. (2)	From [6]
8.9	0.014	1.31	2.1 ^a	0.88	+5.6	+7.5
6.8	0.026	1.81	2.1 ^a	0.80	+9.5	+12.9
5.7	0.036	1.85	2.1 ^a	0.63	+13.2	+14.8
4.1	0.055	2.04	2.7 ^b	0.76 ^c	+19.6	+20.6

Note. Experimental data were taken from Table 4 in [6].

^a Stokes radius of the compact form of the nuclease [22,23].

^b Deduced from the radius of gyration of 2.1 nm [23] for the swollen state of the enzyme.

^c Calculated on the basis of the same asymmetry factor ($S = 1.31$) and the consequent value of 2.16 nm for b .

Although the initial impetus for the determination of protein valence was undoubtedly the academic challenge, the requirement of a magnitude for the net charge of a protein in the assessment of thermodynamic nonideality effects on protein interactions [24–28] has rekindled interest in the problem. On the grounds that quantitative allowance for effects of thermodynamic nonideality in the concentrated biological environment is a prerequisite for complete understanding of physiological control at the molecular level [29,30], the determination of protein valence becomes a matter of some urgency. The demise of the moving boundary apparatus [2] has left the capillary technique as the main procedure for direct measurement of electrophoretic mobility and, hence, protein valence by this means. It is hoped that this study may arouse further interest in the use of capillary zone electrophoresis for the measurement of protein valence and, hence, provide stimulus for the more detailed studies required to resolve the issues raised by the current observations.

Acknowledgment

The financial support of the Biotechnology and Biological Science Research Council (United Kingdom) is gratefully acknowledged.

References

- [1] D.J. Winzor, Determination of the net charge (valence) of a protein: a fundamental but elusive parameter, *Anal. Biochem.* 325 (2004) 1–20.
- [2] A. Tiselius, A new apparatus for electrophoretic analysis of colloidal mixtures, *Trans. Faraday Soc.* 33 (1937) 524–531.
- [3] D. Rodbard, A. Chrambach, Estimation of molecular radius, free mobility, and valence using polyacrylamide gel electrophoresis, *Anal. Biochem.* 40 (1971) 95–134.
- [4] P.D. Grossman, J.C. Colbourn (Eds.), *Capillary Electrophoresis: Theory and Practice*, Academic Press, San Diego, 1992.
- [5] S. Hjertén, High-performance electrophoresis: elimination of electroosmosis and solute sorption, *J. Chromatogr.* 347 (1985) 191–198.
- [6] F. Kálmán, S. Ma, R.O. Fox, C. Horváth, Capillary electrophoresis of *S. nuclease* mutants, *J. Chromatogr. A* 705 (1995) 135–154.
- [7] J. Gao, F.A. Gomez, R. Härter, G.M. Whitesides, Determination of the effective charge of a protein in solution by capillary electrophoresis, *Proc. Natl. Acad. Sci. USA* 91 (1994) 12027–12030.
- [8] H.A. Abramson, M.H. Gorin, L.S. Moyer, The polar groups of proteins and amino acid surfaces in liquids, *Chem. Rev.* 24 (1939) 345–366.
- [9] H.A. Abramson, L.S. Moyer, M.H. Gorin, *Electrophoresis of Proteins*, Reinhold, New York, 1942.
- [10] P. Debye, E. Hückel, Theory of electrolytes: I. Lowering of freezing point and related phenomena, *Phys. Z.* 24 (1923) 185–206.
- [11] D.C. Henry, The cataphoresis of suspended particles: I. The equation of cataphoresis, *Proc. R. Soc. Lond. A* 133 (1931) 106–129.
- [12] E.J. Cohn, J.T. Edsall, *Proteins, Amino Acids, and Peptides as Dipolar Ions*, Reinhold, New York, 1943.
- [13] T.M. Laue, B.D. Shah, T.M. Ridgeway, S.L. Pelletier, in: S.E. Harding, A.J. Rowe, J.C. Horton (Eds.), *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, Royal Society of Chemistry, Cambridge, UK, 1992, pp. 90–125.
- [14] J.C. Laurent, J. Killander, A theory of gel filtration and its experimental verification, *J. Chromatogr.* 14 (1964) 317–330.
- [15] G.K. Ackers, A new calibration procedure for gel filtration columns, *J. Biol. Chem.* 242 (1967) 3237–3238.
- [16] J.R. Whitaker, Determination of molecular weights of proteins by gel filtration on Sephadex, *Anal. Chem.* 35 (1963) 1950–1953.
- [17] P. Andrews, Estimation of protein molecular weights by Sephadex gel filtration, *Biochem. J.* 91 (1964) 222–233.
- [18] W.R. Taylor, J.M. Thornton, R.J. Turnell, An ellipsoidal approximation of protein shape, *J. Mol. Graph.* 1 (1983) 30–38.
- [19] S.E. Harding, J.C. Horton, S. Jones, J.M. Thornton, D.J. Winzor, COVOL: an interactive program for evaluating second virial coefficients from the triaxial shape or dimensions of rigid macromolecules, *Biophys. J.* 76 (1999) 2432–2438.
- [20] J.A. Dean, *Lange's Handbook of Chemistry*, 13th ed., Marcel Dekker, New York, 1986.
- [21] D. Shortle, A.K. Meeker, Mutant forms of Staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation, *Proteins: Struct. Funct. Genet.* 1 (1986) 81–89.
- [22] J.M. Flanagan, M. Kataoka, D. Shortle, D.M. Engelman, Truncated Staphylococcal nuclease is compact but disordered, *Proc. Natl. Acad. Sci. USA* 89 (1992) 748–752.
- [23] L.D. Ward, D.J. Winzor, Adaptation of gel electrophoresis for the quantitative study of specific ion binding: interaction of phosphate with ovalbumin, *Arch. Biochem. Biophys.* 209 (1981) 650–654.
- [24] P.R. Wills, D.J. Winzor, Thermodynamic nonideality and sedimentation equilibrium, in: S.E. Harding, A.J. Rowe, J.C. Horton (Eds.), *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, Royal Society of Chemistry, Cambridge, UK, 1992, pp. 311–330.
- [25] P.R. Wills, M.P. Jacobsen, D.J. Winzor, Direct analysis of solute self-association by sedimentation equilibrium, *Biopolymers* 38 (1996) 119–130.
- [26] J. Behlke, O. Ristau, Analysis of the thermodynamic nonideality of proteins by sedimentation equilibrium experiments, *Biophys. Chem.* 76 (1999) 217–225.
- [27] P.R. Wills, D.R. Hall, D.J. Winzor, Interpretation of thermodynamic nonideality in sedimentation equilibrium experiments on proteins, *Biophys. Chem.* 84 (2000) 217–225.
- [28] P.R. Wills, D.J. Winzor, Studies of solute self-association by sedimentation equilibrium: allowance for effects of thermodynamic nonideality beyond the consequences of nearest-neighbor interactions, *Biophys. Chem.* 91 (2001) 253–262.
- [29] S.B. Zimmerman, A.P. Minton, Macromolecular crowding: biochemical, biophysical, and physiological consequences, *Annu. Rev. Biophys. Biomol. Struct.* 22 (1993) 27–65.
- [30] A.P. Minton, The influence of macromolecular crowding and molecular confinement on biochemical reactions in physiological media, *J. Biol. Chem.* 276 (2001) 10577–10580.