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Examination of the Discrepancy between Size Estimates for Ovalbumin from Small-Angle X-ray Scattering and Other Physicochemical Measurements

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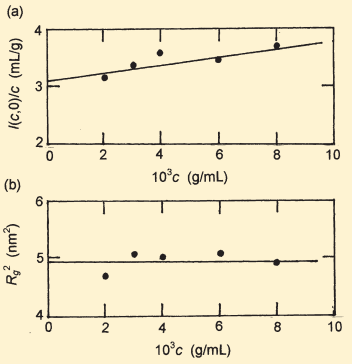
ABSTRACT: Examination of the solution behavior of ovalbumin by small-angle X-ray scattering, dynamic light scattering, and analytical ultracentrifugation methods confirms its existence as a 44-kDa monomer in 20 mM phosphate, pH 7.0, thereby contradicting the discord introduced by published SAXS studies in favor of a dimeric state for this protein at neutral pH. Although the theoretical interpretation of SAXS measurements considers the consequences of thermodynamic nonideality arising from the repulsive interactions between molecules only if they give rise to a positive second virial coefficient, the fact that A_2 is negative for the present system does not account for the earlier findings.



1. INTRODUCTION

Until the final decade of the 20th century, the macromolecular state of ovalbumin in aqueous buffer solution was not in dispute. Measurements of its molecular weight by the traditional procedures of osmometry,[1] static light scattering,[2] and analytical ultracentrifugation[3−6] had established the existence of ovalbumin as a 42−46-kDa monomeric species well before a calculated molecular mass of 43 kDa emerged from the amino acid sequence of the single polypeptide chain:[7] that value increases to 44 kDa after account is taken of the octasaccharide sequence (six mannose and two *N*-acetylglucosamine residues) that is attached to Asn-292. Indeed, the inclusion of ovalbumin as the 43-kDa marker protein in calibration kits for empirical methods of molecular weight determination is testimony to its monomeric status over a wide range of pH values and ionic strengths.

On the other hand, a contrary viewpoint has emerged from a series of small-angle X-ray scattering (SAXS) measurements by Matsumoto and co-workers,[8−10] who proposed a dimeric state for ovalbumin in 0.5−300 mM phosphate buffers (pH 7.0) on the basis of the radius of gyration inferred from Guinier analysis.[11] Recent confirmation of that radius of gyration for the protein in 150 mM NaCl[12] by the same method has prompted this examination of the conflict between conclusions from SAXS measurements and those from other physicochemical procedures about the molecular state of ovalbumin at neutral pH.

2. EXPERIMENTAL SECTION

2.1. Materials. Commercial preparations of ovalbumin from Sigma-Aldrich (grade V A5503) and Fischer Scientific (BP2535) were both supplied as salt-free lyophylized powders with a nominal purity of 98%, the description accorded the Sigma-Aldrich preparations used in the published SAXS studies of this protein.[8−10,12] A stock solution of the Fischer Scientific ovalbumin preparation (10 mg/mL) in phosphate buffer, pH 7.0, I = 0.044 M (0.012 M Na_2HPO_4−0.008 M NaH_2PO_4) was subjected to centrifugal filtration through a 0.1-μm filter (Millipore) before appropriate dilution with more of the same buffer to obtain ovalbumin solutions with concentrations in the range of 0.5−10 mg/mL.

2.2. Dynamic Light Scattering. In accordance with the procedure described previously,[13] measurements of the Stokes radius of ovalbumin were obtained with a Nano-S dynamic light scattering system (Malvern Instruments Ltd., Malvern, U.K.) that employs a 633-nm laser and a fixed scattering angle (173°) that is sufficiently high to justify neglect of the contribution from rotational diffusion effects to the autocorrelation files. Traditional analysis thus yields the translational diffusion coefficient[14] and, hence, through the Stokes−Einstein relationship, the Stokes

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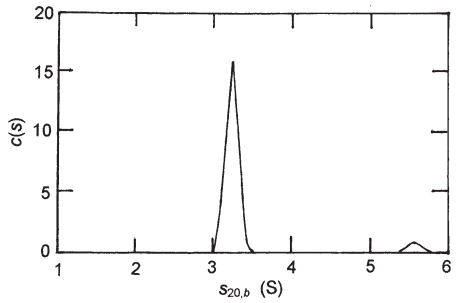

Figure 1. Sedimentation coefficient distribution obtained by SEDFIT analysis[15,16] of Rayleigh interference records of a sedimentation velocity experiment (50000 rpm) on a 1 mg/mL solution of the Sigma-Aldrich preparation of ovalbumin in 20 mM phopshate buffer, pH 7.0.

radius R, the parameter that is calculated by the software supplied with the instrument.

2.3. Analytical Ultracentrifugation. Sedimentation velocity experiments were conducted at 20 °C and 50000 rpm in a Beckman XL-I analytical ultracentrifuge. Rayleigh interference records of the resulting concentration distributions were analyzed by the SEDFIT program[15,16] to obtain $c(s)-s$ distributions, as well as the sedimentation and diffusion coefficients for ovalbumin in phosphate buffer (pH 7.0, I = 0.044 M).

2.4. Small-Angle X-ray Scattering. SAXS data were obtained with a Rikagu instrument described previously[13] that employs an X-ray beam with a wavelength (λ) of 1.54 Å. Scattering intensities at angle 2θ, $I(c,q)$], where $q = 4\pi(\sin\theta)/\lambda$ is the scattering vector, were subjected to Guinier analysis

$$\ln I(c,q) = \ln I(c,0) - \frac{R_g^2 q^2}{3} \qquad (1)$$

for $0.008 \le q \le 0.10$ Å^{-1} to determine estimates of the radius of gyration (R_g) from experiments on ovalbumin with concentrations in the range $0.002 \le c \le 0.008$ g/mL. In addition, linear extrapolation of the same normalized data sets to zero scattering angle was used to determine $I(c,0)$ for each protein concentration and, hence, to evaluate of the light scattering second virial coefficient (A_2) from the expression[17−19]

$$I(c,0) = \frac{I(0,0)}{1 + 2A_2Mc + \ldots} \approx I(0,0)(1 - 2A_2Mc) \qquad (2)$$

by nonlinear least-squares analysis to obtain best-fit estimates of A_2M and $I(0,0)$ as curve-fitting parameters.

3. RESULTS AND DISCUSSION

We begin this examination of the macromolecular state of ovalbumin at neutral pH with characterization of its solution behavior by dynamic light scattering and sedimentation velocity, the latter being chosen to assess the homogeneity of the preparation with respect to molecular size. Such a check on sample purity is a prerequisite for SAXS measurements, the interpretation of which relies heavily on the presumed absence of any aggregated protein.

3.1. Macromolecular State and Purity of Ovalbumin. To check the purity of commercial ovalbumin, a preparation obtained from Sigma-Aldrich, who supplied the material used in the earlier SAXS studies,[8−10,12] was subjected to analytical ultracentrifugation at 50000 rpm. The sedimentation coefficient distribution obtained by SEDFIT[15,16] for a 1 mg/mL solution in 20 mM phosphate buffer, pH 7.0, is presented in Figure 1, which signifies the presence of a minor contaminant ($s_{20,b} \approx 5.8$ S), as well as the predominant 3.3 S species. On the grounds that this analysis is consistent with the manufacturer's specification of 98% for the purity of the preparation, it seems probable that a similar situation pertains to other commercial ovalbumin preparations for which the same level of purity is specified.

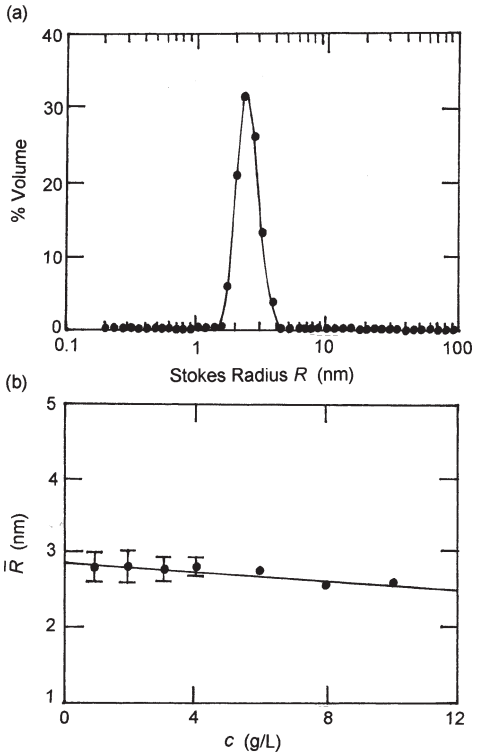

Figure 2. Check on the macromolecular state of the Fischer Scientific preparation of ovalbumin by dynamic light scattering. (a) Apparent size distribution for a 10 g/L solution of ovalbumin in 20 mM phosphate buffer, pH. 7.0. (b) Concentration dependence of the mean hydrated radius.

Table 1. Summary of Effective Radii (R) Deduced from Published Hydrodynamic and Thermodynamic Measurements

measured parameter	conditions	R (nm)	ref(s)
sedimentation coefficient	pH 7.5, I = 0.10	3.00	5
diffusion coefficient		2.94	20, 21
diffusion coefficient	pH 4.6, I = 0.16	2.97 (±0.05)	22
diffusion coefficient	pH 7.5, I = 0.16	2.89	23
second virial coefficient[a]	pH 7.5, I = 0.10	2.92	6
second virial coefficient[b]	pH 4.6, I = 0.20	2.84	24
second virial coefficient[a]	pH 8.4, I = 0.16	2.80	25
second virial coefficient[a]	pH 4.6, I = 0.10	2.88	26

[a] Obtained by sedimentation equilibrium. [b] Obtained by size exclusion chromatography.

The results of a dynamic light scattering experiment on a 10 mg/mL solution of ovalbumin in phosphate buffer (pH 7.0, I = 0.044 M) are presented in Figure 2a and provide a reasonably well-defined estimate of 2.85 nm for the apparent Stokes radius. Repetition of such experiments at a series of concentrations

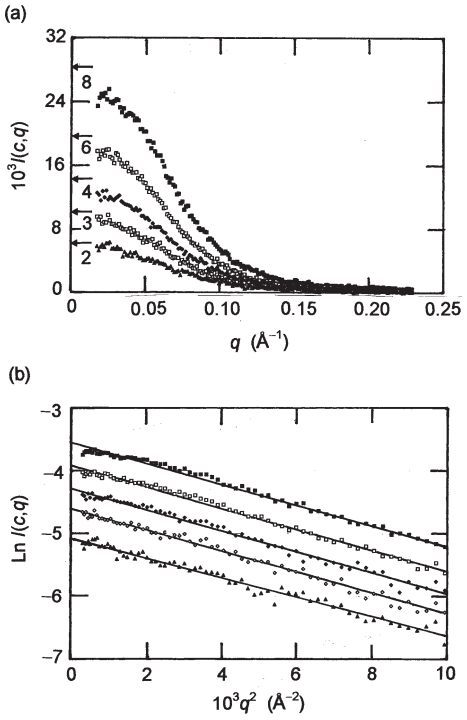

Figure 3. Angular dependence of scattering in SAXS measurements on the indicated concentrations (g/L) of the Fischer Scientific preparation of ovalbumin in 20 mM phosphate buffer, pH 7.0. (a) Intensity, $I(c,q)$ as a function of the scattering vector q. (b) Replot of the results in Guinier format (eq 1). The horizontal arrows in panel a denote the values of $I(c,0)$ inferred from the ordinate intercepts in panel b.

yielded an estimated Stokes radius of 2.88 (±0.06) nm from the ordinate intercept of the concentration dependence of the mean hydrated radius $\overline{R}$ (Figure 2b). This finding compares favorably with values deduced from published studies of ovalbumin by hydrodynamic and thermodynamic studies (Table 1) and thus confirms the conclusion drawn from Figure 1 that the 44-kDa monomeric species is the predominant component of commercial preparations of this protein. In that regard, it should be noted that the level of ovalbumin contamination indicated by the sedimentation coefficient distribution in Figure 1 would give rise to a disparity of only 0.02 nm in the mean Stokes radius from that of monomer, a difference encompassed by the experimental estimate of $\overline{R}$.

Having reconfirmed the monomeric state of ovalbumin at neutral pH, we proceeded with a SAXS study under the same conditions to check published reports of a dimeric state for ovalbumin from such measurements.[8−10,12]

3.2. Consideration of SAXS Data for Ovalbumin. Results of SAXS measurements for the Fischer Scientific preparation of ovalbumin in 20 mM phosphate buffer (pH 7.0) are presented as the dependence of intensity on scattering vector q (Figure 3a) and also as Guinier plots (Figure 3b) to facilitate estimation of $I(c,0)$ from their logarithmic counterparts, the ordinate intercepts in Figure 3b: those estimates of $I(c,0)$ are denoted by horizontal arrows in Figure 3a. Their analysis according to eq 2 is presented in Figure 4a, where the positive slope signifies a negative value of the light scattering second virial coefficient,[17−19] the estimate of $-2\,(\pm 1) \times 10^{-4}$ ml mol g^{-2} for A_2 being comparable to that obtained for isoelectric ovalbumin at a similar ionic strength.[27] Although a negative second virial

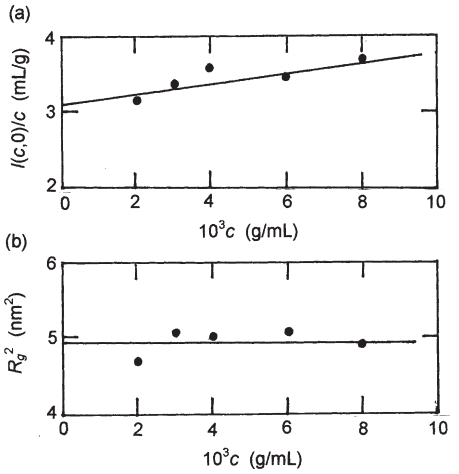

Figure 4. Evaluation of the light scattering second virial coefficient (A_2) and radius of gyration (R_g) for ovalbumin in 20 mM phosphate buffer, pH 7.0. (a) Concentration dependence of the normalized scattering intensity at zero angle (see eq 2). (b) Corresponding dependence of R_g^2 inferred from the slopes of Guinier plots.

coefficient can be indicative of protein self-association, the results presented in Figures 1 and 2 preclude that possibility: it must therefore reflect dominance of the negative protein−buffer contribution to A_2 over that arising from the necessarily positive second virial coefficient for protein self-interaction.[27−29]

Evaluation of A_2 allows calculation of the extent of thermodynamic nonideality prevailing in the SAXS experiments. Specifically, nonideality is incorporated into SAXS analysis through the structure function, $S(c,0)$, defined as

$$S(c,0) = \frac{I(c,0)/c}{I(0,0)} = \frac{1}{1 + 2A_2Mc + \ldots} \qquad (3)$$

which is clearly a rearranged form of eq 2. On the grounds that these calculated estimates of $S(c,0)$ vary only between 1.04 and 1.14 for the present experiments with $0.002 \le c \le 0.008$ g/mL, it is assumed that no serious error in the value of R_g should arise from traditional Guinier analysis (eq 1) because of its implicit assumption of a value of unity for $S(c,0)$. Conformity with this assumption is evident from Figure 4b, which summarizes the concentration dependence of the apparent values of R_g^2 deduced from the slopes of the Guinier plots (eq 1). In the absence of any significant concentration dependence, the individual values were averaged to obtain an estimate of 4.92 (±0.16) nm^2 for R_g^2. The consequent value of 2.22 (±0.4) nm for the radius of gyration corresponds to a Stokes radius of 2.86 (±0.5) nm for ovalbumin, which agrees well with the estimates obtained from the dynamic light scattering measurements (see above). This conclusion is not altered by adopting the viewpoint[17] that the slope of a Guinier plot defines $R_g^2/(1 + 2A_2Mc)$, in which case the estimate of the Stokes radius increases to 2.98 nm—a value still within the range reported in Table 1. We therefore conclude that a monomeric state for ovalbumin is also indicated by the SAXS measurements.

Although we have achieved the desired consistency between size estimates for ovalbumin by SAXS and other traditional procedures, these findings necessitate the exploration of possible reasons for the discrepancies that prompted this investigation. Heterogeneity with respect to molecular size of the commercial preparation of ovalbumin (Sigma-Aldrich grade V) used by

Matsumoto and Chiba[8] is seemingly precluded by their determination of a molecular mass of 43.7 kDa from the ordinate intercept of a Debye plot (Figure 2 of Matsumoto and Chiba[8]), a value in good agreement with their estimate of 44 kDa for the subunit size by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Because the molar mass obtained by static light scattering is a weight-average quantity, any gross contamination of the protein preparation by aggregates would have been manifested as an estimated molar mass considerably larger that of the monomer. However, the presence of a minor contaminant, as in the current Sigma-Aldrich preparation of ovalbumin (Figure 1) would not have been detected by the Debye plot, where (as in the present Figure 2) any change in ordinate intercept from that for the monomer would have been within the envelope of experimental uncertainty inherent in the estimate of $1/M$. No check of protein purity was made in the later SAXS study of ovalbumin.[12]

The static light scattering data reported by Matsumoto and Chiba[8] also signified a negative second virial coefficient for ovalbumin in 20 mM phosphate buffer (pH 7), but the estimate of -100×10^{-4} mL mol g^{-2} for A_2 inferred from that Debye plot is 50-fold higher than the present value. In that regard, the high value is predicated on an upper limiting protein concentration of 1 g/L in the Debye plot (Figure 2 of Matsumoto and Chiba[8]), which seems rather low for a static light scattering study of that vintage. Nevertheless, the light scattering second virial coefficient is unquestionably negative; however, as noted above, a lack of allowance for effects of thermodynamic nonideality in those circumstances leads to only a slight underestimation of molecular size. Consequently, the reported overestimates of R_g^2 by Guinier analysis of their SAXS measurements did not arise from the neglect of thermodynamic nonideality effects. We are thus left with an unresolved inconsistency between the present and earlier findings.

3.3. Detailed Analysis of SAXS Results Reflecting Negative Virial Coefficients. The determination of a negative light scattering second virial coefficient reflecting protein−buffer interactions precludes extension of the SAXS analysis to include the more detailed treatment applied to bovine serum albumin[31] and the recent study of ovalbumin[12] in accordance with the relationship[32]

$$I(c,q) = (N_A/M)c(\Delta\rho)^2V^2P(q)\,S(c,q) \tag{4}$$

N_A is Avogadro's number, M is the molar mass of the protein, and V is its molecular volume: the scattering contrast $(\Delta\rho)$ is the scattering by the solution corrected for that of solvent, and $P(q)$ is the form factor, which takes into account the scattering from a single protein molecule after orientational averaging. Although the magnitude of $S(c,q)$, the structure factor that takes into account the effects of thermodynamic nonideality, is a function of shape as well as size, it is invariably calculated on the basis of the mean spherical approximation whereby the protein molecule is assigned an effective radius R for a sphere with the same molecular volume V—an approximation necessitated by the unavailability of statistical-mechanical expressions for other shapes of molecules bearing net charge. It is the calculation of $S(c,q)$ that presents problems.

Allowance for the effects of thermodynamic nonideality in the molecular interpretation of SAXS data for proteins under conditions of moderate ionic strength ($I < 0.3$ M) has been based on the assumption that the potential of mean force between two nonassociating protein molecules separated by distance r is described adequately by the expression[31,33,34]

$$U(r) = U_{HS}(r) + U_{SC}(r) \tag{5}$$

where $U_{HS}(r)$ is the hard-sphere potential related to the excluded-volume effect and $U_{SC}(r)$ is the screened Coulomb potential. Such action follows McMillan−Mayer theory[35] for the statistical-mechanical interpretation of nonideality in terms of the virial coefficients for self-interaction that appear in the classical expansion of osmotic pressure as a power series in molar concentration c, of protein, namely

$$\frac{\Pi}{RT} = \frac{c}{M} + B_{22}\left(\frac{c}{M}\right)^2 \tag{6}$$

B_{22} is the molar osmotic second virial coefficient for protein self-interaction, the statistical-mechanical interpretation of which leads to the following approximate description in terms of effective radius R and net charge Z[36]

$$B_{22} = \frac{16\pi N_A R^3}{3} + \frac{Z^2(1 + 2\kappa R)}{4I(1 + \kappa R)^2} - \frac{1000Z^4\kappa^3}{128\pi N_A I^2(1 + \kappa R)^4} + \ldots \tag{7}$$

where κ, the Debye−Hückel inverse screening length (cm^{-1}), can be calculated from the ionic strength I as $3.27 \times 10^7\sqrt{I}$. Inasmuch as eq 7 provides a means of calculating the osmotic second virial coefficient only in situations where the series converges, B_{22} is necessarily positive.

The problem with this approach to the treatment of thermodynamic nonideality in SAXS studies is the omission from eq 3 of any allowance for a negative contribution to the potential of mean force from protein−cosolute effects. On the grounds that $U_{HS}(r)$ and $U_{SC}(r)$ both reflect repulsive interactions between protein molecules and hence dictate a positive value for B_{22}, the experimental determination of light scattering second virial coefficients (A_2) for buffered protein solutions that are negative signifies that the protein−cosolute contribution to the potential of mean force must be the dominant term. Although neglect of this contribution in eq 5 can certainly be accommodated by adding another term to cover protein−buffer interactions, there are no statistical-mechanical counterparts of eq 7 to describe thermodynamic nonideality reflecting the negative deviations from Raoult's law exhibited by ions (buffer salts, electrolytes). In other words, the description of thermodynamic nonideality on the statistical-mechanical basis of excluded volume can be applied only to species (proteins and uncharged solutes) for which nonideality gives rise to positive deviations from Raoult's law.

Inasmuch as failure to take into account any attractive contributions to the potential of mean force between protein molecules must inevitably lead to error in calculated magnitudes of the structure factor, $S(c,q)$, an alternative approach might be to substitute values based on experimental estimates of the second virial coefficient (A_2). Indeed, there seems to be no alternative for systems with a dominant protein−buffer contribution to nonideality. Furthermore, the reliability of detailed SAXS structural analysis would be enhanced considerably by experimental confirmation of the calculated second virial coefficient even in situations where A_2 is positive: SAXS measurements reflecting significant but not dominant nonideality contributions from

protein−buffer interactions would then be recognized by the calculation of an osmotic second virial coefficient (B_{22}) that overestimates the light scattering second virial coefficient (A_2).

4. CONCLUDING REMARKS

This reinvestigation of the macromolecular state of ovalbumin by small-angle X-ray scattering and hydrodynamic methods has yielded consistent evidence for its existence as the monomeric 44-kDa species at neutral pH and, thereby, has been unable to reproduce the discordant conclusion in favor of a dimeric state that has emerged from earlier SAXS studies of the protein.[8−10,12] Attention has also been drawn to a deficiency in the current procedure for incorporating the effects of thermodynamic nonideality into the analysis of SAXS measurements because of its misidentification of the light scattering second virial coefficient (A_2) as its osmotic counterpart for protein self-interaction (B_{22}). Inability to calculate the missing protein−buffer contribution to A_2 has led to the recommendation that greater attention be paid to the experimental measurement of the light scattering second virial coefficient as part of any future SAXS study—not only to allow recognition of situations where detailed analysis of SAXS measurements is precluded by dominance of the protein−buffer contribution (A_2 negative) but also to verify the insignificance of its contribution to systems for which the measured light scattering virial coefficient is positive.

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