Light-scattering from detergent-complexed biological macromolecules

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1. A theory is described for Rayleigh light-scattering from solutions of detergent-complexed macromolecules applicable to measurements carried out under conditions of Donnan equilibrium. 2. The theory shows that when scattering measurements are made on detergent-solubilized macromolecules in the presence of detergent micelles the apparent M_r is dependent on the extent of detergent binding and effective charge on the detergent-macromolecule complex and the micellar charge and aggregation number. 3. Equations are given for the apparent M_r of the macromolecule under limiting conditions of high salt and low salt concentration. 4. Low-angle laser-light-scattering measurements were made on lysozyme complexed with sodium n-dodecyl sulphate both in the absence and in the presence of detergent micelles. 5. These experimentally obtained data were used in conjunction with the detergent-binding isotherm to test the theory at high ionic strength. 6. Light-scattering measurements were also made on detergent-saturated complexes as a function of ionic strength and pH. 7. The results are in reasonable accord with both the qualitative and the quantitative predictions of the theory.

Light-scattering is among the few physical techniques that can be used for determinations of absolute M_r values of macromolecules. The determination of the M_r values of very hydrophobic macromolecules, e.g. membrane proteins or glycoproteins, which aggregate or are insoluble in aqueous media, are usually obtained by relative methods such as polyacrylamide-gel electrophoresis in the presence of a solubilizing detergent. Such estimations give only the M_r values of reduced polypeptide chains relative to standards, and under some circumstances can give incorrect results (Maddy, 1976).

In principle the light-scattering technique can be applied to macromolecules solubilized or complexed by detergents provided that the weight concentration of the uncomplexed macromolecule is known and that both light-scattering and refractive-index measurements are made relative to solvent in Donnan equilibrium with the solubilized solute (Eisenberg, 1976). Recently this approach has been tested with globular proteins complexed with SDS (Takagi *et al.*, 1980; Midgley & Jones, 1982) and applied to membrane proteins (Miyake & Takagi, 1981; Kameyama *et al.*, 1982). We have

found, however, that when the free detergent concentration in equilibrium with a protein-detergent complex exceeds the critical micelle concentration (c.m.c.) of the detergent the measured M_r values are very seriously in error. We report these observations together with a theoretical approach to the scattering of light from detergent-solubilized macromolecules in the presence of detergent micelles.

Theory

The coherent scattering from charged multicomponent macromolecular systems was first considered by Zernike (1918), and numerous extensions to this initial treatment have been made (see, e.g., Mysels, 1955; Vrij & Overbeek, 1962). For a macromolecule of concentration c_1 (molecules per unit volume) in Donnan equilibrium with small- M_r components of chemical potential μ_s , the excess Rayleigh factor R_θ over that of the Donnan solution in the absence of solute 1 can be written (Vrij & Overbeek, 1962):

$$R_\theta = \frac{2\pi^2 n_0^2}{N\lambda^4} RT \frac{\left[\sum_{i=2} \psi_i (\partial c_i / \partial c_1)_{\mu_s, T} \right]^2}{(\partial \pi / \partial c_1)_{\mu_s, T}} c_1 \quad (1)$$

Abbreviation used: SDS, sodium n-dodecyl sulphate.

where λ is the wavelength of light, R the gas constant, T the absolute temperature and n_0 the solvent refractive index. π is the osmotic-pressure difference between the macromolecule solution and Donnan solution in equilibrium with it (hereinafter called the Donnan 'solvent'). The summa-

tioned that the micelle-monomer equilibrium ensures that the micelles are effectively a diffusible species.

The osmotic-pressure difference between solution of complex and Donnan 'solvent' is then given by:

$$\pi = RT[m_c + m_c p_c + m_m + m_m p_m + 2x - (m'_m + m'_m p_m + 2y)] \quad (3)$$

tion in eqn. (1) is over the product of the refractive index increments (ψ_i) for each diffusible compon-

Differentiation of eqn. (3) with respect to complex concentration m_c leads to:

$$\left(\frac{\partial \pi / RT}{\partial m_c}\right) = 1 + p_c + (1 + p_m) \left[\left(\frac{dm_m}{dm_c}\right) - \left(\frac{dm'_m}{dm_c}\right) \right] + 2 \left[\left(\frac{dx}{dm_c}\right) - \left(\frac{dy}{dm_c}\right) \right] \quad (4)$$

ent and $(\partial c_i / \partial c_1)_{\mu_s, T}$, which represents the adsorption of diffusible species (i) on the macromolecule. To a reasonable approximation the summation can be replaced by the refractive-index increment

The derivatives of x and y with respect to m_c can be obtained from the Donnan relation (eqn. 2) since at equilibrium the differential of each side is zero, so that it can be shown that eqn. (4) becomes:

$$\begin{aligned} \left(\frac{\partial \pi / RT}{\partial m_c}\right) = 1 + p_c + \left(\frac{dm_m}{dm_c}\right) \left(1 + p_m - \frac{2xp_m}{(m_c p_c + m_m p_m + 2x)}\right) \\ + \left(\frac{dm'_m}{dm_c}\right) \left(\frac{2yp_m}{(m'_m p_m + 2y)} - (1 + p_m)\right) - \frac{2x}{(m_c p_c + m_m p_m + 2x)} \end{aligned} \quad (5)$$

measured relative to the Donnan 'solvent' (Ooi, 1958), i.e.:

$$\sum_{i=2} \psi_i \left(\frac{\partial c_i}{\partial c_1}\right)_{\mu_s, T} \equiv \left(\frac{dn}{dc_1}\right)_{\mu_s, T}$$

Thus to apply eqn. (1) we require the derivative of the osmotic pressure (π) with respect to the macromolecular solute concentration.

For a detergent-complexed macromolecule in the presence of detergent micelles we consider the Donnan equilibrium between the complex solution [(detergent-complexed macromolecule concentration m_c , effective charge p_c) containing micelles (concentration m_m , effective charge p_m) and supporting electrolyte including monomeric detergent, x] and micellar solution [micelle concentration m'_m , effective charge p_m and supporting electrolyte, y]. Then, assuming all the macromolecule is complexed:

$$x(m_c p_c + m_m p_m + x) = y(m'_m p_m + y) \quad (2)$$

where the concentrations are expressed in terms of moles per unit volume.

In this way we have avoided the use of activity coefficients by assuming ideal behaviour and ascribing deviations from ideality to the effective charges on the complexes and micelles (Mysels, 1955; Prins & Hermans, 1956). It should also be

We consider two limiting cases of eqn. (5) by way of simplification. In the presence of an added salt (e.g. NaCl) $x \gg m_c p_c + m_m p_m$ and $y \gg m'_m p_m$:

$$\left(\frac{\partial \pi / RT}{\partial m_c}\right)_{\text{salt}} = 1 + \left[\left(\frac{dm_m}{dm_c}\right) - \left(\frac{dm'_m}{dm_c}\right) \right] \quad (6)$$

In the absence of salt (i.e. 0 salt) to a first approximation $x = y = 0$:

$$\begin{aligned} \left(\frac{\partial \pi / RT}{\partial m_c}\right)_{0 \text{ salt}} = \\ 1 + p_c + (1 + p_m) \left[\left(\frac{dm_m}{dm_c}\right) - \left(\frac{dm'_m}{dm_c}\right) \right] \end{aligned} \quad (7)$$

The osmotic-pressure derivatives given by eqns. (6) and (7) can be related to the light-scattering equation (1) by the equation:

$$\frac{Kg_1}{R_\theta} = \frac{1}{M_1} \left(\frac{\partial \pi / RT}{\partial m_1}\right) = \frac{1}{M_1} \left(\frac{\partial \pi / RT}{\partial m_c}\right) \quad (8)$$

where for plane-polarized incident light of wavelength λ :

$$K = \frac{4\pi^2 n_0^2}{N \lambda^4} \left(\frac{dn}{dg_1}\right)_{\mu_s, T}^2$$

and g_1 is the concentration of macromolecular solute (relative molecular mass M_1 , concentration m_1 moles per unit volume) expressed as 'dry' mass per unit volume and, since all the macromolecule is complexed with detergent, $m_1 = m_c$.

The derivative of m'_m with respect to m_c can be obtained from the total mass balance of detergent. If the average number of detergent molecules bound per macromolecule is $\bar{v}$ and the aggregation number of detergent micelles is n , then:

$$\bar{v}m_c + nm_m + nm'_m + \text{c.m.c.} = \text{constant} \quad (9)$$

Therefore:

$$\left(\frac{dm'_m}{dm_c}\right) = -\frac{1}{n} \left[\bar{v} + n \left(\frac{dm_m}{dm_c}\right) \right] \quad (10)$$

From eqns. (6), (7), (8) and (10) it follows that:

$$\left(\frac{Kg_1}{R_\theta}\right)_{\text{salt}} = \frac{1}{M_1} \left[1 + \frac{\bar{v}}{n} + 2 \left(\frac{dm_m}{dm_c}\right) \right] + 2A_2g_1 \dots \quad (11)$$

$$\left(\frac{Kg_1}{R_\theta}\right)_{0 \text{ salt}} = \frac{1}{M_1} \left\{ 1 + p_c + (1 + p_m) \left[\frac{\bar{v}}{n} + 2 \left(\frac{dm_m}{dm_c}\right) \right] \right\} + 2A_2g_1 \dots \quad (12)$$

where a second virial coefficient (A_2) term has been added to allow for complex-complex interactions. Mass balance within the complex solution requires that:

$$\left(\frac{dm_m}{dm_c}\right) = -\frac{\bar{v}}{n} \quad (13)$$

Hence the apparent M_r values ($M_{\text{app.}}$) for the two limiting cases are given by:

$$(M_{\text{app.}})_{\text{salt}} = \frac{M_1}{(1 - \bar{v}/n)} \quad (14)$$

$$(M_{\text{app.}})_{0 \text{ salt}} = \frac{M_1}{\left[1 + p_c - \frac{\bar{v}}{n} (1 + p_m) \right]} \quad (15)$$

At high salt concentration the effective charges on the complex and micelles will be zero as a result of ionic screening, so that eqn. (14) is seen to be a limiting form of eqn. (15). Also, since $\bar{v}$ can reach a limiting value when the complex is saturated with detergent whereas n can continue to increase with increasing salt concentration, in the limit of $n \gg \bar{v}$ ($M_{\text{app.}})_{\text{salt}} \rightarrow M_1$. Eqns. (14) and (15) show that in the presence of detergent micelles light-scattering

will not give the M_r value of the macromolecule even when both the refractive-index increment and the light-scattering measurements are made with respect to the Donnan 'solvent', owing to the interdependence of concentration fluctuations between macromolecule-detergent complex and detergent micelles. In order to test the above theory, we have chosen the system lysozyme plus SDS. Complex-formation in this system has been previously investigated, and SDS is one of the most widely used detergents for protein solubilization (Jones & Manley, 1979, 1980, 1981).

Experimental

Materials

Lysozyme (mucoprotein glucosylhydrolase, EC 3.2.1.17) (grade I, 3 × crystallized) was obtained from Sigma Chemical Co., London S.W.6, U.K.,

and SDS (especially pure grade) from BDH Chemicals, Poole, Dorset, U.K.

The buffers used were as follows: pH 3.2, 50mM-glycine/HCl; pH 7 and 8.0, 5mM- and 10mM-sodium phosphate; pH 9.0 and 10.0, 50mM-glycine/NaOH. All the buffers contained 0.02% NaN_3 , and the ionic strengths were adjusted as required by the addition of NaCl. All the buffer constituents were of analytical grade, and the solutions were prepared in double-distilled water.

Sample preparation

Lysozyme samples for light-scattering were purified by gel filtration on a Sephadex G-200 column (with the exception of the experiments to investigate the pH effect). The column was equilibrated with the required buffer system, and the lysozyme-containing fractions (detected by their 280nm absorbance) were eluted and combined. Lysozyme solutions covering the required concentration range were prepared and collectively dialysed (see below) against the buffer. Light-scattering measurements were made on each solution and the diffusate buffer. In the experiments in which the free SDS concentration was varied in the range 1–3 mM the lysozyme was passed down a Sephadex G-200 column in glycine buffer (50mM, pH 3.2, 0.2M-NaCl, 5mM-SDS). The lysozyme-containing fractions were combined, and a series of solutions

covering the required concentration were dialysed against enough SDS to give the required free SDS concentrations. Dialysis was carried out in bags cut from Spectrapor membrane tubing with M_r cut-off 6000–8000 [MSE (Fisons), Crawley, Sussex, U.K.]. The free SDS concentration after dialysis was measured by use of the Rosaniline hydrochloride method of Karush & Sonnenberg (1950).

Equilibrium dialysis

The binding of SDS to lysozyme was measured as previously described (Jones & Manley, 1979) by use of Spectrapor bags as above. The lysozyme concentration was 0.125% (w/v) and the equilibration time was in excess of 96 h.

Light-scattering

Light-scattering measurements were made with a Chromatix KMX-6 light-scattering photometer with an He/Ne laser source of wavelength 633 nm. The photometer cell was thermostatically controlled by circulating water, and measurements were made in the low-angle mode $\theta < 6^\circ$. Solutions were continuously circulated through the filter and cell assembly until recorded signal minima reached a constant value. For most solutions 0.2 μ m Millipore filters were used to clarify the solutions. Before scattering measurements were made on lysozyme/SDS solutions it was necessary to ensure that the light-scattering cell and ancillary circulating tubes were thoroughly cleaned by extensively flushing with a dilute solution (approx. 1%, w/v) of SDS.

Refractive-index increments were measured on a Brice-Phoenix differential refractometer with a tungsten light-source in combination with a 633 nm interference filter. The refractometer cell was thermostatically controlled by circulating water.

Ultracentrifugation

Sedimentation-equilibrium measurements to determine the M_r value (M_1) of lysozyme complexed with SDS in the presence of a free SDS concentration above the critical micelle concentration were made in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ solvent mixtures covering a range of solvent density (ρ). The method is based on the equation of Reynolds & Tanford (1976):

$$M_1(1 - \phi'\rho) = M_1[(1 - \bar{v}_p\rho) + \delta_{\text{SDS}}(1 - \bar{v}_{\text{SDS}}\rho)] \quad (16)$$

where $\bar{v}_p$ and $\bar{v}_{\text{SDS}}$ are the partial specific volumes of lysozyme and SDS respectively and δ_{SDS} is the number of grams of SDS bound per gram of lysozyme. ϕ' is the partial specific volume of lysozyme in Donnan equilibrium with the solvent. Samples of lysozyme (approx. 0.1%, w/v) were dialysed for 5 days in 30 mM-SDS plus 0.15 M-NaCl made up in

water, 3:1, 1:1 and 1:3 mixtures (by weight) of $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ and pure $^2\text{H}_2\text{O}$.

Sedimentation analysis was carried out with interference optics to determine the concentration (c)–distance (r) profile, which was used to determine $M(1 - \phi'\rho)$ at an angular velocity ω [16200 rev./min (1.696×10^3 rad/s) or 20406 rev./min (2.137×10^3 rad/s)] by using the procedure of Nazarian (1968) based on the equation:

$$M(1 - \phi'\rho) = \frac{2RT}{\omega^2} \cdot \frac{d(\ln c)}{dr^2} \quad (17)$$

M_1 was obtained by extrapolation of the plot of $M_1(1 - \phi'\rho)$ versus ρ to a solvent density $\rho = (\bar{v}_{\text{SDS}})^{-1}$, at which point $M_1(1 - \phi'\rho)$ is independent of the bound SDS. The partial specific volume of lysozyme was taken to be 0.688 cm³/g (Tanford, 1967), and that of SDS to be 0.874 cm³/g (Dohnal *et al.*, 1980).

Critical micelle concentration

The critical micelle concentrations of SDS in the buffer solutions were measured at 25°C by conductivity by using a glass conductivity cell with platinum electrodes in conjunction with a Wayne-Kerr Universal Bridge (B224).

Results and discussion

M_r of lysozyme

M_r of lysozyme in several buffer systems was determined by light-scattering. Fig. 1 shows typical data at pH 3.2 and ionic strength 0.009, which gave an M_r of 18600 ± 380 ($dn/dc = 0.161 \pm 0.0008$, $A_2 = 3.20 \times 10^{-3} \pm 0.06 \times 10^{-3}$ m³·kg⁻²·mol), which is higher than the known value of 14306 (Canfield, 1963). This discrepancy almost certainly arises from a small degree of association. Dimerization of lysozyme in neutral and alkaline solutions is well documented (Sophianopoulos & Van Holde, 1964). In the presence of SDS at a free concentration equal to the critical micelle concentration (5 mM in 5 mM-phosphate, pH 7.0) the M_r was found to be 14118 ± 415 ($dn/dc = 0.299 \pm 0.004$, $A_2 = 5.08 \times 10^{-3} \pm 0.51 \times 10^{-3}$ m³·kg⁻²·mol), in agreement with the known value. This result demonstrates that complexing of lysozyme with SDS dissociates the aggregates and that the correct M_r of the protein is obtained from light-scattering provided that measurements are made relative to the Donnan solvent and in the absence of micellar detergent.

M_r of lysozyme in the presence of micelles

Table 1 gives light-scattering data for lysozyme in the presence of a high (saturating) concentration

of SDS as a function of ionic strength. In these experiments the free SDS concentrations are well above the critical micelle concentrations of SDS. The results show that the apparent M_r values are all in excess of the known M_r but decrease with increasing ionic strength. This observation is qualitatively in agreement with eqn. (14), since the aggregation number of surfactant micelles increases with ionic strength so that $M_{app.} \rightarrow M_1$ when $n \gg v$.

The negative second virial coefficients at higher ionic strength would appear to imply reversible association between the SDS-complexed lysozyme molecules. In order to demonstrate that the complexes present were in fact monomeric, equilibrium ultracentrifugation in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ solvent mixtures was carried out at an SDS concentration

of 30 mM (i.e. on the saturated complexes in the presence of detergent micelles) (Fig. 2). A lower detergent concentration was used in the ultracentrifugation experiments to diminish the effect of detergent micelles on the schlieren pattern. These data gave an M_r of 15620, clearly indicating that the high M_r values observed by light-scattering are not due to association of the complex but have a fundamental origin.

To test eqn. (14) systematically it is necessary to find conditions where the micelle aggregation number is known and the number of detergent molecules bound per protein molecule can be controlled. The lysozyme/SDS system at an ionic strength of 0.2 meets these requirements, as shown by the binding curve in Fig. 3. The critical micelle concentration of SDS in this buffer is 0.72 mM, and detergent binding to lysozyme increases as the micellar (free SDS) concentration is raised until saturation.

We chose three nominal free SDS concentrations (1, 2 and 3 mM) at which to make light-scattering measurements at Donnan equilibrium, as a function of lysozyme concentration. The precise free SDS concentrations after dialysis were assayed at the end of the experiment and are indicated by

Fig. 1. Low-angle light-scattering from lysozyme in the absence and in the presence of SDS at 25°C

Experimental conditions: ○, glycine buffer, pH 3.2, ionic strength 0.009; ●, phosphate buffer, pH 7.0, ionic strength 0.009, plus 5 mM-SDS.

Fig. 2. Sedimentation equilibrium of lysozyme in the presence of SDS as a function of solvent density at 20°C. Experimental conditions: lysozyme concentration 0.1% (w/v), SDS concentration 30 mM, plus 0.15 M-NaCl. The arrow denotes the solvent density equal to the reciprocal of the partial specific volume of SDS.

Table 1. Low-angle light-scattering data for lysozyme as a function of ionic strength in the presence of micellar SDS. Experimental conditions: temperature 30°C, pH 3.2, SDS concentration 0.2 M.

Ionic strength*	(dn/dc)	$M_{app.}$	$10^3 \times A_2$ ($\text{m}^3 \cdot \text{kg}^{-2} \cdot \text{mol}$)
0.0088	0.161 ± 0.003	82100 ± 12000	0.907 ± 0.095
0.05	0.178 ± 0.007	40300 ± 11000	6.40 ± 0.073
0.10	0.210 ± 0.008	23200 ± 6100	-1.08 ± 0.010
0.15	0.233 ± 0.002	19200 ± 2700	-1.60 ± 0.014

* The ionic strength does not include a contribution from the SDS.

Fig. 3. Binding of SDS to lysozyme ($\bar{v}$ = mol of SDS/mol of lysozyme) as a function of the free SDS concentration at 25°C

Experimental conditions: lysozyme concentration 0.125% (w/v), glycine buffer, pH 3.2, ionic strength 0.2119. The lower arrow denotes the critical micelle concentration (c.m.c.) of SDS, and the upper arrows denote the concentrations at which light-scattering measurements were made.

Fig. 4. Low-angle light-scattering from lysozyme in the presence of SDS at 25°C

Experimental conditions: glycine buffer, pH 3.2, ionic strength 0.2119. $\circ$, $[\text{SDS}]_{\text{free}}$ 1.18 mM; $\bullet$, $[\text{SDS}]_{\text{free}}$ 1.80 mM; $\square$, $[\text{SDS}]_{\text{free}}$ 2.72 mM.

the arrows in Fig. 3. The light-scattering data are shown in Fig. 4 and the results are tabulated in Table 2. Similar results were obtained at pH 7.0, 8.0, 9.0 and 10.0. But in neutral and alkaline solutions the second virial coefficients were positive.

The aggregation numbers (n) for SDS micelles as a function of ionic strength have been measured by numerous groups and compared by Anacker *et al.* (1964). At an ionic strength of 0.21 a reasonable range for n is 100 ± 10 . In Fig. 5 three theoretical curves for the variation of M_{app} with free SDS concentration for values of n of 105, 107 and 110 are plotted in accordance with eqn. (14) by using the known values of $\bar{v}$ as a function of free SDS concentration. The experimental values of M_{app} are in reasonable accord with the theoretical curves for values of n in the range 105–110, so that within the experimental errors in both $\bar{v}$ and n eqn. (14) can be considered to explain satisfactorily the observed 2–3-fold increase in M_{app} with increasing micelle concentration.

Conclusions

The results show that the application of light-scattering to the determination of the M_r of a detergent-solubilized protein when carried out under Donnan equilibrium conditions gives the correct M_r value only in the absence of micellar detergent. In the presence of detergent micelles substantially higher M_r values are obtained.

A theoretical analysis of the latter situation that takes into account the interdependence of concen-

Fig. 5. Apparent M_r of lysozyme as a function of free SDS concentration

The three continuous curves were calculated from eqn. (14) for the values of micelle aggregation numbers indicated.

Table 2. Low-angle light-scattering data for lysozyme at constant ionic strength in the presence of micellar SDS
Experimental conditions: temperature 25°C, pH 3.2, ionic strength 0.2119.

$[\text{SDS}]_{\text{free}}$ (mM)	dn/dc	$\bar{v}$	M_{app}	$10^3 \times A_2$ ($\text{m}^3 \cdot \text{kg}^{-2} \cdot \text{mol}$)
1.18	0.345 ± 0.006	58	30230 ± 1060	-3.88 ± 0.42
1.80	0.345 ± 0.007	67.5	36400 ± 1840	-1.82 ± 0.40
2.72	0.350 ± 0.007	68.5	41650 ± 2170	-1.43 ± 0.25

tration fluctuations between protein-detergent complexes and detergent micelles shows that the apparent M_r is a function of detergent binding and micellar aggregation (at high ionic strength) and also the effective charges on the complex and micelles (at low ionic strength). The apparent M_r values of lysozyme in the presence of SDS can be adequately accounted for in terms of the proposed theory. In the application of the light-scattering technique to the determination of the M_r values of solubilized macromolecules light-scattering measurements are best restricted to systems in which the free detergent concentration is below the critical micelle concentration if erroneous results are to be avoided.

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