The Interaction between β -Lactoglobulin and Sodium *n*-Dodecyl Sulphate

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(Received 17 September 1975)

1. The binding of sodium *n*-dodecyl sulphate to β -lactoglobulin was studied in the pH range 3.5–7.0 by equilibrium dialysis, ultracentrifugation and microcalorimetry. 2. At low binding concentrations (<30 bound surfactant anions per protein molecule) the complexes formed aggregates in solution. 3. At higher binding concentrations aggregation does not occur at low ionic strength (0.01 mol/litre), but continues at high ionic strength (0.1 mol/litre). 4. At 25°C the enthalpy of interaction of sodium *n*-dodecyl sulphate with β -lactoglobulin can be interpreted as the sum of the enthalpies of formation of a complex with 2 bound surfactant anions, with an enthalpy change of $-9.5 \text{ kJ} \cdot \text{mol}^{-1}$ of bound surfactant, and complexes containing at least 22 bound surfactant anions, with limiting enthalpies per bound surfactant anion of $-12.4 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 3.5 and $-3.25 \text{ kJ} \cdot \text{mol}^{-1}$ at pH 5.5. 5. The binding of surfactant and the enthalpy of interaction at pH 3.5 are not significantly affected by the addition of 8 M-urea. 6. The data indicate that at low binding concentrations the interaction is of an ionic nature, and is accompanied by a conformational change in the protein.

The interaction of surfactants at very low concentrations (approximately millimolar) with globular proteins frequently leads to the disruption of their tertiary structure and the formation of protein-surfactant complexes. The mechanism of surfactant denaturation involves the binding of the surfactant ions to sites on the protein molecule, which results in unfolding and further binding, often in a co-operative fashion. There have been several reviews on protein-surfactant interaction (Steinhardt & Reynolds, 1969; Jones, 1975; Waehneltdt, 1975) and on the use of surfactants in the solubilization of membrane proteins (Helenius & Simons, 1975); the latter is a significant application of the study of protein-surfactant interaction.

For many globular proteins the complexes formed with the anionic surfactant sodium *n*-dodecyl sulphate are discrete species consisting of a single protein molecule and bound surfactant ions. It is the property of this surfactant to complex with proteins and to bring about the dissociation of proteins with non-covalently linked subunits into discrete species that gives it such value in polyacrylamide-gel electrophoresis. However, it has been suggested that, for the milk protein β -lactoglobulin and for some proteins near their isoelectric points, co-operative binding can induce an association of the protein to form *n*-dodecyl sulphate-complexed aggregates (Ray & Chatterjee, 1967; Ray, 1968). Further, electrophoretic measurements indicate that the binding of *n*-octylbenzene-*p*-sulphonate anions to β -lactoglobulin occurs in three stages (Hill & Briggs, 1956). A

first stage in which 2 surfactant anions are bound, a second stage in which approx. 22 anions are bound co-operatively and a third micellar-like binding stage, i.e. there are no intermediate complexes between those with 2 and those with approx. 22 bound anions. It has been found that β -lactoglobulin crystallizes with two tightly bound dodecyl sulphate anions (McMeekin *et al.*, 1949). The binding of these two anions has been studied calorimetrically at pH 5.49 in water; binding occurs with an enthalpy change of $-36 \text{ kJ} \cdot \text{mol}^{-1}$ of sodium *n*-dodecyl sulphate (Lovrien & Anderson, 1969).

Bovine β -lactoglobulin consists of two non-covalently linked subunits of mol.wt. 17500 (Townend & Timasheff, 1957). Three genetic variants are known, A, B and C; A and B differ in two amino acid residues, and B differs from C in one residue (McKenzie *et al.*, 1972). At a low temperature (below 25°C) the protein undergoes reversible self-association to a tetramer (mol.wt. approx. 140000) within the pH range 3.5–5.5 (Townend & Timasheff, 1957, 1960*a,b,c*; Chu *et al.*, 1975). The enthalpy of tetramer formation at 5°C and pH 4.65 is $-33 \text{ kJ} \cdot \text{mol}^{-1}$ in 0.1 M-acetate (Atha & Ackers, 1974). The self-association is a consequence of the very high hydrophobicity of the protein (Bigelow, 1967). The protein also engages in several conformation changes as a function of pH (Timasheff *et al.*, 1966).

The complexity of the behaviour of β -lactoglobulin and especially the unusual features of its interaction with surfactant anions led us to investigate this interaction in more detail, with particular emphasis

on the thermal effects of binding and the reported aggregation process induced by binding.

Experimental

Materials

Sodium *n*-dodecyl sulphate was prepared as described by Jones *et al.* (1973). β -Lactoglobulin was obtained from Sigma (London) Chemical Co., London S.W.6, U.K., and was used as supplied. It contains both genetic variants A and B. Urea (analytical grade obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K.) was recrystallized from aq. 95% (v/v) ethanol, and stored in a desiccator.

Three buffers were used: an acetate buffer, pH 3.5, *I* 0.01 mol/litre (0.010 mol of sodium acetate/litre + 0.182 mol of acetic acid/litre); a phosphate buffer, pH 5.5, *I* 0.01 mol/litre (0.0001842 mol of Na_2HPO_4 /litre + 0.009446 mol of NaH_2PO_4 /litre); and a phosphate buffer, pH 7.0, *I* 0.005 mol/litre (0.00120 mol of Na_2HPO_4 /litre + 0.00150 mol of NaH_2PO_4 /litre). A buffer of pH 5.5 and *I* 0.1 mol/litre was obtained by adding NaCl to the above (pH 5.5) phosphate buffer.

Methods

Microcalorimetry. Enthalpy measurements were made with an LKB 10700 batch microcalorimeter, which utilizes the twin-vessel principle, each vessel being divided into two compartments (Wadsö, 1968). The instrument was calibrated electrically. The reaction vessel was charged with 1 g of β -lactoglobulin solution (0.45%, w/v) and 1 g of sodium *n*-dodecyl sulphate solution of the required concentration (range 0–10 mM). The reference vessel was charged with 1 g of buffer and 1 g of a surfactant solution identical with that in the reaction vessel. The vessels were filled by means of syringes, which were accurately weighed before and after discharge. On mixing, the enthalpy of dilution of the surfactant solutions cancel. The enthalpy of dilution of the protein on dilution from 0.45% (w/v) to 0.225% (w/v) at pH 3.5 and 25°C was $-5.6 \pm 1.1 \text{ kJ} \cdot \text{mol}^{-1}$, which is almost negligible compared with the enthalpy of interaction of the surfactant with the protein.

Equilibrium dialysis. An apparatus based on the design of Weder *et al.* (1971) was used as previously described (Jones *et al.*, 1975). Membranes were cut from Spectrapor membrane tubing no. 132655 [MSE (Fisons), Manor Royal, Crawley, Sussex, U.K.], with molecular-weight cut-off 6000–8000. The free surfactant concentration was assayed by the Rosaniline hydrochloride method of Karush & Sonenberg (1950). Calibration curves were obtained in each buffer system and in the presence of 8M-

urea at pH 3.5. In the dialysis experiments the protein concentration was 0.06% (w/v), made up by weight.

Sedimentation analysis. Measurements of sedimentation velocity were made in cells with 12 mm Epon centrepieces with 4° sector angles, by using an AnD rotor in a Spinco model E analytical ultracentrifuge operating at 56100 rev./min (229100g, r_{av} . 6.51 cm) at 20°C. Schlieren optics were used, and photographs were taken at 8 min intervals.

U.v. difference spectra. Measurements were made in the range 270–350 nm with a Perkin-Elmer model 402 double-beam recording spectrophotometer, with an expansion setting of $\times 5$. The instrument reading was adjusted to zero with β -lactoglobulin solutions in both cuvettes, and difference spectra were obtained by adding portions of the surfactant solutions to the reference cuvette. The surfactant concentrations were in the range 2–6 mM, and the final protein concentration was 0.26% (w/v).

All the measurements reported refer to surfactant concentrations below the critical micelle concentration of sodium *n*-dodecyl sulphate in the various media. The calculations were made by assuming a mol.wt. of 35000 for β -lactoglobulin (Townend & Timasheff, 1957).

Results and Discussion

Binding measurements

Fig. 1 shows the number of *n*-dodecyl sulphate anions bound per molecule of β -lactoglobulin ($\bar{\nu}$) as a function of the logarithm of the free surfactant concentration at 25°C, pH 3.5 and 5.5, and in the presence of 8M-urea at pH 3.5. The steep rises in these curves are characteristic of co-operative binding. At the lower binding concentrations $\bar{\nu}$ depends on pH; binding is greater at lower pH and is not influenced by the addition of 8M-urea. The pH-dependence of $\bar{\nu}$ is similar to that previously observed by Ray (1968), who found that less surfactant was bound to cow β -B lactoglobulin at pH 7.5 than at pH 5.4; however, addition of 8M-urea decreased the values of $\bar{\nu}$ at pH 5.4. These results were obtained at *I* 0.10 mol/litre. The binding data reported in Fig. 1 at pH 5.5 and *I* 0.01 mol/litre are in good agreement with Ray's (1968) data at pH 5.4 and *I* 0.10 mol/litre, so that it may be concluded that pH has a much greater influence on surfactant binding to β -lactoglobulin than does ionic strength, and that urea affects binding at high pH but not at low pH. Takagi *et al.* (1975) have reported data for reduced and carboxyamidomethylated β -lactoglobulin at pH 7 and *I* 0.08 mol/litre that also show co-operative binding at a free sodium *n*-dodecyl sulphate concentration of approx. 1 mM.

Fig. 1. Binding of sodium *n*-dodecyl sulphate to β -lactoglobulin ($\bar{v}$) as a function of the logarithm of the free surfactant concentration at 25°C

●, Acetate buffer, pH 3.5, *I* 0.01, protein concentration 0.06% (w/v). ■, Acetate buffer, pH 3.5, plus 8M-urea, *I* 0.01, protein concentration 0.06% (w/v). ○, Phosphate buffer, pH 5.5, *I* 0.01, protein concentration 0.06% (w/v). All the measurements were made by equilibrium dialysis as described in the Experimental section.

Fig. 2. Sedimentation coefficients (*s*) for the system β -lactoglobulin plus sodium *n*-dodecyl sulphate as a function of the total surfactant concentration and the number of surfactant molecules bound per protein molecule ($\bar{v}$) at 20°C

●, ○, Phosphate buffer, pH 5.5, *I* 0.01, protein concentration 0.5% (w/v). ▲, △, Phosphate buffer, pH 5.5, *I* 0.1, protein concentration 0.5% (w/v). The error bars denote the standard deviations derived from the slopes of the plots of the distance of the peaks in the schlieren patterns from the centre of rotation versus time. The average standard deviation of the points without error bars was 0.04. ■, Phosphate buffer, pH 5.5, *I* 0.01 plus 8M-urea, protein concentration 0.5% (w/v). *s* values were corrected for the density and viscosity of 8M-urea. The $\bar{v}$ axis does not apply to the data in 8M-urea.

Sedimentation analysis

Fig. 2 summarizes the results of sedimentation analysis of solutions at pH 5.5 and *I* 0.01 and 0.10 mol/litre. The values of $\bar{v}$ shown on this and subsequent Figures were calculated from the binding data by construction of plots of $\bar{v}$ as a function of total surfactant concentration, as in previous work (Jones *et al.*, 1975).

Considering first the data at *I* 0.01 mol/litre (solid and open circles) over the range of total surfactant concentration 0.80–1.5 mM there are two peaks in the schlieren patterns. At 0.80 mM the slow-moving peak has the larger area, but as the total surfactant concentration is increased its area decreases until at 1.5 mM the fast-moving peak has the larger area. The resolution of the smaller peak at each end of the range is not high, so that the *s* values cannot be determined with great precision. The *s* values calculated from the maximum ordinate of the slow peak are very close to those of the native protein, but those of the fast peak are very much larger, and pass through a maximum with a value of approx. 8S. As the total surfactant concentration is increased above 1.5 mM no slow peak can be resolved, and the *s* values corresponding to the

'fast' peak decrease to values less than those of the original slow peak.

At *I* 0.10 mol/litre (solid and open triangles) we again observe a region of co-existence of two peaks, but, in contrast with the results at the lower ionic strength, the *s* values corresponding to the fast peak remain approximately constant with increasing total surfactant concentration. At the higher surfactant concentrations the peaks are broad, indicating polydispersity. Our data at *I* 0.10 mol/litre are very similar to those of Ray & Chatterjee (1967), who did not report any measurements at *I* 0.01 mol/litre.

There are considerable interpretative difficulties associated with schlieren patterns for interacting systems (Cann, 1970). Ray & Chatterjee (1967) attributed the slow peak to β -lactoglobulin, with approx. 2 bound surfactant ions, and the fast peak to an aggregate of complexes, with approx. 22 bound anions per protein molecule, with the binding of the 22 anions occurring in a highly co-operative fashion. High *s* values of approx. 8S can only arise from aggregation, since Nelson (1971) showed that the *s* values of proteins saturated with *n*-dodecyl sulphate anions are usually less than those of native proteins. Thus the sedimentation analysis unequivocally illus-

Fig. 3. Enthalpy of interaction of sodium *n*-dodecyl sulphate with β -lactoglobulin as a function of the total surfactant concentration and the number of surfactant molecules bound per protein molecule ($\bar{v}$) at 25°C

○, Acetate buffer, pH 3.5, 1.01, protein concentration 0.225% (w/v). ●, Acetate buffer, pH 3.5, plus 8M-urea, 1.01, protein concentration 0.225% (w/v). The values denote the numbers of measurements, and the error bars the range.

trates that aggregation occurs and that it is ionic-strength-dependent; at 1.01 mol/litre the aggregates dissociate with increasing surfactant concentration, whereas they are stable at 1.01 mol/litre. We can also assume that, since initially there is a significant amount of free surfactant present in the system, the amount of free protein is very small, and hence both schlieren peaks can be predominantly attributed to protein-surfactant complexes. Hill & Briggs (1956) have presented convincing evidence that in the initial stages of binding only two species exist with 2 and approx. 22 bound surfactant anions. Our thermochemical data, discussed below, can also be interpreted on this basis, and hence the assignment of the two schlieren peaks to these complexes and their aggregate respectively would be consistent with the present data and those of Hill & Briggs (1956).

Thermochemical analysis

Figs. 3 and 4 show the results of the microcalorimetry measurements. The enthalpy changes on interaction of the surfactant with β -lactoglobulin depend on pH. The curve of ΔH at a total surfactant concentration of 2 mm (Fig. 4) shows a dramatic decrease with increasing pH. At pH 3.5 the final solutions in the microcalorimeter, after mixing, were turbid, presumably owing to the aggregation of surfactant-protein complexes, and on standing a gelatinous precipitate was formed. The calorimetric measurements were made over a period of approx. 30 min. The thermograms had horizontal baselines, indicating that no significant slow thermal effects arising from precipitation could be detected. As a

Fig. 4. Enthalpy of interaction of sodium *n*-dodecyl sulphate with β -lactoglobulin as a function of total surfactant concentration, number of surfactant molecules bound per protein molecule ($\bar{v}$) and pH at 25°C

○, Phosphate buffer, pH 5.5, 1.01, protein concentration 0.225% (w/v). ●, Phosphate buffer, pH 7.0, 1.005, protein concentration 0.225% (w/v). The values of $\bar{v}$ relate to the data at pH 5.5. ■, Enthalpy of interaction at a total surfactant concentration of 2 mm as a function of pH.

first approximation we shall assume that the thermal effects observed at pH 3.5 refer to the initial interaction of the surfactant with the protein, and do not contain a significant contribution arising from the subsequent precipitation process. All the solutions at pH 5.5 and 7.0 were optically clear.

Fig. 5 shows the enthalpy of interaction per molecule of surfactant bound (ΔH_b) as a function of $\bar{v}$ at pH 3.5 and 5.5. At pH 3.5 ΔH_b becomes constant above $\bar{v}$ approx. 25, and decreases below this value. At pH 5.5, ΔH_b decreases rapidly between $\bar{v}=0$ and approx. 20; it decreases very slowly thereafter, and appears to be approaching a limiting value. The contrasting trends at the two pH values below $\bar{v}$ approx. 25 and the approximate constancy of ΔH_b at higher values of $\bar{v}$ could be explained if ΔH_b is the sum of two enthalpy terms, one arising from the enthalpy of formation of a complex with 2 bound surfactant molecules (ΔH_2), which is independent of pH, and another arising from the enthalpy of formation of complexes with $\bar{v} \geq 22$ (ΔH_{lim}). It is reasonable to expect that for co-operative binding the enthalpy of interaction per molecule of surfactant bound would remain approximately constant. If this is so then it should be possible to calculate ΔH_b from the following equation,

$$\Delta H_b = \frac{1}{\bar{v}} \{ 2[P] \Delta H_2 + ([SDS]_{total} - [SDS]_{free} - 2[P]) \Delta H_{lim} \}$$

Fig. 5. Enthalpy of interaction per molecule of bound sodium *n*-dodecyl sulphate (ΔH_b) as a function of the number of surfactant molecules bound per β -lactoglobulin molecule ($\bar{v}$) at 25°C

●, Acetate buffer, pH 3.5, 1.0. The error bars denote the maximum range. ○, Phosphate buffer, pH 5.5, 1.0. The solid lines were calculated as described in the text.

where SDS is sodium dodecyl sulphate and P is the protein. At pH 5.5 the experimental points extrapolate to between $-9 \text{ kJ} \cdot \text{mol}^{-1}$ and $-11 \text{ kJ} \cdot \text{mol}^{-1}$ at $\bar{v} = 0$. The curve in Fig. 5 with $\Delta H_2 = -9.5 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta H_{\text{lim}} = -3.25 \text{ kJ} \cdot \text{mol}^{-1}$ fits the experimental data reasonably well. At pH 3.5 the curve drawn was calculated with $\Delta H_2 = -9.5 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta H_{\text{lim}} = -12.4 \text{ kJ} \cdot \text{mol}^{-1}$. In this case the fit below $\bar{v} = 25$ is poor and outside the limits of error. The turbidity observed at pH 3.5 indicates that much larger aggregates are formed at this pH, and it is possible that a significant contribution from the enthalpy of aggregation might exist. Such a contribution need only be small, and could be relatively more significant at low values of $\bar{v}$, when the observed enthalpies are lower than at higher values. For example, at $\bar{v} = 10$ the observed enthalpy of interaction is $-94.5 \text{ kJ} \cdot \text{mol}^{-1}$ (Fig. 3), and an endothermic contribution of $17.5 \text{ kJ} \cdot \text{mol}^{-1}$ would be sufficient to bring the value of ΔH_b on to the calculated curve. An alternative possibility is that, at pH 3.5, ΔH_2 is less negative than $-9.5 \text{ kJ} \cdot \text{mol}^{-1}$, but no better agreement at low values of $\bar{v}$ is obtained if ΔH_2 is put equal to approx. $-5 \text{ kJ} \cdot \text{mol}^{-1}$, and the agreement at higher values of $\bar{v}$ is much worse. We conclude that a value of $\Delta H_2 = -9.5 \text{ kJ} \cdot \text{mol}^{-1}$ is in fair agreement with the data at both pH values. This value contrasts with a value of $\Delta H_2 = -36 \text{ kJ} \cdot \text{mol}^{-1}$ measured in pure water (Lovrien & Anderson, 1969); as the latter authors point out, a difference between values in water and in buffer solution would be expected because the protein binds buffer ions, and ΔH_2 corresponds to an ion-exchange process.

Fig. 6. Molar difference extinction coefficient at 300nm for β -lactoglobulin as a function of the number of sodium *n*-dodecyl sulphate molecules bound ($\bar{v}$) at 25°C

●, Both spectrophotometer cuvettes contained 3 ml of a solution of 2.6 g of β -lactoglobulin/litre in phosphate buffer, 1.0, pH 5.5. The reference cuvette also contained sodium *n*-dodecyl sulphate in the range 2–6 mM.

Nature of the binding process

The marked dependence of the enthalpy of interaction of sodium *n*-dodecyl sulphate with β -lactoglobulin on pH suggests that the interaction is initially of an ionic nature, as has been found for other proteins (Burkhard & Stolzenberg, 1972; Tipping *et al.*, 1974; Oakes, 1974). It is noteworthy that the net charge on β -lactoglobulin changes from approx. +20 at pH 3.5 to approx. -10 at pH 7.0 (Nosaki *et al.*, 1959). At higher binding concentrations the co-operative nature of binding is more characteristic of hydrophobic bonding, but, surprisingly, the extent of binding at pH 3.5 is not affected by the presence of 8M-urea, nor has this denaturant any significant effect on the magnitudes of the enthalpies of interaction (Fig. 3). The curve of the enthalpy of interaction in 8M-urea oscillates about the curve for the enthalpy in the absence of urea. The errors in the enthalpy measurements in 8M-urea are much larger than those in buffer alone, and the oscillations in the curve are probably not significant. As was the case with buffer alone at pH 3.5, after being mixed in the microcalorimeter the solutions were turbid, and precipitation occurred on standing.

β -Lactoglobulin dissociates into its two subunits and adopts a random-coil conformation restricted only by disulphide bonds in 8M-urea (Skerjanc & Lapanje, 1969). It is apparent that the addition of surfactant to the protein in 8M-urea at pH 3.5 brings about the aggregation of the subunits, and that the species formed are essentially the same as those formed in buffer alone.

The situation at pH 5.5 is clearly different. The sedimentation analysis shows that at this pH the protein is dissociated into its subunits in 8M-urea.

The sedimentation coefficients are approximately one-half those of the native protein (Fig. 2), and no aggregates are formed. Recalling that 8M-urea has no effect on the extent of surfactant binding at pH 3.5, but decreases binding at pH 5.4 (Ray & Chatterjee, 1967), it seems reasonable to deduce that, when the protein has a high charge (approx. +20 at pH 3.5), the initial ionic interactions predominate, whereas when the protein has a low charge (approx. -2 at pH 5.5) less surfactant is initially bound, and the ability of urea to weaken hydrophobic bonds becomes manifest.

Addition of the surfactant to β -lactoglobulin at pH 5.5 gives rise to a difference spectrum with an absorption band at 300 nm presumed to arise from a change in environment of tyrosine or tryptophan residues. The variation of the molar extinction coefficient at 300 nm with $\bar{\nu}$ is shown in Fig. 6. There is a steep rise in $\Delta\epsilon_{300}$ up to $\bar{\nu}$ approx. 25 and a slower rise thereafter. The change in $\Delta\epsilon_{300}$ below $\bar{\nu}$ approx. 25 is attributed to the formation of the complex containing 22 bound surfactant ions. One interpretation of the difference spectrum is that the protein has undergone a conformational change on binding surfactant, as previously suggested (Hill & Briggs, 1956).

We thank Mrs. L. Hall for experimental assistance and the Science Research Council for a grant for the purchase of the LKB microcalorimeter.

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