The Interaction between Bovine Serum Albumin and Surfactants

By MALCOLM N. JONES,* HENRY A. SKINNER† and EDWARD TIPPING†‡

*Department of Biochemistry and †Department of Chemistry, University of Manchester,
Manchester M13 9PL, U.K.

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1. Potassium *n*-decyl phosphate binds exothermically to bovine serum albumin at pH 7.0 to form a specific complex containing approx. 60 phosphate anions. 2. The formation of the complex is accompanied by changes in the u.v. difference spectrum of the protein. 3. At higher phosphate concentrations (above 0.4 mM) surfactant molecules continue to be bound, and the protein undergoes a gross change in conformation. 4. *n*-Dodecyltrimethylammonium bromide binds endothermically to bovine serum albumin at pH 7.0 but the extent of binding for a given free surfactant concentration is less than for the phosphate surfactant. 5. Binding is accompanied by a small change in the specific viscosity and by changes in the u.v. difference spectrum of the protein. 6. It is suggested that over the surfactant concentration ranges studied *n*-decyl phosphate ions first bind to the C-terminal part of the protein and then to the more compact N-terminal part whereas *n*-dodecyltrimethylammonium ions bind only to the C-terminal part of bovine serum albumin.

The study of the nature of the interactions between proteins and surfactants provides insight into the action of surfactants as denaturants and as solubilizing agents for membrane proteins; systems of this type serve as models for the interactions that occur between membrane proteins and lipids. The interaction between globular proteins and *n*-alkyl sulphates has been extensively investigated (Steinhardt & Reynolds, 1969). The enthalpies of interaction between selected globular proteins and sodium *n*-dodecyl sulphate have been measured by microcalorimetry and the results were interpreted in terms of an initial interaction between the anionic head group of the surfactant and positive sites on the protein molecule, followed by hydrophobic interactions at higher binding levels (Tipping *et al.*, 1974). The interaction between proteins and cationic surfactants has received less attention but several studies are currently being reported (Birdi, 1973; Kaneshina *et al.*, 1973; Jones *et al.*, 1973; Nozaki *et al.*, 1974).

The aim of the present investigation was to study the interaction between bovine serum albumin and selected surfactants containing the same head groups as those occurring in natural membrane phospholipids. The surfactants chosen were *n*-dodecyltrimethylammonium bromide and potassium *n*-decyl phosphate. The phosphate ester head groups

occur in phosphatidic acid, and are covalently linked to the trimethylammonium groups in phosphatidylcholine. A satisfactory synthesis and isolation procedure was developed for the *n*-decyl phosphate surfactant; it was found, however, that only the potassium salt is appreciably soluble in aqueous media. This latter surfactant binds extensively to bovine serum albumin, the process being exothermic; in contrast, *n*-dodecyltrimethylammonium bromide binds endothermically to the protein.

Experimental

Materials

n-Dodecyltrimethylammonium bromide. The preparation of this has been described previously (Jones *et al.*, 1973).

Potassium *n*-decyl phosphate. This was prepared by the reaction between phosphorus oxychloride and *n*-decanol followed by neutralization of the reaction mixture with KOH. To a solution of freshly distilled POCl₃ (43.2 ml) in chloroform (400 ml), *n*-decanol (90 ml) was added dropwise with gentle stirring. The reaction mixture was refluxed for 4 h. After cooling, the mixture was transferred to a separating funnel and diluted threefold with water. The mixture was neutralized by the addition of aq. 10% (w/v) KOH with phenolphthalein as indicator. The aqueous layer was separated and saturated with KHCO₃. The potassium *n*-decyl phosphate was then extracted with butan-1-ol (four times with 600 ml portions).

‡ Present address: Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London W.1, U.K.

The combined butan-1-ol extracts were then distilled under vacuum to remove the water, which caused potassium *n*-decyl phosphate to precipitate from solution. After distillation the mixture was stored at 5°C overnight and the crude potassium *n*-decyl phosphate was filtered off and three times recrystallized from methanol (Found: C, 43.3; H, 7.6; K, 14.9; P, 11.8; $C_{10}H_{21}OPO_2(OH)K$ requires C, 43.5; H, 8.0; K, 14.2; P, 11.2%)

The critical micelle concentration, as measured by the conductance method in potassium phosphate buffer, $I = 0.005$, at pH 7.0 and 25°C, was $4.45 \times 10^{-3} M$. The surface tension-concentration curve showed no minimum and gave a critical micelle concentration of $4.62 \times 10^{-3} M$. The titration curve of potassium *n*-decyl phosphate at a concentration below the critical micelle concentration gave a pK_a of 3.3, and hence at pH 7.0 the surfactant is essentially a 1:1 electrolyte.

It should be noted that both the lithium and sodium salts of *n*-decyl phosphate esters are insoluble in water at 25°C and hence potassium *n*-decyl phosphate may only be used in media free of these cations. The solubility of the salts follows the sequence $K^+ > Na^+ > Li^+$ (the reverse of the order found for the *n*-alkyl sulphate esters).

Bovine serum albumin. This was type F (fatty-acid free) obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., and was used as supplied.

Buffers. Two buffers were used. A sodium phosphate buffer, pH 7.0, I 0.005 mol/litre (0.00120 mol of Na_2HPO_4 /litre + 0.00150 mol NaH_2PO_4) and a potassium phosphate buffer, pH 7.0, I 0.005 mol/litre (0.00120 mol of K_2HPO_4 /litre + 0.00150 mol of KH_2PO_4 /litre).

Water. Deionized water was distilled from alkaline $KMnO_4$ and then redistilled.

Methods

U.v. difference spectra. Measurements were made in the range 230–310 nm with a Perkin-Elmer model 402 double-beam recording spectrophotometer with an expansion setting of times five. The instrument reading was adjusted to zero with bovine serum albumin solutions in both cuvettes, and difference spectra were obtained by adding portions of the surfactant solutions to one cuvette. The final bovine serum albumin concentration was 3.5 g/litre.

Viscometry. Viscosity measurements were made by using a Ubbelohde suspended-level viscometer for the bovine serum albumin-*n*-dodecyltrimethylammonium bromide system and a 'Fica 52 Automatic Capillary Viscometer' (Applied Research Laboratory Ltd., Wingate Road, Luton, Beds., U.K.) for the bovine serum albumin-potassium-*n*-decyl phosphate system. Both instruments were thermo-

statically controlled at 25°C. The specific viscosity of a solution is given by

$$\eta_{sp.} = (t - t_0)/t_0$$

where t and t_0 refer to the flow times of the solution and solvent respectively. The average values were based on measurements of at least ten flow times (t) on a series of solutions of fixed protein concentration (1.0 g/litre) and variable surfactant concentrations. The reproducibility was $\pm 0.2s$ for the Ubbelohde viscometer and $\pm 0.04s$ for the Fica viscometer.

Microcalorimetry. A Beckman 190B microcalorimeter was used, and calibrated as previously described (Jones *et al.*, 1971). For measurements on the bovine serum albumin-*n*-dodecyltrimethylammonium bromide system, the reaction cell was charged with 0.1500 (± 0.0004) g of bovine serum albumin solution (concn. 3.0%, w/v) in the drop well, and with 15.0 (± 0.01) g of *n*-dodecyltrimethylammonium bromide solution of known concentration in the annular space. The reference twin cell was charged similarly with the bovine serum albumin solution, but with 15.0 (± 0.01) g of buffer solution in the annular space. For the bovine serum albumin-potassium *n*-decyl phosphate system, double the amount of bovine serum albumin solution was placed in the drop wells. The dilution of bovine serum albumin after mixing was the same in both cells, so that the enthalpy of dilution of protein was presumed to cancel.

Equilibrium dialysis. An MSE (Fisons) 'Dianorm Dialysis System' was used to determine the amount of surfactant bound to the protein at 25°C. This apparatus, based on the design of Weder *et al.* (1971), consists of a parallel arrangement of Teflon bicompartment cells of volume approx. 1 ml between which the membranes are clamped. The membranes were cut from 'Spectrapor' membrane tubing no. 132680, molecular-weight cut-off 12000–14000. They were soaked overnight in distilled water and washed with buffer before use. The bovine serum albumin-surfactant solution was added to one compartment and after equilibrium was attained (approx. 24 h) the free surfactant concentration was assayed in the protein-free compartment. For the bovine serum albumin-*n*-dodecyltrimethylammonium bromide system the assay method of Few & Ottewill (1956), involving the formation of the *n*-dodecyltrimethylammonium bromide Orange II dye complex, was used. For the bovine serum albumin-potassium *n*-decyl phosphate system the Rosaniline hydrochloride method of Karush & Sonenberg (1950) was used. In the dialysis experiments the protein concentration was 0.06% (w/v).

In all calculations the molecular weight of the bovine serum albumin was taken as 66000 (Spahr & Edsall, 1964).

Fig. 1. Binding of potassium *n*-decyl phosphate to bovine serum albumin as a function of free surfactant concentration

$\bar{v}$ = number of surfactant molecules bound/protein molecule. All measurements were made in potassium phosphate buffer, $I = 0.005$, pH 7.0 at 25°C as described in the Experimental section.

Fig. 2. Enthalpy of binding of potassium *n*-decyl phosphate to bovine serum albumin as a function of the total surfactant concentration and the number of surfactant molecules bound per protein molecule ($\bar{v}$)

Left-hand axis (○) enthalpy of binding measured in potassium phosphate buffer, $I = 0.005$, pH 7.0 at 25°C. Protein concentration, 0.06% (w/v). The inset is a qualitative resolution of the enthalpy of binding into two components, where the negative sign indicates binding and the positive sign, unfolding. The arrow indicates the critical micellar concentration.

Results and Discussion

Bovine serum albumin–potassium *n*-decyl phosphate interaction

Fig. 1 is a plot of the number of *n*-decyl phosphate anions bound per molecule of bovine serum albumin ($\bar{v}$) as a function of the free potassium *n*-decyl

phosphate concentration in solution, as measured by equilibrium dialysis. The curve initially rises very steeply, reaching a plateau at $\bar{v} \approx 60$. This behaviour is very similar to that found with bovine serum albumin *n*-dodecyl sulphate, where initially 50–60 ions are bound to form a specific complex (Putnam & Neurath, 1945), and suggests that the phosphate head group interacts with the same sites as the sulphate head group.

To clarify the presentation of all subsequent data, the binding data have been used to calculate $\bar{v}$ in solutions of known total surfactant and protein concentrations. If it is assumed that $\bar{v}$ is not a function of protein concentration then plots of $\bar{v}$ as a function of total surfactant concentration, for a given protein concentration, can be constructed and used to determine $\bar{v}$ in a given system (Jones *et al.*, 1973).

Fig. 2 shows that the enthalpy change on the formation of the complex is negative. The magnitude (approx. 300 kJ·mol⁻¹) is comparable with the enthalpy change found for the binding of 60 *n*-dodecyl sulphate ions to bovine serum albumin (approx. 330 kJ·mol⁻¹). However, between $\bar{v} = 60$ and 160 the enthalpy change remains constant implying that the further binding of these 100 anions occurs athermally. Above $\bar{v} = 160$ the enthalpy curve falls, indicating that an endothermic process is contributing to the overall enthalpy change. In this respect the enthalpy curve qualitatively resembles that for the sodium *n*-dodecyl sulphate–ribonuclease A interaction (Jones *et al.*, 1973). The final rise in the enthalpy curve occurs as the total surfactant concentration in the solution approaches the critical micelle concentration.

Addition of potassium *n*-decyl phosphate to bovine serum albumin gives rise to a u.v. difference spectrum with absorption bands at 243 nm and 290 nm, corresponding to changes in the local environment of aromatic amino acid residues. Fig. 3 shows that the difference molar extinction coefficient of the 243 nm band changes abruptly during the binding of the first 60 surfactant anions. The decrease in $\Delta\epsilon_{243}$ above $\bar{v} \approx 30$ is somewhat puzzling, but it could imply that the local environment of the phenylalanine residues is becoming more hydrophobic by virtue of neighbouring bound surfactant. We have observed a smaller maximum in the $\Delta\epsilon$ versus $\bar{v}$ plot for ribonuclease A and sodium *n*-dodecyl sulphate (Jones *et al.*, 1973). Although the difference molar extinction coefficient must go to zero at $\bar{v} = 0$ we observed a difference spectrum at a total surfactant concentration of 0.3×10^{-4} M (where the value of $\bar{v}$ is less than 1) which implies that the binding of the first surfactant ion changes the environment of aromatic residues in the protein.

Fig. 3. Molar difference extinction coefficient at 243 nm and specific viscosity for bovine serum albumin as a function of the number of potassium *n*-decyl phosphate molecules bound

●, $\Delta \epsilon_{243}$. Both spectrophotometer cuvettes contained 3 ml of a solution of 3.5 g of bovine serum albumin/litre in potassium phosphate buffer, $I = 0.005$, pH 7.0 at 25°C. One cuvette also contained potassium *n*-decyl phosphate in the range 0–1 mM. ○, $\eta_{sp.}$ in the above buffer. The protein concentration was 0.1% (w/v) and the potassium *n*-decyl phosphate concentration was in the range 0–5 mM.

Also shown in Fig. 3 is the change in the specific viscosity ($\eta_{sp.}$) with the extent of binding. Only a small change in $\eta_{sp.}$ occurs during the binding of the first 40 surfactant ions after which the curve rises monotonically. This suggests that no very large conformation change occurs during the formation of the specific complex, but on further binding the protein undergoes a gross change in conformation. A particularly noteworthy feature of the *n*-decyl phosphate anion is its ability to induce this gross conformation change in contrast with *n*-decyl sulphate anions, which bind to bovine serum albumin but do not appreciably unfold it (Reynolds *et al.*, 1967). The critical micelle concentration of potassium *n*-decyl phosphate (4.5 mM) is lower than that of sodium *n*-decyl sulphate (33 mM) (Mukerjee & Mysels, 1971) so that the phosphate surfactant has a greater tendency to form an aggregated state.

Bovine serum albumin–*n*-dodecyltrimethylammonium bromide interaction

Fig. 4 shows the number of *n*-dodecyltrimethylammonium cations bound per molecule of bovine serum albumin as a function of the free surfactant concentration in solution, as measured by equilibrium dialysis. There are substantial differences between the binding data reported in the literature for this system. Few *et al.* (1955) give a curve for a 1% (w/v) bovine serum albumin solution (at an ionic strength of 0.1) which shows saturation at $\bar{v} \approx 109$ at a free concentration of *n*-dodecyltrimethyl-

ammonium bromide of 0.01 M, but under very similar conditions Kaneshina *et al.* (1973) found $\bar{v} \approx 160$ at this free surfactant concentration. Both these studies were made by using Visking dialysis tubing. We were unable to get reproducible data with Visking tubing in this system but our protein concentration was 17 times lower than that used by these workers. We have made binding experiments at higher surfactant concentrations than those covered by Fig. 4, which show that the protein does not saturate at a value of $\bar{v} \approx 109$ but continues to bind more surfactant.

In contrast with potassium *n*-decyl phosphate,

Fig. 4. Binding of *n*-dodecyltrimethylammonium bromide to bovine serum albumin as a function of free surfactant concentration

$\bar{v}$ = number of surfactant molecules bound/protein molecule. All measurements were made in sodium phosphate buffer, $I = 0.005$, pH 7.0 at 25°C as described in the Experimental section. The numerals denote overlapping points.

Fig. 5. Enthalpy of binding of *n*-dodecyltrimethylammonium bromide to bovine serum albumin as a function of the total surfactant concentration and the number of surfactant molecules bound per protein molecule ($\bar{v}$)

Left-hand axis (○) enthalpy of binding measured in sodium phosphate buffer, $I = 0.005$, pH 7.0 at 25°C. Protein concentration 0.03% (w/v).

Fig. 6. Molar difference extinction coefficient at 243 nm and specific viscosity for bovine serum albumin as a function of the number of *n*-dodecyltrimethylammonium molecules bound

●, $\Delta\epsilon_{243}$. Both spectrophotometer cuvettes contained 3 ml of a solution of 3.5 g of bovine serum albumin/litre in sodium phosphate buffer, $I=0.005$, pH 7.0, at 25°C. One cuvette also contained *n*-dodecyltrimethylammonium bromide in the range 0–5 mM. ○, η_{sp} , in the above buffer. The protein concentration was 0.1% (w/v) and the *n*-dodecyltrimethylammonium concentration was in the range 0–5 mM.

much less surfactant is bound for a given free concentration despite the longer chain length of the trimethylammonium compound. This observation agrees with the other studies on protein-cationic surfactant interactions cited in the introduction, and illustrates the important role of the charged head group.

Fig. 5 shows the enthalpy change on interaction of *n*-dodecyltrimethylammonium bromide with bovine serum albumin. The enthalpy change is endothermic in contrast with the exothermic enthalpy change observed on interaction of anionic surfactants with bovine serum albumin.

Fig. 6 shows the changes in the molar extinction coefficient at 243 nm and the specific viscosity as a function of the extent of binding. The viscosity data imply only a small change in conformation as binding proceeds, whereas the difference spectra indicate environmental changes of the aromatic residues.

Hilak *et al.* (1974) have proposed a model for the structure of the bovine serum albumin molecule which accounts for the behaviour of the protein on proteolytic hydrolysis and is in accord with X-ray-scattering data. In this model the molecule has a compact structure at neutral pH in the N conformation, and on acid denaturation to the F

conformation only the C-terminal section of the molecule becomes unfolded and the N-terminal moiety remains compact without loss of helix content. This model differs in detail from that proposed by Vijai & Foster (1967) who postulated the existence of a cleft between two compact regions in the N conformation. On acid denaturation to the F conformation the cleft between the two compact regions (or lobes) opens up but the two lobes of the molecule initially remain folded. Thus the two models differ in the compactness of the C-terminal lobe and its susceptibility to denaturation.

If we consider the data in the light of the model of Hilak *et al.* (1974) then the initial interaction between bovine serum albumin and the *n*-decyl phosphate anion could involve the binding of the surfactant mainly to the C-terminal section of the molecule bringing about its unfolding, the compact N-terminal moiety remaining intact. With increasing concentration of surfactant anions, the more stable compact N-terminal part of the molecule begins to unfold, exposing new binding sites. Such a two-stage unfolding process could give rise to an overall enthalpy curve of the form observed (as shown in the inset of Fig. 2) and would be consistent with our present data and with those for the interaction between sodium *n*-dodecyl sulphate and bovine serum albumin (Tipping *et al.*, 1974).

In contrast, *n*-dodecyltrimethylammonium bromide perhaps binds readily only to the more easily unfolded C-terminal part of the bovine serum albumin molecule, and that over the range of concentration studied here does not penetrate appreciably into the more compact N-terminal part. In this respect it is worth recalling that the relatively compact ribonuclease A molecule is not denatured by *n*-dodecyltrimethylammonium bromide (Jones *et al.*, 1973). If both lobes of the bovine serum albumin molecule have comparable resistance to unfolding then neither would be denatured by *n*-dodecyltrimethylammonium bromide although this surfactant might penetrate the cleft between the two lobes. In this respect it is not possible to unequivocally differentiate between the two models although on balance the data is slightly in favour of the model of Hilak *et al.* (1974).

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