

Detergents as Probes of Hydrophobic Binding Cavities in Serum Albumin and Other Water-Soluble Proteins

Ulrich Kragh-Hansen,* Florence Hellec,[†] Béatrice de Foresta,[‡] Marc le Maire,[‡] and Jesper V. Møller[†]

Departments of *Medical Biochemistry and [†]Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark; and [‡]Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, CEA, CNRS-URA 2096 et Université Paris Sud-LRA 17V, Centre d'Etudes de Saclay, F-91191 Gif-sur-Yvette, Cedex, France

ABSTRACT As an extension of our studies on the interaction of detergents with membranes and membrane proteins, we have investigated their binding to water-soluble proteins. Anionic aliphatic compounds (dodecanoate and dodecylsulfate) were bound to serum albumin with high affinity at nine sites; related nonionic detergents (C₁₂E₈ and dodecylmaltoside) were bound at seven to eight sites, many in common with those of dodecanoate. The compounds were also bound in the hydrophobic cavity of β -lactoglobulin, but not to ovalbumin. In addition to the generally recognized role of the Sudlow binding region II of serum albumin (localized at the IIIA subdomain) in fatty acid binding, quenching of the fluorescence intensity of tryptophan-214 by 7,8-dibromododecylmaltoside and 12-bromododecanoate also implicate the Sudlow binding region I (subdomain IIA) as a locus for binding of aliphatic compounds. Our data document the usefulness of dodecyl amphipathic compounds as probes of hydrophobic cavities in water-soluble proteins. In conjunction with recent x-ray diffraction analyses of fatty acid binding as the starting point we propose a new symmetrical binding model for the location of nine high-affinity sites on serum albumin for aliphatic compounds.

INTRODUCTION

Detergent molecules are characterized by the presence of segregated hydrophobic and hydrophilic moieties, a property that allows them to self-associate into micellar structures above a certain concentration termed the critical micellar concentration (CMC). The ability to form micelles is the basis for their successful use in solubilization and purification of membrane proteins as a result of their ability to shield hydrophobic protein and lipid surfaces against contact with aqueous solvent (Tanford and Reynolds, 1976; Tanford, 1980; Møller et al., 1986; le Maire et al., 2000). At non-solubilizing concentrations, i.e., at detergent activities below the CMC, the amphipathic properties of detergents also serve useful purposes as a means to permeabilize and perturb membrane structure without solubilization (Heleinius and Simons, 1975; Andersen et al., 1983; McIntosh and Davidson, 1984; Huang et al., 1985; Champeil et al., 1986; Lasch, 1995). This is mainly the result of hydrophobic partitioning of individual detergent molecules at lipid and protein-lipid interfaces (de Foresta et al., 1996; Kragh-Hansen et al., 1993, 1998). However, binding of detergent to Ca²⁺-ATPase probably also takes place at discrete sites at the membrane protein surface (Froud et al., 1986; le Maire et al., 1987; de Foresta et al., 1994).

From data on water-soluble proteins there is evidence that in general nonionic and bile salt detergents (in contrast to sodium dodecyl sulfate (SDS)) bind only weakly, if at all

(Nozaki et al., 1976; le Maire et al., 1980). An exception is serum albumin, a protein that is specialized in the binding of fatty acids, bilirubin, steroids, and a large number of dyes and drugs (Peters, 1996; Kragh-Hansen, 1990; Carter and Ho, 1994). Studies on the crystalline structure of serum albumin have indicated that the protein is built from three homologous domains (I - III), each of which consist of two subdomains (A and B), with distinct helical folding patterns that are connected by flexible loops (He and Carter, 1992). The α -helices of each subdomain line a number of hydrophobic cavities, some of which have been shown to serve as binding pockets for myristate (Curry et al., 1998) and other ligands, particularly in subdomains IIA and IIIA (He and Carter, 1992; Carter and Ho, 1994). Binding data obtained with detergents have shown that serum albumin interacts with Triton X-100 (Makino et al., 1973; Sukow et al., 1980; Tribout et al., 1991), deoxycholate (Makino et al., 1973), and β -octylglucoside (Wasylewski and Kozik, 1979) at a large number of sites, but the relation of this binding compared with that of other ligands is unknown.

In the present investigation we have examined the nature of the interaction of selected detergents with serum albumin and other water-soluble proteins (β -lactoglobulin and ovalbumin). This was done with several purposes in mind. First, we wanted to study detergent-protein interactions more directly than can be done by perturbation of membrane proteins where effects exerted via protein or protein-lipid interactions are not easily distinguished (Kragh-Hansen et al., 1998). Another consideration arose from the realization that the polar properties of detergent molecules ensure sufficient water solubility so that realistic binding isotherms can be obtained below the CMC to test their suitability as probes of hydrophobic environments in proteins and membranes. A third consideration was to obtain

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Address reprint requests to Dr. Jesper V. Møller, University of Aarhus, Department of Biophysics, Ole Worms Alle 185, DK-8000 Aarhus C, Denmark. Tel.: 45-8942-2938; Fax: 45-8612-9599; E-mail: jvm@biophys.au.dk.

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information on the location of the bound detergent molecules; this issue is of some practical interest in relation to the recent use of detergents to prevent nonspecific aggregations during three-dimensional crystallization of water-soluble proteins, presumably by covering hydrophobic patches on the protein surface (McPherson et al., 1986; Arluison et al., 1999). The compounds used as ligands included four nonionic detergents ($C_{12}E_8$, Triton X-100, dodecylmaltoside, and 7, 8-dibromododecylmaltoside), one ionic detergent (SDS), and two fatty acid anions (dodecanoate and 12-bromododecanoate). These compounds are all characterized by a similar size of their hydrophobic moiety but have widely different polar headgroups. Binding characteristics of the nonbrominated compounds were studied by equilibrium dialysis and, in conjunction with the brominated derivatives, by intrinsic protein fluorescence to obtain information on the following questions. To what extent does binding of the compounds to proteins take place at discrete and common hydrophobic cavities rather than nonspecifically at the protein surface? What is the role of the hydrophobic moiety as a determinant of binding characteristics, as opposed to electrostatic charge and bulkiness of the polar headgroup? To what extent are cooperative interactions involved in detergent binding? It was anticipated that answers to some or all of these questions would help to evaluate the nature of the interaction of detergents with proteins. As the study progressed it became clear that the C_{12} ligands were indeed useful as probes of hydrophobic environments at discrete binding sites in proteins. On the basis of available x-ray structural data this allowed us to proceed to propose a comprehensive binding model for the large number of binding sites on serum albumin.

MATERIALS AND METHODS

Materials

Human serum albumin (97% pure) was obtained from AB Kabi (Stockholm, Sweden). The protein was defatted with charcoal in dilute sulfuric acid, pH 3.0, at 0°C (Chen, 1967), dialyzed extensively against deionized water, lyophilized, and stored at -20°C until use. The residual fatty acid content was determined enzymatically to be less than 0.1 mol/mol of protein (Kragh-Hansen and Vorum, 1993). Other proteins used were bovine β -lactoglobulin and chicken ovalbumin, which were bought from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

For the measurement of bound detergent the following labeled compounds were used: octaethyleneglycol[1- ^{14}C]dodecyl monoether ($C_{12}E_8$) and [1- ^{14}C]dodecyl- β -D-maltoside, synthesized from [1- ^{14}C]dodecanol as previously described (Kragh-Hansen et al., 1993), obtained from Centre d'Etudes Nucleaires de Saclay (Gif-sur-Yvette, France); Triton[phenyl- 3H (N)] X-100 from New England Nuclear Corp. (Boston, MA); and dodecyl[^{35}S]sulfate (SDS) and [1- ^{14}C]dodecanoate from The Radiochemical Centre (Amersham, UK). Whenever necessary, radiolabeled detergents were purified by gel chromatography on agarose or Sephadex columns. This was done after addition of unlabeled carrier detergent to separate the radioactive detergent that elutes with the micelle peak ahead of the radioactive degradation products that appear in the eluate, corresponding to the total volume of the column (see Mokus et al., 1998, for further details). The resultant preparations, 100% pure as evidenced by thin layer chromatog-

raphy, were then used directly for the binding experiments. Unlabeled detergents were obtained from the following sources: $C_{12}E_8$ from Nikko Chemicals (Tokyo, Japan); Triton X-100 from Serva (Heidelberg, Germany); SDS from Merck (Darmstadt, Germany); dodecyl- β -D-maltoside (DM) from Biochemica Boehringer (Mannheim, Germany); and dodecanoic acid from Fluka AG (Buchs, Switzerland). The purity of the detergents used was assessed by electrospray mass spectrometry as previously described (Kragh-Hansen et al., 1998). The following brominated compounds were used in conjunction with studies of protein intrinsic fluorescence: 12-bromododecanoic acid (BrDod) from Aldrich (Steinheim, Germany) and 7,8-dibromododecyl- β -D-maltoside (Br $_2$ DM), synthesized as previously described (de Foresta et al., 1996).

Equilibrium dialysis

Detergent binding was quantitated by the use of Dianorm equilibrium dialyzers (Dianorm Geräte, München, Germany). The dialysis cells, with half-cell volumes of 250 μ l carved into cylindrical disks, were assembled pair-wise, together with flat pieces of separating cellophane tubing. For this we used Visking 18/32, ϕ = 1.8 cm, with a molecular mass cutoff of 8000, except in experiments with SDS where Visking 20/32, ϕ = 1.8 cm, with a slightly higher molecular mass cutoff of 10,000–12,000, was used. In all cases the dialysis medium contained 0.01 M Tes (pH 7.5), 0.1 M NaCl, 0.1 mM $CaCl_2$ (except in experiments involving dodecanoate or dodecylsulfate where $CaCl_2$ was omitted), with addition of sodium azide (1 mM) and gentamicin sulfate (20 μ g/ml) to prevent bacterial growth. Control experiments showed that the presence of the antibiotic did not affect binding. Two types of experiments were performed. In *cis*-experiments, 225 μ l of dialysis medium, containing labeled and unlabeled detergent at different concentrations together with a constant concentration of protein (usually 5 mg/ml), was injected into the right-side compartment of the cells. In *trans*-experiments, protein and detergent were added separately to the left and the right half-cell compartment, respectively. After closure of the cell compartments the apparatus was placed in a temperature-controlled water bath at 20°C, and the cells were rotated for 3–6 days. The cell compartments were then emptied, and radioactivity was determined by liquid-scintillation counting. We found that although generally equilibrium was obtained in our experiments some detergent was lost during dialysis. This loss resulted in the following recoveries (\pm SD): $C_{12}E_8$, $88 \pm 6\%$ (n = 148); Triton X-100, $87 \pm 6\%$ (n = 106); DM, $93 \pm 7\%$ (n = 89); SDS, $86 \pm 6\%$ (n = 232); dodecanoate, $89 \pm 12\%$ (n = 281). The loss must be supposed to be caused by adsorption to the plastic of the cell compartments, as it could not be accounted for by adsorption to the cellophane membranes, which amounted to only 1–3%. Concentrations on the *trans* side, indicative of the level of unbound detergent, were obtained from duplicate samples in two to five full binding experiments with an accuracy (SEM) of $\pm 7\%$, with a range of 5–9% in the experiments with different ligands. The levels of bound detergent, calculated from the *cis* data after subtraction of radioactive counts from the *trans* side, were obtained with an accuracy of ± 4 (2–6)%.

Intrinsic fluorescence

The intrinsic fluorescence properties of serum albumin, attributable to the single tryptophan residue at amino acid residue 214 (Minghetti et al., 1986), were used to study the nature of the interaction of the detergents with the protein. In these experiments we recorded in 1-cm quartz cells fluorescence emission spectra of defatted human serum albumin at 20°C after excitation at 290 nm on a Shimadzu RF-5301PC spectrofluorometer. The protein was dissolved at a concentration of 0.5 mg/ml (corresponding to 7.5 μ M) in the same medium as used for the equilibrium dialysis experiments (0.01 M Tes, 0.1 M NaCl, with or without 0.1 mM $CaCl_2$, pH 7.5). Both excitation and emission slits were set at a bandwidth of 3 nm, and the emission spectra of the protein-containing samples were subtracted

for buffer blanks. Ligand-induced changes of the emission spectra were recorded following addition of small volumes of stock solutions of brominated and nonbrominated ligands to the cuvette and subjected to spectral analysis as described in the Results.

Calculations

We found that in most cases our binding data, measured at relatively low concentrations of unbound ligand (from 0 up to 0.1–0.3 mM), could be analyzed in terms of binding models with two classes of non-interacting binding sites, according to the following equation:

$$\bar{v}_L = \frac{n_1 K_1 [L]}{1 + K_1 [L]} + \frac{n_2 K_2 [L]}{1 + K_2 [L]} \quad (1)$$

In this equation, \bar{v}_L is the average number of ligands bound per protein molecule, $[L]$ is the concentration of free ligand, and n_1 , K_1 , and n_2 , K_2 are the number of binding sites and association constants of the first and second binding class, respectively. Calculations were initiated by the use of reciprocal Klotz plots (Kragh-Hansen, 1990) and refined by nonlinear, least-squares analysis with the aid of the MULTI program (Yamasaki et al., 1996). The intrinsic association constants for the serum albumin dodecanoate interaction were also transformed into stoichiometric constants on the basis of the formulae given by Klotz and Hunston (1979).

Inhibition of binding of ligand L by ligand I was analyzed on the basis of a strictly competitive binding scheme, with no interactions between sites, assuming that at a given site, x, there is either no effect of I (Eq. 2a) or there is competitive displacement of L by I (Eq. 2b):

$$\bar{v}_{L(x)} \begin{cases} = \frac{K_{L(x)} [L]}{1 + K_{L(x)} [L]} & (2a) \\ = \frac{K_{L(x)} [L]}{1 + K_{L(x)} [L] + K_{I(x)} [I]} & (2b) \end{cases}$$

In Eqs. 2a and 2b, $\bar{v}_{L(x)}$ and $K_{L(x)}$ is the binding ratio and association constant of the ligand L, respectively, at site x (represented by either K_1 or K_2 in Eq. 1), and $K_{I(x)}$ is the association constant of ligand I at a common binding site (likewise represented by either K_1 or K_2 for I from Eq. 1). In these experiments complete isotherms for ligand L were obtained in the presence of a high and constant concentration of I. The applicability of Eqs. 2a and 2b to account for the experimental binding curves was then evaluated by summing up contributions from all L-sites, assuming various values for the number of common binding sites. Furthermore, to analyze whether the primary binding sites of L and I were common we tested what combinations of strong and weak sites for L and I would best fit the data.

RESULTS

Binding of neutral detergents

Various aspects of our protein binding data obtained with the Dianorm system on the nonionic detergents $C_{12}E_8$, dodecylmaltoside, and Triton X-100 are shown in Figs. 1–3. In all cases equilibrium was established after dialysis for 3–6 days as indicated by agreement between *cis* and *trans* data when detergent had been added either to the protein-containing (open symbols) or protein-free (closed symbols) compartment of the dialysis cells at the start of the dialysis. This was also the case at the highest concentrations used when the passage of detergent across the cellophane membrane was impeded by the

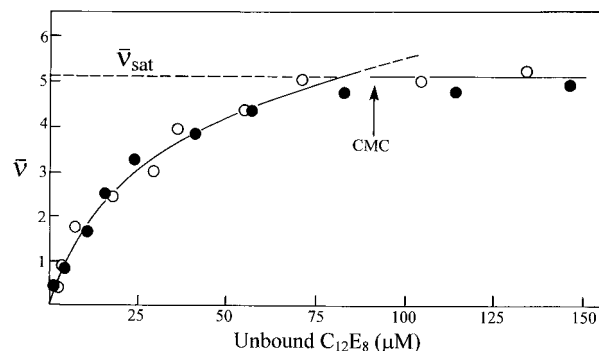


FIGURE 1 Binding isotherm of $C_{12}E_8$ to defatted human serum albumin at 20°C, as determined by Dianorm dialysis at pH 7.5 and $\mu = 0.12$. Albumin was present at a concentration of 75 μ M (5 mg/ml), whereas $C_{12}E_8$ was added to the half-cell compartment at concentrations varying from 38 to 530 μ M. The open symbols show data obtained after *cis* equilibration for 3 days, when $C_{12}E_8$ was added to the same half-cell compartment as albumin; the closed symbols represent *trans* data after equilibration for 3 days, obtained with $C_{12}E_8$ added to the protein-free half-cell compartment. Binding (\bar{v}) in this and the following figures is calculated as the number of moles of ligand bound per mole of protein. Note that there is equilibration both below and above the CMC (90 μ M), with binding reaching an upper level at the CMC. The solid curve below the CMC has been made by use of the n and K values given in Table 1 and Eq. 1.

presence of detergent micelles in one of the cell compartments. Thus, as can be seen from Fig. 1, human serum albumin interacts with $C_{12}E_8$ at several sites, but binding levels off to reach a plateau value at the CMC (90 μ M); i.e., there is no evidence for micellar or cooperative interaction of serum albumin with the detergent.

Fig. 2 compares the binding properties of defatted human

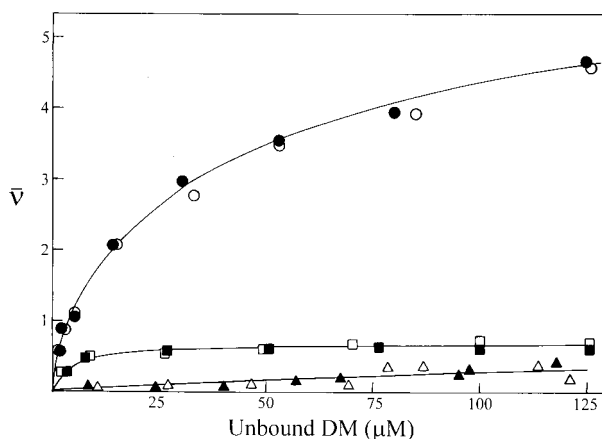


FIGURE 2 Binding isotherms of dodecylmaltoside at 20°C to defatted human serum albumin (\circ , \bullet), β -lactoglobulin (\square , \blacksquare), and ovalbumin (\triangle , \blacktriangle) at pH 7.5 and $\mu = 0.12$. The experiments were performed as described in the legend to Fig. 1, with protein present at a concentration of 5 mg/ml and dodecylmaltoside added to either the *cis* (\circ , \square , \triangle) or *trans* (\bullet , \blacksquare , \blacktriangle) half-cell compartment at initial concentrations varying from 48 to 625 μ M. The solid curves for albumin and β -lactoglobulin have been made by use of the n and K values given in Table 1 and Eq. 1. The curve for ovalbumin has been drawn from the nK value given in the text.

serum albumin, β -lactoglobulin, and ovalbumin with dodecylmaltoside. Although both dodecylmaltoside and $C_{12}E_8$ interact with serum albumin at several sites, the binding stoichiometry for β -lactoglobulin and ovalbumin remains below unity. For ovalbumin, binding is proportional to the concentration of unbound detergent over a wide range, suggesting the presence of a large number of binding sites (n) with a very low affinity for detergent ($nK = 4 \times 10^3 \text{ M}^{-1}$ for both dodecylmaltoside and $C_{12}E_8$). On the other hand, for β -lactoglobulin, the binding data can be accounted for in terms of one binding site only, but with a high binding affinity for C_{12} ligands. Analysis of the binding isotherms of serum albumin and β -lactoglobulin on the basis of Eq. 1 is summarized in Table 1. The calculations indicate the presence of one high-affinity binding site for both $C_{12}E_8$ and dodecylmaltoside on serum albumin, with association constants $> 10^5 \text{ M}^{-1}$ and six to seven secondary binding sites with association constants ~ 30 times lower than K_1 . By comparison, the binding site on β -lactoglobulin has affinities for $C_{12}E_8$ and dodecylmaltoside 4–5 times lower than the primary binding site on serum albumin. The interaction of β -lactoglobulin with detergent is in accordance with the known properties of the protein: thus, β -lactoglobulin, belonging to the lipocalin family has been shown to bind an appreciable number of ligands, including fatty acids, inside a hydrophobic pocket of the protein (Qin et al., 1998). Our data indicate a stoichiometry for this interaction that is slightly lower than unity ($n = 0.85$), which as can be seen from Table 1 was also a characteristic for the binding of dodecanoate by β -lactoglobulin. We examined the possibility of whether this evidence of incomplete binding could be attributed to the presence of endogenous (fatty acid) ligand by subjecting our β -lactoglobulin preparation to the same charcoal defatting procedure as was applied to serum albumin (Materials and Methods). However, we found that this pretreatment had no effect on the binding properties of β -lactoglobulin. Because, in addition, the preparation appeared to be homogeneous both by gel electrophoresis and HPLC size exclusion chromatography, we assume that the incomplete ligand binding calculated for β -lactoglobulin probably either reflects some uncertainty in the determination of protein concentration or is an indication of the

presence of part of the protein in a nonfunctional or denatured state.

In contrast to the alkyl chain detergents, the binding isotherm of Triton X-100 to human serum albumin shown in Fig. 3 is only slightly hyperbolic and does not provide evidence of an approach toward saturation at the CMC (0.25 mM). The binding analysis suggests the presence of discrete binding sites with an affinity of $5.5 \times 10^3 \text{ M}^{-1}$, which is a somewhat lower value than for $C_{12}E_8$ and dodecylmaltoside in the second binding class. In Table 1 they are therefore categorized as belonging to the second class to indicate the absence on serum albumin of binding sites with a very high affinity for Triton X-100. On the other hand, our data indicate that, in addition to these four sites, Triton X-100 is bound at a large number of perhaps 12 additional binding sites with a very low affinity (600 M^{-1}). Previously, for Triton X-100, Makino et al. (1973) also reported the presence of four primary binding sites on serum albumin, but with somewhat higher association constants ($2.2 \times 10^4 \text{ M}^{-1}$ at pH 9.2) and no secondary sites. On the other hand, Sukow et al. (1980), studying the interaction of Triton X-100 with bovine serum albumin both by equilibrium dialysis and calorimetry, proposed a relatively high-affinity binding of the first two detergent molecules with positive cooperativity and the presence of 15 weak and thermodynamically equivalent sites. The binding data given by the latter investigators, which were obtained at pH 7.2 and $\mu = 0.050$, are replotted as the broken line in Fig. 3. It can be seen that their data, apart from the cooperative feature at low values of \bar{v} , are quite similar to ours.

Binding of dodecanoate by serum albumin, β -lactoglobulin, and ovalbumin

As shown in Fig. 4 agreement between *cis* and *trans* binding data for dodecanoate was obtained after dialysis for 3 days

TABLE 1 Detergent binding sites on human serum albumin and β -lactoglobulin

Protein	Ligand	n_1	$K_1 (\text{M}^{-1})$	n_2	$K_2 (\text{M}^{-1})$
Albumin	$C_{12}E_8$	1	7.7×10^5	7	2.6×10^4
β -Lactoglobulin	$C_{12}E_8$	0.85	2.1×10^5		
Albumin	Dodecylmaltoside	1	4.7×10^5	6	1.3×10^4
β -Lactoglobulin	Dodecylmaltoside	0.85	2.4×10^5		
Albumin	Dodecanoate	1a	8.0×10^6	7	9.5×10^4
		1b	5.5×10^5		
β -Lactoglobulin	Dodecanoate	0.85	1.0×10^5		
Albumin	Dodecylsulfate	5	2.4×10^5	4	1.8×10^4
Albumin	Triton X-100			4	5.5×10^3 *

*The analysis also indicated a low-affinity binding class, corresponding to 12 sites with an association constant of 600 M^{-1} .

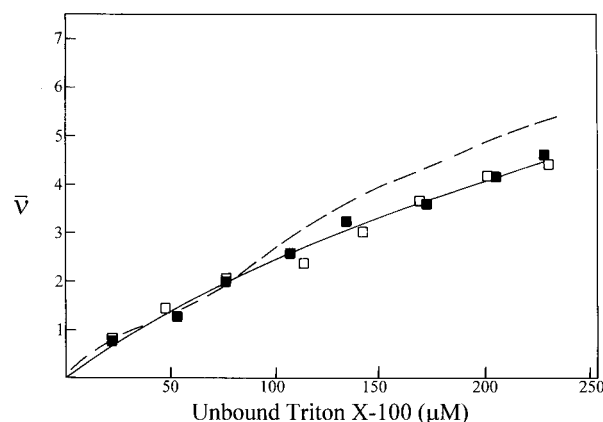


FIGURE 3 Binding isotherm of Triton X-100 at 20°C to defatted human serum albumin at pH 7.5 and $\mu = 0.12$. Initial concentrations of Triton X-100 varied from 100 to 960 μM . *Cis* and *trans* data are shown by the open and closed symbols, respectively. The broken curve shows data obtained by Sukow et al. (1980) on bovine serum albumin at pH 7.2 and $\mu = 0.050$. The solid curve has been made by use of the n and K values given in Table 1 and Eq. 1.

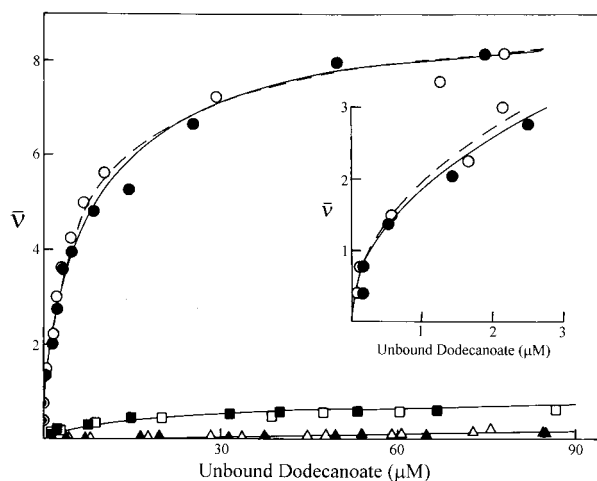


FIGURE 4 Binding isotherms of dodecanoate at 20°C to defatted human serum albumin (○, ●), β -lactoglobulin (□, ■), and ovalbumin (△, ▲) at pH 7.5 and $\mu = 0.12$. *Cis* (○, □, △) and *trans* (●, ■, ▲) experiments were obtained by dialysis for 3–6 days at protein concentrations of 5 mg/ml and with initial dodecanoate concentrations varying from 12 to 630 μ M in the case of β -lactoglobulin and ovalbumin, and from 12 to 2400 μ M for human serum albumin. The inset shows binding of dodecanoate at very low concentrations of unbound dodecanoate, corresponding to the range for the two high-affinity sites for the fatty acid. The solid curves for albumin and β -lactoglobulin have been made by use of the n and K values given in Table 1 and Eq. 1. The curve for ovalbumin has been drawn from the nK value given in the text. The broken curves represent binding data published by Pedersen et al. (1986), analyzed on the basis of the following stoichiometric constants, corresponding to each stoichiometric step: 1) 8.31×10^6 M^{-1} (9.22×10^6 M^{-1}); 2) 1.67×10^6 M^{-1} (1.11×10^6 M^{-1}); 3) 2.88×10^5 M^{-1} (4.47×10^5 M^{-1}); 4) 6.18×10^5 M^{-1} (2.39×10^5 M^{-1}); 5) 1.07×10^2 M^{-1} (1.43×10^5 M^{-1}); 6) 1.98×10^8 M^{-1} (8.91×10^4 M^{-1}); 7) 9.25×10^1 M^{-1} (5.45×10^4 M^{-1}); 8) 1.43×10^7 M^{-1} (3.06×10^4 M^{-1}); 9) 1.86×10^3 M^{-1} (1.32×10^4 M^{-1}); 10) 1.94×10^4 M^{-1} (0). The values in parentheses are calculated on the basis of the values for intrinsic constants given in Table 1.

at levels of unbound dodecanoate up to 0.1 mM (pH 7.5, $\mu = 0.11$). Under these conditions dodecanoate was bound to serum albumin at several sites. On the other hand, binding of the fatty acid to the other proteins examined (β -lactoglobulin and ovalbumin) was low, and only in the case of β -lactoglobulin was there evidence for the presence of a discrete binding site. At levels of dodecanoate above 0.1 mM we consistently observed deviations between *cis* and *trans* data, indicating that under these conditions the dialysis technique was not suitable for binding measurements, due to restricted passage of the fatty acid across the dialysis membranes. This was also the case when we extended the equilibration for 6 days (data not shown). These findings suggest aggregation of fatty acid anions to take place above 0.1 mM. However, the size of these aggregates must be small. This we conclude because we could prepare water-clear solutions of dodecanoate up to a fatty acid concentration of 1 mM at pH 7.5 and $\mu = 0.12$. These are medium conditions in which dodecanoate, according to an investigation by Cistola et al. (1988), does not form classical

micelles and where phase separation, when it occurs, results in the formation of nonsoluble bilamellar sheets.

Table 1 shows that the serum albumin binding data can be analyzed in terms of two strong sites in the first binding class (termed 1a and 1b) and seven sites in the second class. Thus, the total number of discrete binding sites corresponds to, or is a little higher than, the number of binding sites observed with the neutral C_{12} detergents. Furthermore, the association constants are higher, especially that corresponding to the first bound site (1a), which has an extraordinarily high affinity that makes it possible to distinguish it from the association constant for the second high-affinity binding site (1b). By contrast, β -lactoglobulin binds only dodecanoate at its high-affinity site with a moderate affinity, comparable to that of dodecylmaltoside. Furthermore, as in the case of dodecylmaltoside and $C_{12}E_8$, binding of dodecanoate by ovalbumin can be considered unspecific (the fatty acid is bound with a low affinity at an unspecified number of sites: $nK = 2.6 \times 10^3$ M^{-1}). The present data on serum albumin accord well with those obtained in a previous study by Pedersen et al. (1986), as indicated by the broken line in Fig. 4. Pedersen et al. (1986) based the analysis of their data on 10 sites, i.e., a slightly higher number than in our analysis, which were evaluated in terms of the stoichiometric constants enumerated in the legend to Fig. 4. However, the stoichiometric model assumes strongly cooperative properties in the binding mechanism, particularly between steps 5 and 6 and between steps 7 and 8 for which we have no evidence (see Discussion). Otherwise, there is good agreement as can be seen by comparison with our data (shown in parentheses in the legend to Fig. 4 after transformation of the intrinsic constants given in Table 1 into stoichiometric constants).

Binding of dodecylsulfate by serum albumin

With dodecylsulfate it was not possible, as previously reported by Ray et al. (1966), to obtain dialysis equilibrium for concentrated albumin concentrations within a reasonable time. To reach equilibrium after 3–6 days required a reduction in albumin concentration from 75 μ M (0.5%, w/v) to 15 μ M and the use of dialysis membranes with a slightly larger molecular mass cutoff (see Materials and Methods). This is probably due to a change in the pore properties of the cellophane membrane, induced by dodecylsulfate or by the dodecylsulfate-albumin complex, because we observed that the passage of radiolabeled nonionic detergent across the cellophane membranes was likewise retarded in the presence of dodecylsulfate.

The inset in Fig. 5 shows high-affinity binding of dodecylsulfate to defatted, native serum albumin at low concentrations of the detergent, up to 0.1 mM. This concentration of unbound detergent is far below the CMC, which at the ionic strength used in our experiments ($\mu = 0.12$) is estimated to be 1.5 mM (Mokus et al., 1998). It can be seen that

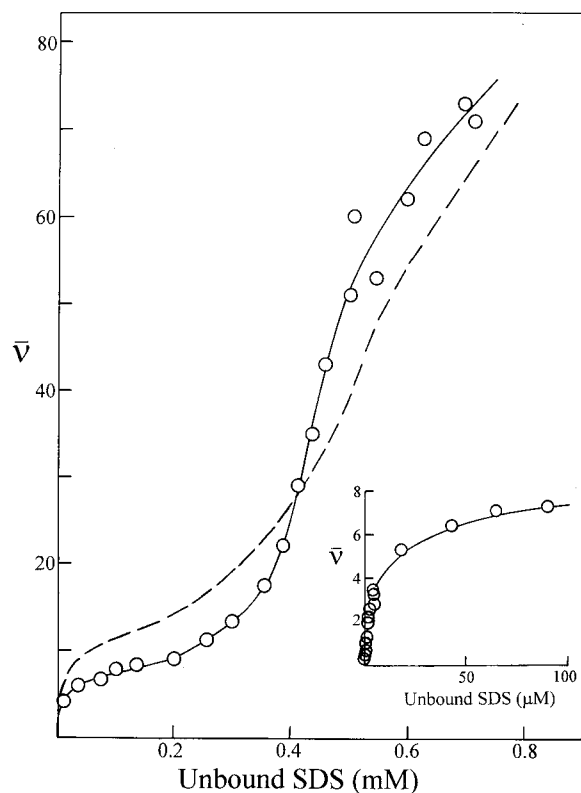


FIGURE 5 Binding isotherm of dodecylsulfate at 20°C to defatted human serum albumin at pH 7.5 and $\mu = 0.12$. *Cis*-binding was measured by dialysis for 3–6 days both at very low (*inset*) and intermediate concentrations, giving rise to unfolding. The initial concentrations of dodecylsulfate varied from 3.5 to 667 μM , whereas the albumin concentration in these experiments was reduced from the usual 75 μM to 15 μM . For comparison, the broken curve shows data obtained with a similar dialysis method by Steinhardt et al. (1971) at a lower ionic strength and pH ($\mu = 0.033$ and pH 6.86). The curve in the inset has been made by use of the n and K values given in Table 1 and Eq. 1.

in the low-concentration range the binding data follow a saturation-like curve that transcends into cooperative binding at higher dodecylsulfate concentrations (main part of Fig. 5). Detailed calculations according to Eq. 1 indicate that high-affinity binding can be described by two binding classes, with five sites in the first class and four sites in the second class (Table 1). The association constants are within the range calculated for the neutral C_{12} detergents. The major difference rests with the presence of five, rather than only one, binding sites in the first binding class. This difference is consonant with the view that although hydrophobic interactions are of primary importance for the binding process, there are secondary contributions to the binding energy arising from electrostatic interactions at four to five sites (Steinhardt and Reynolds, 1969; Tanford, 1972).

High-affinity binding of dodecylsulfate to native human serum albumin has previously been studied by Halfman and Nishida (1972) and Steinhardt et al. (1971). On the basis of fluorescence experiments Halfman and Nishida (1972) pro-

posed detergent binding of dodecylsulfate to two binding classes ($n_1 = 4$ and $K_1 = 2.8 \times 10^6 \text{ M}^{-1}$; $n_2 = 8$ and $K_2 = 10^5 \text{ M}^{-1}$, 0.01 M phosphate buffer containing 0.15 M KCl, pH 7.5). By contrast, Steinhardt et al. (1971), studying dodecylsulfate binding by equilibrium dialysis, reported the presence of only one class of high-affinity sites ($n = 8$ and $K = 2.2 \times 10^6 \text{ M}^{-1}$) but at a lower ionic strength (phosphate buffer, $\mu = 0.033$, pH 6.86; see the broken curve in Fig. 5), which may have contributed to a steeper binding isotherm as the result of increased electrostatic interactions.

Competitive binding of C_{12}E_8 and dodecanoate

The fact that all C_{12} compounds have approximately the same number of binding sites on serum albumin suggests that many of these could be common. We have tested this hypothesis in competition experiments where fixed concentrations of dodecanoate were added to albumin solutions, containing various concentrations of C_{12}E_8 . Dodecanoate was chosen as an inhibitor of detergent binding in these experiments because of its high association constants for binding to serum albumin and the possibility of adding this compound at high concentrations without the formation of micelles. As can be seen from Fig. 6, distinct inhibition of

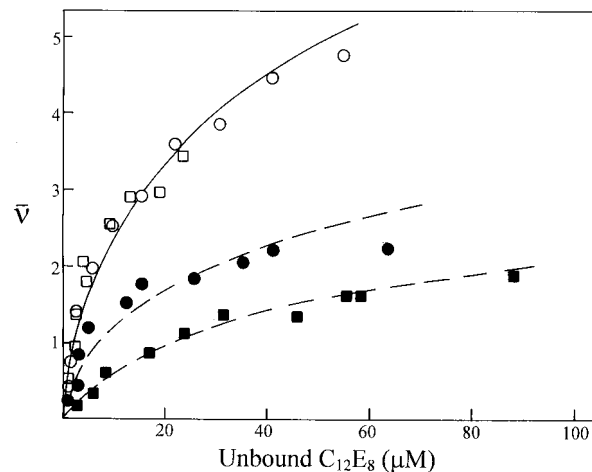


FIGURE 6 Inhibitory effect of dodecanoate at 20°C on binding of C_{12}E_8 to defatted human serum albumin at pH 7.5 and $\mu = 0.12$. *Cis*-binding isotherms of C_{12}E_8 were obtained by varying the concentration of the detergent in the presence of 0.5 mM (●) or 0.9 mM (■) dodecanoate. The unfilled symbols refer to corresponding *cis* binding data obtained in the absence of fatty acid. The solid curve has been made by use of the n and K values given in Table 1 and Eq. 1. The broken curves are simulations of C_{12}E_8 binding, assuming seven common binding sites for C_{12}E_8 and dodecanoate with the association constants indicated in Table 1, competing in the following way: dodecanoate at sites 1a and 1b with two C_{12}E_8 sites in the second class; four dodecanoate sites in the second class with four C_{12}E_8 sites in the second binding class; and one dodecanoate binding site in the second class with the high-affinity site for C_{12}E_8 . With this binding scheme the concentration of unbound dodecanoate was calculated to vary between 20 and 75 μM when added at a total concentration of 0.5 mM and between 250 and 335 μM when added at a total concentration of 0.9 mM.

$C_{12}E_8$ binding was observed that was dependent on the concentration of fatty acid added (0.5 or 0.9 mM). The binding isotherms were subjected to analysis on the basis of a strictly competitive binding scheme (Eq. 2b). The broken curves shown in Fig. 6 represent the best fits to the binding data that we could obtain based on the association constants given in Table 1. It appears from our simulations that the inhibitory effect of dodecanoate on $C_{12}E_8$ binding can be accounted for on the basis of at least seven common binding sites, but with the important proviso that the high-affinity site for $C_{12}E_8$ is different from the two high-affinity sites for dodecanoate. Otherwise, it becomes impossible to simulate the pronounced curvature of the $C_{12}E_8$ binding isotherm persisting at low concentrations of $C_{12}E_8$. This is especially the case when the total concentration of dodecanoate is 0.5 mM; this level of dodecanoate corresponds to an unbound concentration of the fatty acid of $\sim 20 \mu\text{M}$ in the absence of $C_{12}E_8$ and $\sim 75 \mu\text{M}$ at the highest level of added $C_{12}E_8$. In combination with the high association constants for dodecanoate in the first binding class at sites 1a and 1b (Table 1), this concentration level of fatty acid would efficiently displace $C_{12}E_8$ from its primary site, if they were common, and hence result in flattening of the binding isotherm. On the other hand, the curvature observed is a predicted feature if high-affinity binding of $C_{12}E_8$ either is not affected by dodecanoate or, what appears more probable, is common with one of the dodecanoate binding sites in the second class of lower affinity. The experiments suggest that, disregarding the possible effect of allosteric interactions (see below), most of the binding sites for the C_{12} ligands on serum albumin can be considered to be located at common or closely situated sites but that individual features of the ligands may be decisive for the affinity with which they are bound at these sites.

Effect of bound ligands on tryptophan fluorescence

From structural three-dimensional data it has been concluded that the high-affinity binding site(s) for a number of diverse ligands are localized within subdomains IIA or IIIA of the albumin molecule (Carter and Ho, 1994; Peters, 1996). These binding regions, in the nomenclature of Sudlow et al. (1975, 1976), correspond to sites I and II, respectively. Both subdomains IIA and IIIA are characterized by the presence of a central cavity, formed from six amphipathic helices arranged in a myoglobin-like fold. We took advantage of the opportunity provided by the presence of a single tryptophan residue (W-214) in human serum albumin (Minghetti et al., 1986), located in the hydrophobic cavity of the IIA subdomain, corresponding to the so-called Sudlow I binding region (cf. Fig. 7), to monitor ligand-induced spectrofluorometric changes of the environment at this site in subdomain IIA. To be able to distinguish effects caused by binding of ligand at site(s) close to W-214 from effects

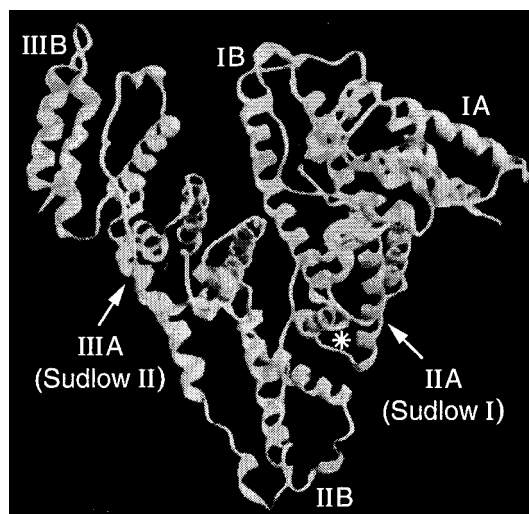


FIGURE 7 Domain organization and main binding regions of serum albumin. The location of the Sudlow I and Sudlow II binding regions and the topology of the IA-B, IIA-B, and IIIA-B domains within the native (heart-shaped) structure of human serum albumin are shown. Furthermore, the position of Trp-214, which is present in the middle of helix H2 in subdomain IIA, is indicated by an asterisk.

caused by binding at other sites, we compared fluorescence emission spectra obtained in the presence of dodecylmaltoside with those obtained with a brominated analog 7, 8-dibromododecyl β -maltoside (Br_2DM); see Fig. 8. This compound has previously proved useful to study tryptophan accessibility in membranous and solubilized sarcoplasmic reticulum Ca^{2+} -ATPase (de Foresta et al., 1996) and in Ca^{2+} -ATPase peptide-detergent complexes (Soulié et al., 1998). Quenching occurs primarily by collisional contacts of bromine atoms with tryptophan, but also has a partly static character under conditions of high medium viscosity (de Foresta et al., 1999). We found that under the standard conditions used for these assays (which correspond to those used in the dialysis experiments) excitation of our defatted human serum albumin preparation at 290 nm resulted in an emission spectrum with a λ_{max} of 341 nm. As can be seen from Fig. 8, addition of increasing concentrations of Br_2DM caused a progressive reduction of the fluorescence intensity, accompanied by a decrease of λ_{max} in the albumin emission spectrum. Thus, the fluorescence was strongly quenched, whereas λ_{max} was reduced from 341 nm to 327 nm by addition of 300 μM Br_2DM (this concentration slightly exceeds the CMC (220 μM) as determined by a micelle dye uptake method (de Foresta et al., 1996)). This suggests nearly complete occupancy of the tryptophan site in subdomain IIA by the detergent molecule, resulting in collisional quenching of the tryptophan residue by the bromine atoms of Br_2DM . Compared with this, addition of dodecylmaltoside gave rise to only a modest reduction of λ_{max} to 337 nm, accompanied by a slight increase, rather than a decrease, in fluorescence intensity, as shown by the dotted and broken curves, labeled DM, in Fig. 8.

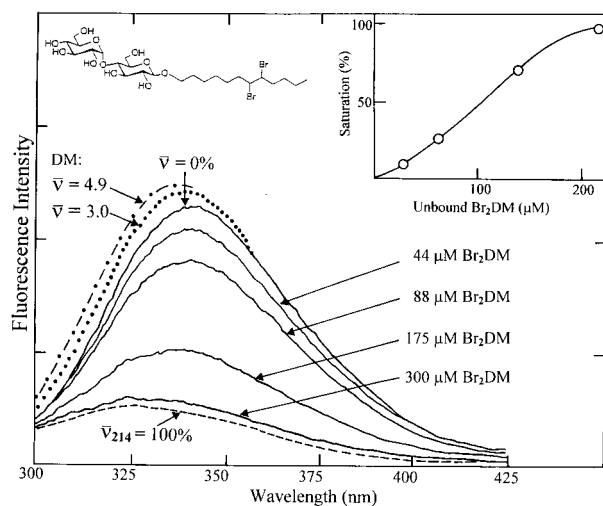


FIGURE 8 Fluorescence emission spectra of human serum albumin in the presence of 7,8-dibromododecylmaltoside and dodecylmaltoside at pH 7.5 and $\mu = 0.12$. The spectra were recorded at 20°C by excitation of 7.5 μ M defatted human serum albumin at 290 nm. The trace labeled $\bar{v} = 0\%$ shows the emission spectrum of albumin in the absence of added ligand. The traces below show the effect of adding 44, 88, 175, and 300 μ M Br₂DM (this will correspond to binding levels \bar{v} of ~ 2.25 , 3.5, 4.75, and 5.5, assuming that Br₂DM binds with similar affinities as dodecylmaltoside). The curve labeled $\bar{v}_{214} = 100\%$ (— — —) shows the curve calculated for complete binding of Br₂DM at the W-214 site. This curve was deduced from the trace recorded in the presence of 300 μ M Br₂DM, assuming nearly complete (96%) saturation of the W-214 region with brominated detergent at this concentration, which just exceeds the CMC (see text; acceptable simulations could be obtained only within a narrow range of saturability (92–100%)). The 0% and 100% curves were used for calculations of binding of Br₂DM at the tryptophan site as shown by the inset. Finally, the broken and dotted curves labeled DM $\bar{v} = 4.9$ and $\bar{v} = 3.0$ show for comparison with Br₂DM the effect of adding 45 and 200 μ M dodecylmaltoside, corresponding to binding levels of 3.0 and 4.9.

In similar experiments 12-bromododecanoate (BrDod) also caused a decrease in λ_{\max} and fluorescence intensity (Fig. 9). However, the quenching with BrDod was not as complete as with Br₂DM, probably because of the presence of only one bromine atom in BrDod as compared with two bromine atoms in Br₂DM. This is consistent with previous observations showing the reduced quenching efficiency of monobrominated as compared with dibrominated compounds (Bolen and Holloway, 1990). In addition, it is possible that the ω -terminal bromine atom of BrDod is located deeper in the protein structure, in a less favorable position for collisional contacts with W-214 than Br₂DM. Assuming binding at the tryptophan site to be nearly maximally saturated at the highest concentration of brominated ligand added (see legends to Figs. 8 and 9) it was possible with both Br₂DM and BrDod to decompose the fluorescence spectral traces into two spectral components, representing 1) albumin with unaltered fluorescence spectrum, i.e., uncomplexed or with ligand only bound at other sites than the tryptophan-containing binding region (no quenching of tryptophan) and 2) albumin with ligand saturating the tryptophan site (maximal quenching of fluorescence). These components are indicated in Figs. 8 and 9 by the traces labeled $\bar{v} = 0\%$ and by the broken curve labeled $\bar{v}_{214} = 100\%$. All the intervening traces, which represent incomplete binding of ligand at the tryptophan site, can be reconstructed by linear combinations of these two curves. The reconstructions are perfect, suggesting that the shape of the W-214 emission spectrum is not significantly affected by brominated ligand bound elsewhere on the albumin molecule. This evidence enabled us to estimate tentative binding isotherms for the interaction of brominated ligand at the tryptophan site as a function of the unbound ligand concentration (see insets in Figs. 8 and 9). From these curves, which is S-shaped in the case of Br₂DM, and hyperbolic for BrDod, it is possible to estimate half-saturation association constants of $\sim 1 \times 10^4$ M⁻¹ for Br₂DM and 3×10^4 M⁻¹ for BrDod for binding at the tryptophan site. It is thus clear by comparison with the data of Table 1 that the W-214 site does not serve as a primary binding locus for the C₁₂ ligands; rather, the association constants at this locus match those obtained for the nonbrominated ligands in the second binding class. The S-shaped isotherm obtained for binding of Br₂DM at the W-214 site could indicate allosteric effects of ligand binding at other sites on the association constant at the tryptophan site. However, as considered below, they

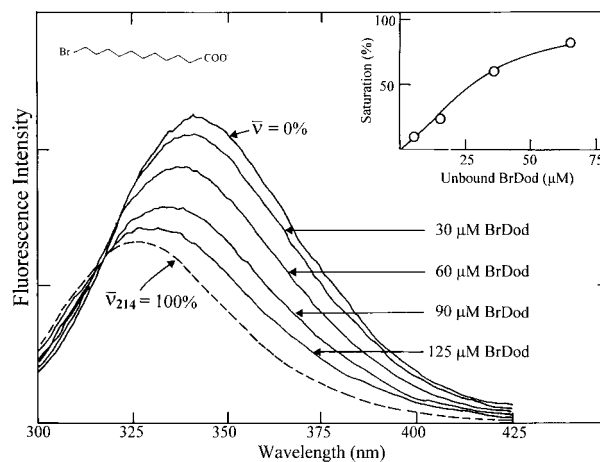


FIGURE 9 Fluorescence emission spectra of human serum albumin in the presence of 12-bromododecanoate at pH 7.5 and $\mu = 0.12$. The spectra were recorded at 20°C by excitation of 7.5 μ M of defatted human serum albumin at 290 nm in the same way as described in Fig. 8. The additions of 30, 60, 90, and 125 μ M BrDod correspond to the binding levels of the fatty acid of 4, 6, 7.4, and 8 mol/mol albumin, assuming similar binding constants for BrDod as for dodecanoate. Note that for low levels of BrDod ($\bar{v} < 3-4$) we could not discern any effect of addition of the fatty acid on the emission spectrum. The broken curve represents the spectrum for complete binding of BrDod at the W-214 site, as deduced from an assumption of 80% binding at the tryptophan site at 125 μ M BrDod. Variations within the 70–90% range will give acceptable reconstructions; these variations, although slightly affecting the λ_{\max} and fluorescence maximum for the BrDod curve corresponding to 100% saturation of the tryptophan site, will only slightly affect the isotherm deduced for binding of BrDod at the tryptophan site shown in the inset.

tophan site (maximal quenching of fluorescence). These components are indicated in Figs. 8 and 9 by the traces labeled $\bar{v} = 0\%$ and by the broken curve labeled $\bar{v}_{214} = 100\%$. All the intervening traces, which represent incomplete binding of ligand at the tryptophan site, can be reconstructed by linear combinations of these two curves. The reconstructions are perfect, suggesting that the shape of the W-214 emission spectrum is not significantly affected by brominated ligand bound elsewhere on the albumin molecule. This evidence enabled us to estimate tentative binding isotherms for the interaction of brominated ligand at the tryptophan site as a function of the unbound ligand concentration (see insets in Figs. 8 and 9). From these curves, which is S-shaped in the case of Br₂DM, and hyperbolic for BrDod, it is possible to estimate half-saturation association constants of $\sim 1 \times 10^4$ M⁻¹ for Br₂DM and 3×10^4 M⁻¹ for BrDod for binding at the tryptophan site. It is thus clear by comparison with the data of Table 1 that the W-214 site does not serve as a primary binding locus for the C₁₂ ligands; rather, the association constants at this locus match those obtained for the nonbrominated ligands in the second binding class. The S-shaped isotherm obtained for binding of Br₂DM at the W-214 site could indicate allosteric effects of ligand binding at other sites on the association constant at the tryptophan site. However, as considered below, they

could also be an indication of the presence of two binding sites in the W-214 region.

The interaction of nonbrominated ligand with serum albumin followed a characteristic pattern that at high binding levels resulted in an increase in fluorescence intensity, accompanied by a reduction in λ_{\max} . This is demonstrated in Fig. 10 with dodecanoate as a representative example. Corresponding to binding involving the primary sites (up to $\bar{\nu} = 2$), there is only a slight increase in fluorescence intensity, whereas λ_{\max} remains unchanged. Addition of more dodecanoate causes a further rise in fluorescence intensity, accompanied by a decrease of λ_{\max} , an effect that reasonably can be attributed to an increased hydrophobicity of the region surrounding the tryptophan site. A biphasic effect on the emission spectra, corresponding to the binding at primary and secondary sites, respectively, was also observed with $C_{12}E_8$ and dodecylsulfate (data not shown). It follows from these results that nonbrominated ligand probably affects tryptophan fluorescence both as the result of direct interaction with this site as well as to a lesser extent by allosteric effects, resulting from interactions at other sites. Furthermore, the correlation between the decrease in λ_{\max} and binding in the second class lends support to the data obtained with brominated ligands by showing that W-214 is not a site for primary interaction with C_{12} ligands.

A summary of the spectral changes observed with nonbrominated ligands is assembled in Table 2 from which it can be seen that both $C_{12}E_8$ and dodecylmaltoside give rise to smaller changes in W-214 fluorescence than dodecanoate and dodecylsulfate. There is little doubt that the more mod-

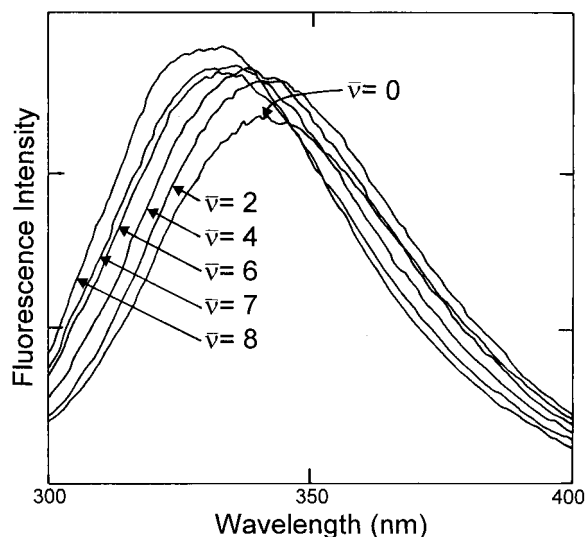


FIGURE 10 Emission spectra of human serum albumin in the presence of dodecanoate at pH 7.5 and $\mu = 0.12$. The spectra were recorded at 20°C by excitation of 7.5 μ M defatted human serum albumin at 290 nm in the same medium as described in Fig. 8. Dodecanoate was added at concentrations of 16, 30, 60, 90, and 125 μ M, which according to the binding isotherm for dodecanoate (Fig. 4 and Table 1) results in binding levels of $\bar{\nu} = 2, 4, 6, 7$, and 8 mol of fatty acid/mol albumin.

TABLE 2 Detergent-induced fluorescence changes of human serum albumin

Ligand	λ_{\max}^* (nm)	I^\dagger	$\bar{\nu}$
None	341	1.00	0
$C_{12}E_8$	333	1.16	5 [‡]
Dodecylmaltoside	333	1.10	5 [‡]
Dodecanoate	331	1.21	8 [§]
Dodecylsulfate	325	1.31	8 [§]

*Emission maximum after excitation at 290 nm.

[†]Ratio between fluorescence intensity at λ_{\max} for liganded versus unliganded albumin.

[‡]Binding level of detergent at the CMC.

[§]Ligand was added to the albumin solutions to give the indicated values of $\bar{\nu}$.

erate effects observed with the nonionic C_{12} detergents, at least in part, reflect the lower levels of detergent binding, resulting from the restrictions imposed by the formation of micelles, which prevents full binding at the secondary sites. It should be noted that the quenching effect of brominated ligands could be only partially reversed by nonbrominated ligand, as shown in Fig. 11, by addition of high concentrations of dodecanoate to an albumin-Br₂DM mixture. Furthermore, under these conditions, λ_{\max} was reduced to a lower level (323 nm) than observed in the presence of either ligand alone. These findings may indicate that dodecanoate can be bound jointly with Br₂DM at the tryptophan site instead of resulting in a fully competitive displacement of the latter from this site.

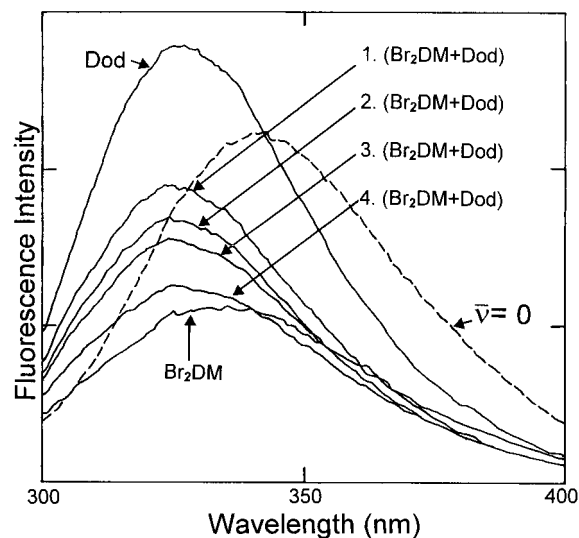


FIGURE 11 Partial reversal of quenching with 7,8-dibromododecylmaltoside by high concentrations of dodecanoate. Human serum albumin and Br₂DM were present at a concentration of 7.5 and 175 μ M, respectively, whereas the dodecanoate concentration was varied as follows: trace 1, 500 μ M; trace 2, 200 μ M; trace 3, 100 μ M; and trace 4: 50 μ M. In the trace labeled Br₂DM there was no addition of dodecanoate, and in the curve labeled Dod, dodecanoate was present at a concentration of 500 μ M without Br₂DM. The broken curve labeled $\bar{\nu} = 0\%$ shows the emission spectrum in the absence of ligands.

DISCUSSION

General aspects of hydrophobic and electrostatic protein-ligand interactions

The present data emphasize the unique binding capabilities of serum albumin, as compared with other water-soluble proteins. Thus, we could analyze detergent binding in terms of seven to eight high-affinity sites for $C_{12}E_8$ and dodecylmaltoside, whereas dodecanoate and dodecylsulfate under the same conditions were bound at nine sites and with a somewhat higher affinity. These observations fit well with the commonly held view that hydrophobicity is the predominant driving force for binding of anionic ligands like fatty acids, but assisted by electrostatic interactions between the carboxylate group and basic amino acid residues (Spector, 1975; Reed, 1986; Cistola et al., 1987; Hamilton et al., 1991; Pedersen et al., 1995). On the other hand, bulkiness of the headgroup such as is a characteristic of polyethylene glycol detergents ($C_{12}E_8$) does not seem to be an obstacle for ligand binding.

The number of high-affinity binding sites for the C_{12} ligands that we have detected in our study is higher than the six sites generally reported for long-chain (Peters, 1996) and medium-chain (Curry et al., 1999) fatty acids. It is also higher than found for most aromatic and heterocyclic compounds of a similar size where the number of high-affinity sites is usually limited to ~ 4 – 6 moles per mole of albumin (Kragh-Hansen et al., 1974; Kragh-Hansen, 1988). This low number was also found in the present study for Triton X-100; in accordance with previous reports (Sukow et al., 1980; Makino et al., 1973), we detected only about four discrete binding sites and with a somewhat lower affinity than for $C_{12}E_8$ and dodecylmaltoside. Probably, the flexibility provided by the dodecyl chain allows $C_{12}E_8$ and dodecylmaltoside molecules more degrees of freedom to insert into the hydrophobic cavities of serum albumin.

With β -lactoglobulin we obtained evidence for binding of $C_{12}E_8$, dodecylmaltoside, and dodecanoate at a single high-affinity site (Table 1). This binding site evidently is located inside the hydrophobic cavity formed by the β -barrel construction of this protein (Qin et al., 1998). By contrast, the same ligands were only nonspecifically bound by ovalbumin, which lacks an internal binding site. These data support the view that dodecanoate and detergents with dodecyl chains can be used as probes of hydrophobic cavities inside water-soluble proteins.

Binding model for serum albumin

With respect to serum albumin, considerable information is available from x-ray diffraction studies on the three-dimensional structure of this protein and the location of various binding sites (He and Carter, 1992; Carter and Ho, 1994; Curry et al., 1998, 1999; Bhattacharya et al., 2000). It has been found that most of the binding sites are placed inside

the amphipathic helices (H1–6 and H7–10) that cluster to form subdomains I–IIIA and I–IIIB. Fig. 12 indicates common features in the construction of subdomains A and B on the albumin molecule. With subdomain IIIA as a prototype Fig. 12 *A* shows that H3 and H4 form the floor, whereas H1–H2 together with H5–H6 form the sides and the roof of the internal cavity. Bound fatty acid is sandwiched between these helices by the formation of tunnels, open at both ends and lined by hydrophobic amino acid residues (Curry et al., 1998). In the case of subdomain IIIA, the interior is able to bind the hydrocarbon chains of two myristate molecules, whereas the carboxylate groups of the ligands interact with nearby basic amino acid residues belonging either to the same or a neighboring subdomain (as exemplified by *Myr-4*, which interacts with R-410 in IIIA, and *Myr-3*, which interacts with R-485 in IIIA and with R-348 in the preceding IIB subdomain). Fig. 12 *B* shows that subdomains B have a similar construction as the A subdomains, except that they are composed of only four amphipathic helices (H7–H10) that are homologous to H1–H4. So far, the ability of a subdomain to concomitantly bind two ligands has been established only for subdomain IIIA (the Sudlow II binding region), which can accommodate two myristate molecules, and subdomain IB, which can accommodate one myristate and one aromatic ligand (Curry et al., 1998). In this model of the description of myristate binding there is the conspicuous absence of binding sites inside subdomains IA, IIB, and IIA, i.e., the Sudlow I binding region, leading to the conclusion that ligand binding by serum albumin is asymmetrical (Curry et al., 1998). This original structural description of fatty acid binding is consistent with a number of protein-chemical studies on bovine serum albumin, obtained by the use of proteolytic fragments and palmitate (Reed et al., 1975, 1976), chemical labeling of lysine residues with affinity derivatives of palmitate (Reed, 1986), and NMR studies with medium- and long-chain ^{13}C -enriched fatty acids (Cistola et al., 1987; Hamilton et al., 1991). All of these studies have led to the suggestion of three high-affinity fatty acid binding regions on serum albumin, localized to subdomains IB, IIIA, and IIIB. However, none of these models account for the potential of serum albumin to bind nine dodecanoate and dodecylsulfate molecules with an appreciable affinity as indicated by our study. To obtain further information on the location of hydrocarbon ligands we investigated the perturbing effect that these compounds exert on the fluorescence properties of the lone tryptophan residue present in human serum albumin as residue W-214. This study included the use of brominated ligands, which previously were shown to severely quench tryptophan fluorescence by direct contact with bound ligand, mainly according to a collisional mechanism (Bolen and Holloway, 1990; de Foresta et al., 1996). We found that both 7,8-dibromododecylmaltoside and 12-bromododecanoate strongly reduced the tryptophan fluorescence of human serum albumin. Furthermore, we found that λ_{max} of trypto-

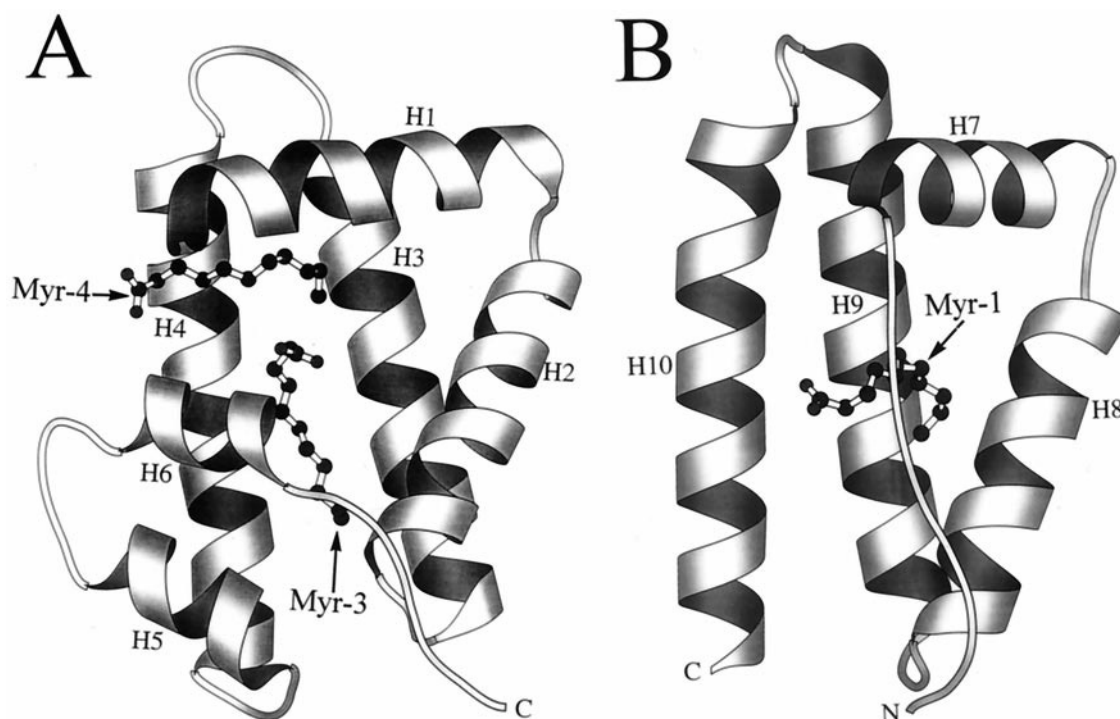


FIGURE 12 Construction and intra-domain binding of fatty acid by the A and B subdomains of serum albumin. (A) The six helices (H1–H6) and (B) the four helices (H7–H10) of the human serum albumin IIIA and IB subdomains, with their connecting loops. In addition, the location of bound myristate, corresponding to the Myr-3, Myr-4, and Myr-1 binding sites, are shown.

phan is reduced by both brominated and nonbrominated ligands, indicative of a decreased polarity of the tryptophan environment. In the tertiary structure of human serum albumin, W-214 is located on the inside of H2 in subdomain IIA. The detailed analysis of the fluorescence data (Figs. 8–11) implicates W-214 as a participant in the binding of dodecyl-containing ligands in the second binding class.

On the basis of the above findings we are therefore in a position to propose subdomain IIA, which already is identified as the structural basis for the Sudlow I site, also to have a binding site for the various dodecyl-containing compounds that we have examined. This conclusion is now confirmed in the most recent study on x-ray diffraction of albumin fatty acid complexes, published after the completion of our study (Bhattacharya et al., 2000), from which it is concluded that the Sudlow binding region I contains a common binding site for short-chain, medium-chain, and long-chain fatty acids. On the basis of homology considerations and three-dimensional structure, other putative binding sites are subdomains IA and IIB. In the most recent publication on serum albumin binding by Bhattacharya et al. (2000) the authors recognized the presence of a dodecanoate and myristate binding site in subdomain IA (designated as site 2'). With respect to subdomain IIB, Curry et al. (1998, 1999) note that the hydrophobic cavity of this subdomain is restricted especially by the presence of bulky aromatic amino acid side chains inside (F-330 and Y-353) the cavity and, as part of a helical stretch in the sequence

linking subdomains IIB and IIIA, just outside the cavity (F-309). Nevertheless, as can be seen from Fig. 13, sequence comparisons indicate strong similarities of IIB with IB and IIIB, which, as discussed above, each have been identified as having a binding site for medium- and long-chain fatty acids. The homology is especially striking between IB and IIB. Thus, in IB there are aromatic residues (Y-138 and Y-161) at exactly the same position within the domain as the aromatic residues that are reported to be obstructive in IIB (F-330 and Y-353). In the description of the IB site given by Curry et al. (1999) it is noted that the Y-138 and Y-161 residues in the liganded state are rotated 90° away to accommodate the incoming myristate molecule. Given the similarity of subdomains IB and IIB both with regard to their secondary and tertiary structure, the potential for binding of dodecyl-containing compounds can be envisaged by similar adjustment of the occluding aromatic residues. As can be seen from Table 3, our proposal of a binding site in subdomain IIB accounts for the presence of nine high-affinity binding sites on serum albumin for dodecanoate and dodecylsulfate suggested by our study. As an alternative to a binding site location in IIB, the ninth binding site could be present in the IB, IIA, IIB, and IIIA inter-domain cleft, located in the middle of the heart-shaped albumin molecule (cf. Fig. 7), which was found to bind two dodecanoate molecules (Bhattacharya et al., 2000), or in the IIIA/IIIB interface region.

Use of dodecyl-containing compounds as probes of hydrophobic cavities in proteins

According to our study on human serum albumin and other proteins, dodecyl-containing amphipathic compounds bind with a high affinity to hydrophobic cavities in water-soluble proteins. They thus commend themselves as probes with which to investigate the hydrophobic properties of the clefts or cavities that form the active site in water-soluble enzymes, the antigen-combining site of antibodies, and a host of extracellular and intracellular proteins that like β -lactoglobulin bind ligands of a hydrophobic nature. Their properties for this purpose may be attributed to a small cross-sectional area and flexibility that permit alkyl-containing compounds to more easily adapt inside hydrophobic cavities than, e.g., aromatic compounds such as Triton X-100. In general, dodecyl compounds, as compared with other alkyl compounds with either a shorter or a longer chain length, seem to be the best compromise between, on the one hand, having the water solubility required to reveal the full extent (capacity) for hydrophobic binding and, on the other hand, having a long enough hydrocarbon chain to maintain a sufficient affinity for proper determination of protein-binding isotherms. Furthermore, the use of brominated derivatives of these compounds will permit the establishment of sensitive and quick fluorescent assays to record collisional and/or static quenching in those cases where a nearby tryptophan residue is present, e.g., to enable studies of changes in hydrophobic properties of active sites during catalysis. Finally, C_{12} ligands with a charged headgroup offer the opportunity to examine the effect of electrostatic interactions at the binding sites. Extension of such methods to membrane proteins is more problematic, due to the nonspecific interaction of hydrophobic compounds with the membrane lipid of biological membranes (Kragh-Hansen et al., 1998). It is nevertheless feasible, and for instance, brominated derivatives of hydrophobic compounds (e.g., sterols and pyrethroids) have previously been used to test the presence of hydrophobic binding sites on the membranous Ca^{2+} -ATPase (Michelangeli et al., 1990a,b). In selected cases, dodecyl-containing compounds may be useful also for this purpose, e.g., for membrane proteins with hydrophobic cavities like P450 (Mueller et al., 1995), which can be prepared as functional oligomeric complexes in the absence of lipid and detergent (Behlke, 1992) or which like the Pgp multidrug resistance protein is involved in high-affinity binding and transport of substrates of this kind (Orlowski et al., 1998).

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