The Binding of Some Long-Chain Fatty Acid Anions and Alcohols by Bovine Serum Albumin*

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ABSTRACT: The binding isotherms of three aliphatic long-chain carboxylates (dodecanoate, decanoate, and octanoate) and a long-chain alcohol (decanol) to native bovine serum albumin have been measured. None of these ligands induces massive disruption of protein structure such as has been observed with dodecyl and tetradecyl sulfates. A summary of the number of sites and association constants for the 13 ligands studied to date in this laboratory (homologous sulfates, sulfonates, and alcohols) is presented and offers support for the previously proposed model for ligand-induced unfolding in proteins. An unexpected difference between carboxylates and the other ligands studied is that the ultraviolet difference spectra resulting from carboxylate binding show *red* rather than blue shifts in the $\pi \rightarrow \pi^*$ transition of the aromatic chromophores.

Carboxylates conform to the other ligands, however, in producing red shifts in the short-wavelength region (230 mμ). It is suggested that the red shifts are produced by tyrosine side chains, and that the sign of the shift is characteristic of protein complexes in which the higher energy tryptophan-associated sites are not also engaged.

Two previous publications in this series (Ray *et al.*, 1966; Reynolds *et al.*, 1967) have presented binding data for two homologous series of long-chain detergent anions and a few selected corresponding alcohols and hydrocarbons to bovine serum albumin (BSA).[1] The number of sites and corresponding association constants in the native and unfolded protein for these ligands were determined. Major protein phase transitions, when they occur, were followed by means of viscosity and optical properties. The significant conclusions of the previous work were as follows. (1) Binding on a limited number of high-energy sites of native BSA is accompanied by small perturbations in optical properties of the protein which are not associated with massive disruption of three-dimensional structure (see also Polet and Steinhardt, 1968). (2) Only dodecyl and tetradecyl sulfates cause unfolding of the native macromolecule, as evidenced by hydrodynamic and optical measurements. (3) When comparing compounds of identical hydrocarbon tail lengths but different polar end groups, the affinity of the protein for the ligand decreases in the order $SO_4^- > SO_3^- >$ OH. (4) The number of high-energy sites on the native protein decreases with decreasing length of the hydrocarbon tail, but the binding energies are not significantly different within the same series (*i.e.*, same polar end group).

The present work includes the binding isotherms of sodium dodecanoate, decanoate, and octanoate, and of decanol to BSA. The optical properties of the protein in the ultraviolet region and the hydrodynamic properties are also given as a function of binding. A complete summary of all the binding studies conducted on BSA in the current as well as previously published work from this laboratory shows that the binding affinities for all the ligands investigated are $SO_4^- > SO_3^- > COO^- = OH$ with the single exception of the shortest chain alcohol, octanol, which shows a much lower binding affinity than octanoate. When the ligand is a sulfate, sulfonate, or carboxylate, the number of high-energy sites on native BSA decreases as the length of the hydrocarbon tail decreases. The three alcohols studied, however, appear to have the same small number of high-energy sites within the limits of experimental error.

The ultraviolet difference spectra of sulfates, sulfonates, and alcohols bound to BSA have been shown to be qualitatively identical up to the occupation of 10–15 binding sites (Polet and Steinhardt, 1968). However, the difference spectra of the carboxylates bound to BSA are much different than those observed for the previously mentioned ligands. No protein phase transitions in the presence of carboxylates, up to very large values of ligand equilibrium concentration, can be detected by hydrodynamic measurements.

Experimental Section

Materials

Carboxylates labeled with ^{14}C were obtained from the Radiochemical Centre, Amersham, England, at

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[1] The abbreviation used in this paper that is not defined in *Biochemistry 5*, 1445 (1966) is: BSA, bovine serum albumin.

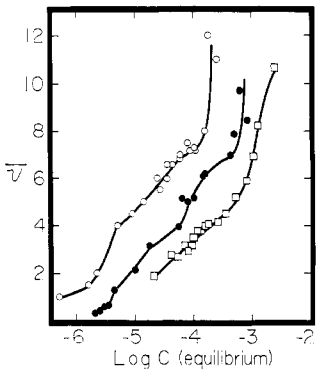

FIGURE 1: Binding isotherms for octanoate, decanoate, and dodecanoate ions and 0.1% BSA at 2°. The lines drawn through the data are not to be construed as more than a visual aid distinguishing the sets. (○) Sodium dodecanoate, (●) sodium decanoate, and (□) sodium octanoate.

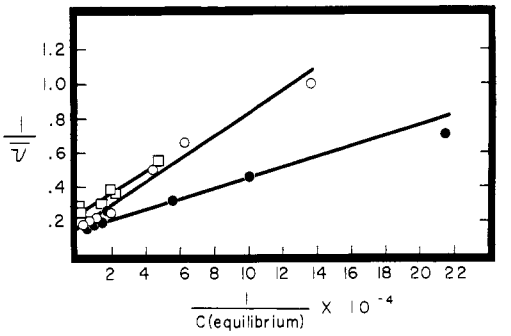

FIGURE 2: Reciprocal plots of isotherms of Figure 1. The reciprocals of the ordinates at $1/C = 0$ gives n, the number of high-affinity sites. The abcissa as given applies to decanoate and octanoate. The exponent should be read 10^{-5} in the case of dodecanoate. Symbols as in Figure 1.

greater than 99% purity by the criterion of thin-layer chromatography.

1-Decanol labeled with ^{14}C was obtained from Baird-Atomics and diluted 1:10 with 1-decanol (Purissima grade) from ICN.

Crystalline BSA (Nutritional Biochemicals, lot 9385) was deionized on a mixed-bed ion-exchange column (Bio-Rad AG 501-X8) containing 1 in. of Dowex 501-X4 at the bottom. In all experiments described herein a 0.1% BSA solution was used. No effort was made to remove any residual fatty acid which may have survived the deionizing procedure. Wishnia and Pinder (1964) and Scatchard *et al.* (1959) have found that protein deionized by the methods given here gives binding data which do not differ significantly from material treated by acid–heptane–isooctane as described by Goodman (1957). J. Cassel in our own laboratory (unpublished data) has made similar observations in the low $\bar{\nu}$ range with picrate ion.

Mukerjee (1965) has measured the dimerization constants of long-chain fatty acids. Calculations based on these constants show that C_{12}, C_{10}, and C_8 carboxylic acids are almost totally in the monomeric form through the concentration range employed in the present measurements to determine the number of high-energy sites and their association constants (*i.e.*, log $C < -4.5$ for dodecanoate, log $C < -3.5$ for decanoate, and log $C < -3.0$ for octanoate).

The preparation of 0.033 ionic strength phosphate buffers has been described previously (Ray *et al.*, 1966). All binding measurements with the carboxylates were carried out at pH 6.8. The binding of decanol to BSA was determined at pH 5.6 at the same ionic strength.

Methods

The procedure for determining binding isotherms with radioactive ligands has been described previously (Ray *et al.*, 1966).

Viscosity measurements were made in Cannon-Fenske viscometers having flow times from 100 to 600 sec. The constant-temperature bath was thermostated at 2 ± 0.005°. Solutions were prepared by calculating the appropriate ligand concentrations from the binding isotherms. No evidence of viscosity dependence on rate of shear was noted. In a previous publication (Reynolds *et al.*, 1967) viscosity measurements were reported for 0.1% BSA solutions containing various amounts of bound long-chain sulfates and sulfonates. The stock solutions in the earlier work (5% BSA) were passed through Millipore filters which contain small amounts of dodecyl sulfate. The specific viscosities of BSA–detergent complexes published by Reynolds *et al.* (1967) were spot checked with solutions not exposed to Millipore filters and were found to be identical. Contamination of the protein solutions with dodecyl sulfate apparently did not occur since large volumes of concentrated solution were passed through the filter and diluted to 0.1% protein prior to measurement. In the present work no solutions were exposed to Millipore filters.

Ultraviolet difference spectra were obtained with 0.1% BSA using a Cary 14 recording spectrophotometer with matched cells of 5-cm (λ 3000–2600 Å) and 1-cm (λ 2600–2200 Å) path length. Some difficulty with light scattering was encountered with the longer path-length cells when carboxylate ligands were used. However, in the difference spectra reported herein, only those spectra were used in which $\Delta\epsilon$ was zero at 3200 Å. In addition, the applicability of Beer's law was determined by examining the difference spectra at the highest $\bar{\nu}$ value reported at 1.0% BSA in 1-cm (λ 3200–2600 Å) and 2-mm (2600–2200 Å) cells.

Results

Binding Isotherms. The binding isotherms at 2° for sodium dodecanoate, decanoate, and octanoate are shown in Figure 1. The lower $\bar{\nu}$ values are accurate within 10%; higher $\bar{\nu}$ values have an uncertainty of ±1. Values of C are uniformly reliable to better than 5%, except where $C < 10^{-6}$ where the uncertainty may rise to 20%. Reciprocal plots ($1/\bar{\nu}$ *vs.* $1/C_{eq}$, where $\bar{\nu}$ = molar ratio of bound ligand to protein and C_{eq} = equilibrium concentration of ligand) are shown in Figure 2. Assuming that the sites are characterized by identical association constants, the intercept at $1/C_{eq} = 0$ gives n, the

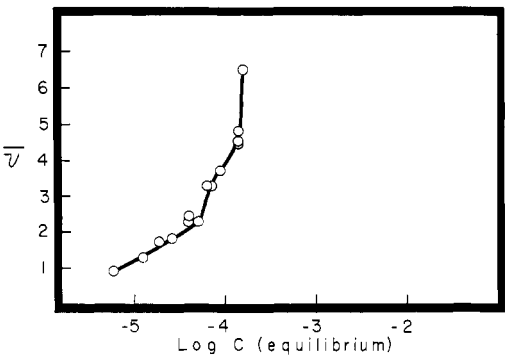

FIGURE 3: Binding isotherm for decanol and BSA at 2°.

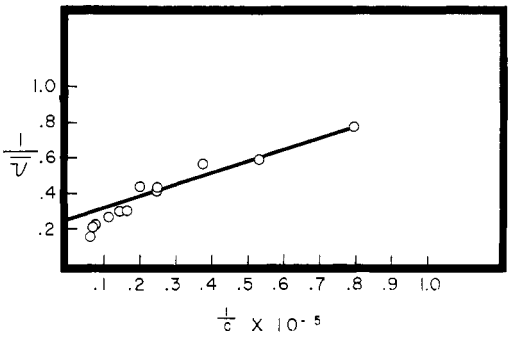

FIGURE 4: Reciprocal plot of isotherm of Figure 3.

number of identical sites on the native protein, and the slope of the line in Figure 2 is $1/nK$, where K is the association constant for the protein sites. As in the previous work from this laboratory, no correction has been made for w, the electrostatic interaction factor. For a further discussion of this point, see Reynolds *et al.* (1968).

The results of the above analysis are presented in Table I together with a compilation of the binding data for the other ligands reported in the previous two papers of this series. It is obvious that the pattern observed previously of decreasing n with decreasing hydrocarbon tail length is also observed for the carboxylate anions. In addition, the association constant is lower for these ligands than for the corresponding sulfates or sulfonates.

The sharp increase in $\bar{\nu}$ at $C_{eq} > 10^{-4}$ M which might suggest a cooperative process does not represent the liberation of additional binding sites by unfolding, a conclusion based on the hydrodynamic data and ultraviolet difference spectra reported in this paper. The binding isotherm and reciprocal plot for the binding of decanol to 0.1% BSA are given in Figures 3 and 4. The values of n and K from these data are included in Table I. Note that the number of sites of all alcohols appears to be identical within experimental uncertainty although the K values decrease with decreasing hydrocarbon tail length. Again, no evidence of protein unfolding is observed by hydrodynamic or optical criteria.

Viscosity. Figure 5 presents the viscosity data for 0.1% BSA as a function of $\bar{\nu}$ for the four ligands investigated. There is no evidence of large shape or volume changes such as were found with dodecyl and myristyl sulfates and which must accompany unfolding of three-dimensional structure.

Ultraviolet Difference Spectra. Figures 6–8 present the ultraviolet difference spectra due to binding by BSA of carboxylate anions. Experimental points are plotted in the figures every 50 Å for ease in following the curves which were calculated from optical density differences at 10-Å intervals along a continuous tracing on the recorder of a Cary Model 14 spectrometer. Significantly, the optical perturbations are very different from those observed with the sulfates and sulfonates (Polet and Steinhardt, 1968). The carboxylates cause an apparent red shift in both the long-wavelength aromatic transitions and the short-wave-

TABLE I: Numbers of High-Energy Binding Sites and Association Constants of BSA for Certain Homologous Long-Chain Anions at 2°.

Ligand	n	K
Tetradecyl sulfate	10–11	1×10^6
Dodecyl sulfate	8–9	1.2×10^6
Decyl sulfate	5–6	1.4×10^6
Octyl sulfate	4–5	6×10^5
Dodecylsulfonate	6	3×10^5
Decylsulfonate	5	9×10^5
Octylsulfonate	3	1×10^5
Dodecanoate (laurate)	6–7	2.3×10^5
	2[a]	1.6×10^{6a}
	5[a]	2.4×10^{5a}
Decanoate	6–7	6×10^4
Octanoate	4–5	5×10^4
	5[b]	1.2×10^{4b}
Dodecanol	4–5	1.5×10^5
Decanol	4–5	7×10^4
Octanol	4–5	3×10^3

[a] Goodman (1958), who also gave data for considerably longer chains, including two unsaturated fatty acids. The highest binding constants given are for stearate [k_1 (two sites) = 8×10^7 and k_2 (five sites) = 8×10^5] and oleate [k_1 (two sites) = 1.1×10^8 and k_2 (five sites) = 4×10^6]. [b] Teresi and Luck (1952).

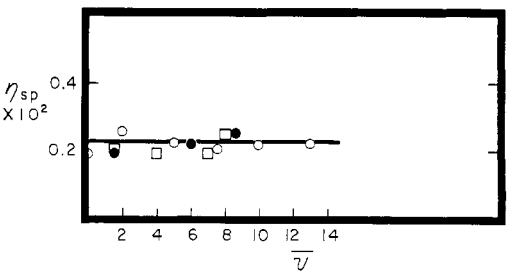

FIGURE 5: Specific viscosities of solutions of BSA–fatty acid systems at various values of the average molal ratio, $\bar{\nu}$ (0.1% BSA). Symbols as in Figure 1.

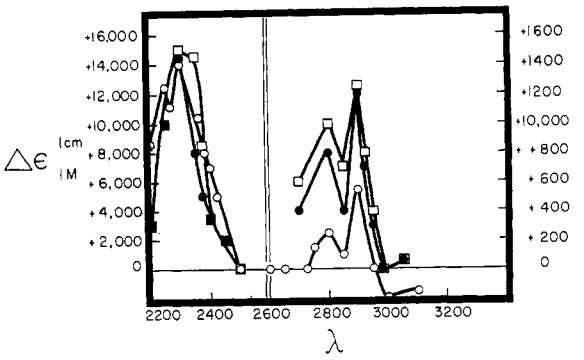

FIGURE 6: Ultraviolet difference spectra of BSA as affected by the average number ($\bar{\nu}$) of octanoate ions bound. λ is given in Å. (○) $\bar{\nu}$ = 3.3, (●) $\bar{\nu}$ = 5.8, and (□) $\bar{\nu}$ = 10.2

length band which is generally considered to be a mixture of aromatic and peptide transitions (Wetlaufer, 1962).

Discussion

The primary purpose in studying the binding of structurally related ligands to BSA has been to relate the ligand–protein interaction to the segment–segment interactions of the protein itself, thus giving some insight into the nature of disruptive forces in protein transitions. Among all the ligands in Table I only dodecyl and tetradecyl sulfate induce disruption of secondary and three-dimensional structure in BSA.

Previous publications (Reynolds *et al.*, 1967; Aoki and Foster, 1958) outlined a model for ligand-induced unfolding which could describe many features of the binding and unfolding data. The success of this model strongly suggests that a protein phase transition comes about as the direct result of the existence of a much larger number of binding sites on the unfolded state than on the native state. The general criterion for ligand-induced unfolding was shown to be $mJ \gg nK$, where n = number of sites on native protein, m = number of sites on unfolded protein, K = affinity constant associated with n, and J = affinity constant associated with m. The only ligands in Table I which cause unfolding of BSA are those with $n > 8$ and $K > 10^6$. In terms of the above model this suggests that m and J decrease more rapidly than n and K, as the

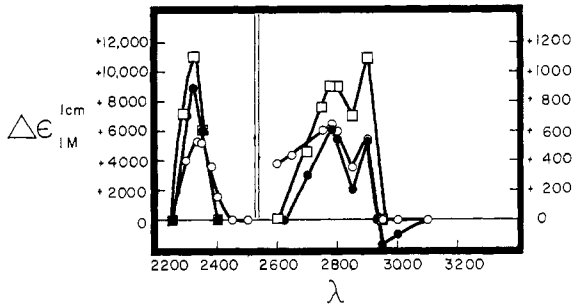

FIGURE 7: Ultraviolet difference spectra of BSA as affected by the average number ($\bar{\nu}$) of decanoate ions bound. λ is given in Å. (○) $\bar{\nu}$ = 2.5, (●) $\bar{\nu}$ = 6, and (□) $\bar{\nu}$ = 9.7.

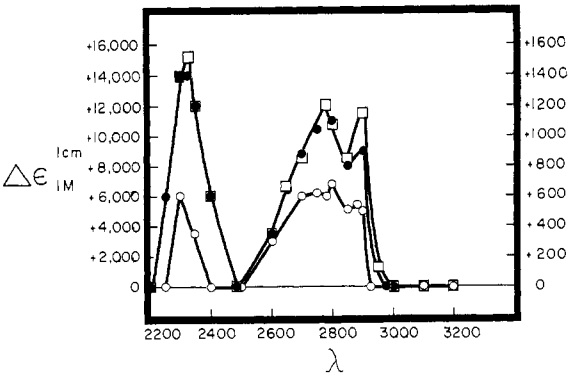

FIGURE 8: Ultraviolet difference spectra of BSA as affected by the average number ($\bar{\nu}$) of dodecanoate ions bound. λ is given in Å. (○) $\bar{\nu}$ = 1.9, (●) $\bar{\nu}$ = 6, and (□) $\bar{\nu}$ = 8.9.

ligand chain length is shortened or as the transition is made from sulfate to other polar heads. mJ is never larger than nK for the ligands investigated except dodecyl and tetradecyl sulfates.

The addition of binding data for carboxylates and alcohols to the previously published data for sulfates and sulfonates further supports the view that neither electrostatic repulsion nor replacement of protein hydrophobic bonds with interactions between the ligand hydrocarbon tail and the protein segments need be invoked as the primary forces involved in protein disruption.

Table I includes data from two other laboratories on two of the compounds reported here. Considering differences in pH and ionic strength, and in the different defatting procedures applied to the protein, the results are in fair agreement with ours. Goodman's severely acid-defatted isooctane-extracted material reveals two sites of much higher affinity; our data agree with his second group of five sites. It is not certain that Goodman's protein has not suffered some irreversible alteration; likewise, it is not certain that his heptane partition equilibrium method does not affect the protein also. Wishnia and Pinder (1964) and Scatchard *et al.* (1959) report that the binding of hydrocarbons and of small ions are not affected by Goodman's treatment, and J. M. Cassel (unpublished) finds no differences in the binding of picrate ion. The present work, however, is the only one in which the results of equilibrium dialysis can be compared with those of his partition procedure with identical ligands.

Simple calculation shows that even with the highest affinity ligands ($K > 10^6$) we have used (Reynolds *et al.*, 1967), there will be little displacement of tightly bound residual fatty acid ($K = 10^8$) on high energy sites (if there are any) until most of the unoccupied sites have been filled by the ligand under study.[2] Thus, the affinity measured in our experiments will tend to be that of the next lowest energy sites, but the number of sites indicated will be that of the sum of the

[2] It is presupposed in the foregoing that still another set of sites of only slightly lower K does not further complicate the analysis.

two sets. The same situation will prevail with the ligands of lower affinity because displacement of the residual palmitate, or stearate (if any is present), will always occur at equilibrium concentrations of ligand which nearly suffice to saturate the second set of sites. Thus, the data for different ligands presented here are fully comparable with one another, since the residual fatty acid content of the proteins remains constant and small. Work with protein fully defatted with charcoal by the method of Chen (1967) has been reported (Sogami and Foster, 1967a,b); the protein appears to be drastically altered with respect to pH-stability properties, when compared with the resin-deionized or acid-extracted crystalline BSA which has been used in all earlier binding studies, including our own.

The protein optical perturbations brought about by binding are similar for all sulfates, sulfonates, and alcohols, and consist of blue shifts in the long-wavelength region (2500–3200 Å) and red shifts below 2500 Å. These have been attributed in part to tyrosine and tryptophan perturbations by Polet and Steinhardt (1968), who also present evidence for the participation of a third chromophore, possibly histidine, in the shorter of the two ultraviolet bands. These investigators concluded that tryptophan was very near the highest energy sites and that the available (unmasked) tyrosines were close to sites of only slightly lower affinity.

However, as the present paper shows, when carboxylate anions are bound to BSA only a red shift is observed in both wavelength regions. The observed spectrum is consistent with a tyrosine red shift, possibly induced by the close proximity of the hydrocarbon tail to the aromatic residues. The difference in sign of the tyrosine shift when carboxylate anions are the ligands, as compared to all other ligands studied, does not necessarily suggest different protein sites for these ligands. Since the carboxylate ions apparently do not bind at the eight sites of highest energy which are associated with the two tryptophan residues (Polet and Steinhardt, 1968), the protein which binds carboxylates has not been subjected to the conformational strains incident to the binding at these sites. Binding at the exposed tyrosine-associated sites has been shown to produce blue shifts (Ray *et al.*, 1966; Polet and Steinhardt, 1968) when the tryptophan-associated sites are engaged. *When the latter are not engaged*, the conformation is such that red shifts result, similar to those reported by Green (1962) for the binding of biotin by avidin.

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