Bovine Serum Albumin

STUDY OF THE FATTY ACID AND STEROID BINDING SITES USING SPIN-LABELED LIPIDS*

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SUMMARY

Three spin-labeled derivatives of stearic acid and two derivatives of palmitic acid have been used to study the structure of the strong fatty acid binding site of bovine serum albumin. The steroid and indole binding sites have been studied using spin-labeled derivatives of androstol and indole, respectively. Paramagnetic resonance and fluorescence quenching data suggest that the fatty acid, steroid, and indole binding sites may be identical.

The mobility of the nitroxyl group at C-8 of palmitic acid bound to albumin at a 1:1 molar ratio is unaffected when the carboxyl group is esterified. When the nitroxyl group is located at C-5 on this acid, its motion is detectably increased by esterification of the carboxyl group but the magnitude of this change is small. This result suggests that the carboxyl group may play a minor role in the binding of fatty acids to the strongest fatty acid binding site of albumin.

When stearic acid derivatives bearing the nitroxide at C-5, C-12, and C-16 are bound to albumin at a ligand to albumin ratio of 1, the order of mobility at 0-30° is C-16 > C-12 > C-5. Although motion at the methyl terminus is always greater than at the COOH terminus in the range 0-60°, a simple monotonic increase in chain motion between the two termini is not observed. Arrhenius plots of the motion parameters for these bound fatty acids show two abrupt changes in slope. The temperature ranges for these changes are 15-23° and 38-45°. These results suggest that when one mole of spin-labeled fatty acid is bound to albumin, the protein undergoes a conformational change in each of these temperature ranges.

Albumin is the most abundant protein in mammalian plasma. This molecule has a molecular weight of about 67,000 (1, 2), and exists as a single polypeptide chain (2). Of the approximately 588 amino acids present in bovine albumin, there are only 2 tryptophanyl, 1 cysteylnyl, and 4 methionyl residues (1, 2). It has been estimated that the protein possesses about 50% α-helical structure which is stabilized by 17 to 18 disulfide bridges (3).

The affinity of the protein for ionic ligands bearing alkyl side chains has been shown to depend on the hydrophobic character of the chain and the sign of the charged group. Molecules with long chains and negative ionic groups such as stearic acid are very tightly bound, whereas molecules containing shorter side chains or positively charged ionic groups such as hexyltrimethylammonium bromide are less tightly bound (4). Many of the anionic ligands have at least two classes of binding sites on albumin which differ by 1 to 2 orders of magnitude in relative binding affinity. Recent studies suggest that arginine and tryptophan are at or near these sites (5). Studies directed toward the identification of those amino acid residues in albumin which are involved in fatty acid binding are currently underway (6).

Albumin also binds a variety of other ligands, such as acetylcholine, acetate, barbiturates, digitonin, penicillin, sulfonamides, and thyroxine (3) and is the principal vehicle for the transport of several lipid classes, including free fatty acids (7), lysolecithin (8), and certain steroids (9). Because of our interest in the mechanisms of lipid binding and transport (10-12), we have undertaken a study of the interaction of bovine albumin with nitroxide-containing derivatives of fatty acids, fatty acid methyl esters, androstol, and indole.

EXPERIMENTAL PROCEDURES

Spin Labels—Spin-labeled fatty acids (12,3-, 10,3-, 7,6-, 5,10-, and 1,14-SLFA* in Fig. 1) were obtained by hydrolysis of the corresponding ester in dioxane-aqueous sodium hydroxide (15) or from Syva (Palo Alto). Spin-labeled derivatives of fatty acid methyl esters (II) were prepared by m-chloroperbenzoic acid oxidation of the corresponding oxazolidine (16). Starting ketesteres were synthesized by standard methods (15). Spin-labeled fatty acids and esters with carbon chains of 16 and 18 carbons were used. All experiments were performed on a variety of spin-labeled lipids.

1 Albumin has also been studied by the use of covalently attached spin labels (13, 14).

2 The abbreviations used are: SLFA, spin-labeled fatty acid; SLFAME, spin-labeled fatty acid methyl ester; SLA, spin-labeled androstol; SLC, spin-labeled carboline; doxyl, 2,2-dimethyl-oxazolidinyl-1-oxyl; HDL, high density lipoprotein; apoLP-Gln-1, apolipoprotein-glutamine 1 is the most abundant apoprotein in human HDL; apoLP-Gln-II, apolipoprotein-glutamine II is the second most abundant apoprotein in human HDL.
androstenol (SLA, III) was prepared as described by Hubbell and McConnell (18). Spin-labeled carbolene (SLC, IV) was prepared by coupling phenylhydrazine with 1-oxyl-2,2,6,6-tetramethylpiperidone-4 followed by cyclization (19) then oxidation (20).

Instrumentation—Electron paramagnetic resonance (EPR) spectra were recorded on a Varian E-12 spectrometer equipped with a variable temperature controller. Acetone and Dry Ice were used to cool the nitrogen. The temperature of the cavity was measured before and after recording the spectrum with a Tri-R electronic thermometer to an accuracy of ±0.5°C. Samples were contained in flat quartz microcells (Varian). The splitting between the two extrema of the EPR spectra could be measured with an accuracy of ±0.3 G. pH was measured with a Radiometer TTT2 pH meter equipped with a combination micro-electrode, type CK2231C. Protein absorption at 278 nm was measured with a Beckman Acta V spectrophotometer. Fluorescence spectra were recorded at 22°C with an Aminco-Bowman spectrofluorometer equipped with an RCA1P28 phototube. Excitation wavelength was 280 nm and slit widths for the excitation and analyzing monochrometers were 2.0 nm.

Analysis of EPR Spectra—For spectra which indicated strong immobilization of spin label motion, the splitting between the low and high field extrema (2Tg) was used as an index for immobilization. For an isotropic ensemble of nitroxides whose correlation times (τc) fall within a certain range, 2Tg increases as the motional freedom of the nitroxide decreases. In the range of motion where 2Tg is measurable, this parameter is useful for comparing various degrees of immobilization. Since 2Tg values were not always quantifiable, calculation of order parameters (S) was not feasible (15).

Albumin—Crystalline bovine serum albumin (fatty acid-poor) was obtained from Miles Laboratories (Lot 21) and used as received, except where indicated otherwise. In a typical experiment, 100 mg of albumin (~1.5 μmol) were dissolved in 3 ml of distilled, deionized water and the pH was adjusted to 7.4. Approximately 1.5 μmol of spin label in ethanol was transferred to a test tube and evaporated to dryness with a stream of nitrogen. The solution of albumin was then added and stirred gently for 3 to 6 hours at 5°C.

For some experiments, defatted (21) monomeric (22) albumin was used. It was prepared in the following manner. One gram of albumin was dissolved in 10 ml of water and to this was added 0.5 g of powdered charcoal (Norit A, MCB). The mixture was adjusted to pH 3.0 with 1 M HCl and was stirred for 2 hours at room temperature. The charcoal was removed by centrifugation (18,000 rpm, 5°C, 20 min). The supernatant was adjusted to pH 8.5 with 1.0 M NaOH. A 2 ml aliquot (200 mg) of this defatted albumin solution was applied to a column (2.5 × 100 cm) of Sephadex G-150 equilibrated with 0.2 M NaCl-0.05 M Tris, pH 8.0, at 5°C.

Comparison of Albumin-bound Spin-labeled Palmitic Acids and Methyl Esters—The resonance spectra of albumin-bound 7,6- and 10,3-spin-labeled fatty acids (A, C) and their corresponding esters (B, D) are shown in Fig. 3. All four spectra indicate strong association of the nitroxide-bearing alkyl chain with albumin. While all these spectra appear similar, there are subtle, but significant spectral differences which distinguish any single spectrum from the others; viz., the total separation between the low and high field extrema (2Tg) and the shape of the high field side of the center field line (Mg). Values of 2Tg for albumin-bound spin-labeled fatty acids and esters are collected in Table I. While the differences in values of 2Tg are small, they...
FIG. 3. EPR spectrum of spin-labeled fatty acids and methyl esters bound to albumin. The low field-high field extrema separation is given in gauss. Spectra are aligned so that the high field extrema coincide. Details of sample preparation are given under “Experimental Procedures.” A, 7,6-SLFA (61.7); B, 7,6-SLFA (61.7); C, 10,3-SLFA (63.0); D, 10,3-SLFA (61.2).

TABLE I
Separation between low field and high field extrema ($2T_{1}$) of EPR spectra of various spin-labeled palmitic acids (I) and esters (II) bound to albumin at 23°C, pH 7.4.

<table>
<thead>
<tr>
<th>Spin label</th>
<th>Doxyl position</th>
<th>$2T_{1}$</th>
<th>Fig. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,6-SLFA (I)</td>
<td>C-8</td>
<td>61.7</td>
<td>A</td>
</tr>
<tr>
<td>7,6-SLFA (II)</td>
<td>C-8</td>
<td>61.7</td>
<td>B</td>
</tr>
<tr>
<td>10,3-SLFA (I)</td>
<td>C-5</td>
<td>63.0</td>
<td>C</td>
</tr>
<tr>
<td>10,3-SLFA (II)</td>
<td>C-5</td>
<td>61.2</td>
<td>D</td>
</tr>
</tbody>
</table>

The temperature dependence of $2T_{1}$ values for each of the bound fatty acids at three selected temperatures are compared in Table II. The data for 10°C and 30°C indicate a monotonic increase in motion of carbon atoms at increasing distances from the COOH terminus. At 50°C, the data suggest that the central portion of the chain is bound more rigidly than either the carboxyl or methyl terminal segments.

Arrhenius plots of the motion parameters ($2T_{1}$) for the bound spin-labeled fatty acids are shown in Fig. 5. For the temperature range 0-60°C, there are two abrupt changes in slope. The lower transition temperature ($t_{1}$) occurs at 15-23°C and the higher ($t_{2}$) at 38-45°C (Table III). The C-5-labeled stearate exhibits the lowest value for both $t_{1}$ (15°C), and for $t_{2}$ (38°C). The $t_{2}$ value for the C-12 analog (45°C) is significantly higher than that of the C-16 derivative (40°C) while $t_{1}$ for the C-12 analog (21°C) is slightly lower than that of the C-16 derivative (23°C).

Circular dichroic and intrinsic fluorescence measurements of albumin as a function of temperature indicated changes in $\psi$ helicity and emission maximum which were too small (<5%) to allow a meaningful comparison with the paramagnetic resonance data.

To demonstrate that the two abrupt changes in slope of Ar-
The presence of the doxyl group on these molecules increases the relative quenching efficiencies of these ligands (Table IV). The absolute magnitudes for this constant indicate that the mechanism whereby these fatty acids quench albumin fluorescence must involve actual binding of these ligands and cannot be due to mere diffusion-controlled collision (29). The relative values of this constant indicate that the association constants ($K_a$) for the natural and spin-labeled fatty acids are of the same order of magnitude (29).

**Comparison of Albumin-bound Spin-labeled Androstol and Spin-labeled Carboline**—In order to determine the interrelationships of the steroid, indole, and fatty acid binding sites, the binding of spin-labeled androstol (SLA, III) and spin-labeled carboline (SLC, IV) to albumin was also examined. When both SLA and protein concentrations are $\sim 10^{-4}$ M, the spectrum shown in Fig. 8A is obtained. This spectrum exhibits a single, broad line component which is similar to that in the spectra of the bound spin-labeled fatty acids. In fact, the splitting ($2T_{11}$) observed for bound SLA is exactly the same as that observed for bound 10,3- SLFA, i.e. 63.0 G (Fig. 3C, Table 1). While bound SLA and SLC give spectra which have comparable hyperfine splittings, there is a subtle difference in their line shapes on the high field side of the center field line $M_0$. This difference may be due to different types of anisotropic motion of the doxyl moiety when the long axes of these two molecules are oriented similarly in the same albumin binding site(s).\(^5\)

When a solution $10^{-4}$ M in albumin and spin-labeled carboline (SLC, IV) was examined, the resulting EPR spectrum (Fig. 8B) contained two components; a narrow line component due to unbound label and a broad component ($2T_{11} = 68.0$ G) due to very tightly bound label. Even when the ligand concentration was decreased to $0.25 \times 10^{-4}$ M, the same type of mixed spectrum was obtained.

The spectra of albumin-bound SLC and SLA at $20^\circ$ differ in two significant respects: first, when the ligand to protein ratio is 1:1 in each case, a narrow line component is not observed with the spin-labeled androstol (III), indicating there is no detectable

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**Table III**

<table>
<thead>
<tr>
<th>Spin label</th>
<th>Doxyl position</th>
<th>$t_1$ (°)</th>
<th>$t_2$ (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12,3-SLFA (I)</td>
<td>C-5</td>
<td>15</td>
<td>38</td>
</tr>
<tr>
<td>5,10-SLFA (I)</td>
<td>C-12</td>
<td>21</td>
<td>45</td>
</tr>
<tr>
<td>1,14-SLFA (I)</td>
<td>C-16</td>
<td>23</td>
<td>40</td>
</tr>
<tr>
<td>SLA (III)</td>
<td>24</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>SLC (IV)</td>
<td>17</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 6. Arrhenius plots of the motion parameters for 12,3-SLFA bound to A, apoHDL; B, apoLP-Gln-I, at pH 7.4. The transition temperatures $T_1$ and $T_2$ for 12,3-SLFA bound to these apolipoproteins are 13–17° and 16°, respectively, above those values exhibited with albumin.

TABLE IV

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_m$</th>
<th>$\Delta \lambda_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12,3-SLFA (I)</td>
<td>2,400</td>
<td>7.5</td>
</tr>
<tr>
<td>5,10-SLFA (I)</td>
<td>11,300</td>
<td>7.0</td>
</tr>
<tr>
<td>1,14-SLFA (I)</td>
<td>4,340</td>
<td>3.5</td>
</tr>
<tr>
<td>SLA (III)</td>
<td>6,000</td>
<td>0.0</td>
</tr>
<tr>
<td>SLC (IV)</td>
<td>10,000</td>
<td>2.0</td>
</tr>
</tbody>
</table>

a The quenching constants ($K_m$) are calculated from the initial slopes of the Stern-Volmer plots (Figs. 7 and 10) where the molar ratio of ligand to bovine serum albumin $\leq 1$.

b The blue shift ($\Delta \lambda_{max}$) is measured relative to the $\lambda_{max}$ of albumin to which no fatty acid is bound, i.e. 343 nm.

c This higher value of $K_m$ is due to enhanced quenching probably caused by the paramagnetic C-5 doxyl group (34) which is closer to the sensitive tryptophan (36) than either of the more distant doxyls at C-12 or C-16.

d Spin-labeled carboline (IV) absorbs in the same region of the ultraviolet spectrum as albumin. Hence, the $K_m$ obtained using this quencher cannot be unambiguously interpreted in terms of SLC binding. That SLC causes a 2.5-nm blue shift is consistent with binding, however.

unbound ligand. With the spin-labeled carboline (IV), however, a narrow line component is observed, indicating that some of this ligand exists in unbound form. Second, the extrema of the broad component in the SLC spectrum are separated by 68.0 G, 4.0 G more than observed in the case of SLA. These results indicate that the association constant for the binding of spin-labeled androstol to albumin is greater than for spin-labeled carboline. However, in the bound state, the carboline analog is more rigidly held than the androstol derivative.

When bound to albumin, both spin-labeled androstol (III) and spin-labeled carboline (IV) exhibit two abrupt changes in slope of Arrhenius plots of their motion parameters (Fig. 9, A and B,}

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FIG. 8. EPR spectra of A, spin-labeled androstol (SLA, III), B, spin-labeled carboline (SLC, IV), in the presence of albumin. The total splitting between the high and low field extrema of Spectrum A is 63.0 G, the same as that observed for 5,10-SLFA (I) bound to albumin. In Spectrum B, the broad line components are due to tightly bound SLC whose motion is strongly hindered ($2T_m = 68.0$ G). The narrow line components are due to unbound SLC. Albumin concentration = 0.5 mM; spin label concentration = 0.1 mM.

FIG. 9. Arrhenius plots of the motion parameters for A, spin-labeled androstol (III); B, spin-labeled carboline (IV) bound to albumin.

The quenching effects of spin-labeled androstol and carboline on the intrinsic fluorescence of albumin are indicated by the Stern-Volmer plots in Fig. 10. With SLA (III), a quenching constant, $K_{sv}$, of 6,000 was determined (Table IV). This value is similar to those values obtained for oleic acid (2400), 5,10-SLFA (1800), and 1,14-SLFA (4340) and suggests that the association constant and site of binding for SLA is similar to that of the fatty acids, even though this steroid does not induce a detectable blue shift of the wavelength of maximum emission, $\lambda_{max,em}$.

Spin-labeled carboline (IV) absorbs in the same region of the ultraviolet spectrum as albumin. Hence, the quenching effect of this ligand is probably due to a combination of its binding, paramagnetic, and absorption properties. Since a determination of the relative contribution of each of these properties to the observed quenching constant (10,000, Table IV) is not possible, this value of $K_{sv}$ cannot be interpreted in terms of a distance-dependent paramagnetic quenching effect (34) as was done for 12,3-SLFA (I). However, the observation that spin-labeled carboline induces a blue shift in the $\lambda_{max,em}$ (2.5 nm, Table IV) does suggest that it binds near 1 of the 2 tryptophan residues of bovine albumin.

**DISCUSSION**

In order to probe different regions of the strong fatty acid binding site of albumin, three stearic acid derivatives bearing the doxyl group at different distances from the carboxyl moiety were used. The spectral results (Table II) indicate that in the range of 0-60°, there is more motion at the methyl terminus of the fatty acid chain than at the COOH terminus. However, there does not appear to be a gradual increase in motion in going from the polar COOH terminus toward the nonpolar hydrocarbon tail as has been observed with spin-labeled fatty acids (either free or in phosphatidylcholine) which have been incorporated into phospholipid multibilayers (31). Rather, the alkyl chain is bound fairly rigidly out to at least C-12. From C-12 to C-16 there is a small, abrupt increase in motion. At 50°, the C-12 region of spin-labeled stearate was immobilized more than either the C-5 or C-16 regions (Table II) indicating that at elevated temperatures, the ends of the molecule are released before the central portion.

Based on chemical modification studies, Jonas and Weber (5) respectively). These transitions occur at 24° and 50° for SLA (III), and at 17° and 45° for SLC (IV) (Table III). While the transition temperatures for bound SLC fall within the range observed for the bound spin-labeled fatty acids (15-23° and 38-40°), those for bound SLA were above these ranges. Significantly, at any temperature in the range 0-50°, SLC is always the most rigidly bound of the ligands since it always exhibits the greatest splitting between the low and high field lines.
have suggested that there are arginine residues at or near the strong hydrophobic anion binding sites of bovine albumin. The implication of their study is that the cationic guanidino groups of these arginine residues form electrostatic or hydrogen bonds with the carboxyl groups of fatty acids. Our results indicate this or a similar interaction is operative but is not the dominant mechanism whereby albumin binds fatty acids. Rather, our results suggest that the entire length of the fatty acid chain is bound by hydrophobic interactions which are somewhat variable along the length of the binding site. This view is supported by the spectral data obtained from the uncharged spin-labeled fatty acid esters (Table 1, Fig. 3). These data show that esterification of the carbonyl group causes no change in the mobility of the C-8 doxyl function, and only a small (albeit reproducible) increase in the mobility of the C-5 doxyl group (1.8 G). That the mobility increase for the doxyl group is greatest for the fatty acyl chain which bears this functionality nearest the carboxyl group is consistent with the view that an arginine side chain interacts with this carboxyl group.

Further information about the preferential interaction of specific regions of fatty acids with albumin was obtained from a study of the temperature dependence of the resonance spectra of the bound doxyl stearates. Results from these studies (Fig. 5, Tables II, III) indicate that the doxyl groups of the bound spin-labeled fatty acids undergo two abrupt changes in motion, the lower of these occurring between 15° and 23° and the higher between 38° and 45°. The temperature required to bring about the lower transition (t₁) is lowest (15°) for the fatty acid with the doxyl group closest to the COOH terminus (12,3-SLFA). It is the same fatty acid which exhibits the lowest temperature (38°) for the higher transition (t₂). This does not mean that the carboxyl end of the fatty acid is bound more loosely than segments of the molecule closer to the methyl terminus. On the contrary, the experiments involving doxyl palmitic acids (Table I) demonstrate that the carboxyl end is the most rigidly bound portion of the molecule. Furthermore, the mobility parameter of albumin-bound doxyl stearates (Table II) is greatest (i.e. the motion is slowest) for the derivative carrying the nitroxide nearest the carboxyl group, up to a temperature of 50°. What this result does mean is that temperature dependent changes in mobility of the bound fatty acid are first observed in the carboxyl portion of the molecule. The transitions t₁ and t₂ may represent small conformational changes in the protein at or near the fatty acid binding site. Such changes could result in decreased interaction between the fatty acid and the protein, thereby allowing new degrees of motional freedom (e.g. trans-gauche isomerization) for the carbon atoms at or near the doxyl moieties. That these transitions are observed first with 12,3-SLFA suggests that these conformational changes in the strong fatty acid binding site begin in the local region of this site which interacts with the carboxyl segment of the molecule. Although the spectra of the bound fatty acids do change with temperature, the net change between 0° and 60° is not particularly great, probably due to stabilization of the tertiary structure of the protein by its 17 to 18 disulfide bonds or by the bound fatty acid itself, or both. That these transitions were not observed by circular dichroism or tryptophan fluorescence suggests the local nature of these structural changes.

Abrupt changes in the slopes for the Arrhenius plots of the motion parameter for albumin-bound spin-labeled fatty acids are also observed for the spin-labeled sterol derivative (SLA, III) and the spin-labeled indole derivative (SLC, IV). Androstyl steroids similar to SLA have been shown previously to bind to bovine albumin with an affinity somewhat lower than that of long chain fatty acids (38). These relative affinities are not surprising when the surface contact areas of these molecules are compared. However, our experiments indicate that once SLA is bound, it is as rigidly held (as strongly immobilized) as the spin-labeled fatty acids. A similar result has been obtained by Chambas et al. (26) with a different nitroxyl steroid. The quenching constant, Kₚ, for SLA (III) (Table IV) is comparable to that of oleic acid and the spin-labeled fatty acids (except 12,3-SLFA which exhibits distance dependent paramagnetic quenching in addition to the normal quenching). This result suggests that SLA is (a) binding at the strong fatty acid binding site, or (b) binding at a different site but in a manner which produces the perturbation of tryptophan emission. A determination of whether this perturbation is due to ligand binding near the affected tryptophan so as to change directly its environment, or is the result of a conformational change induced indirectly by ligand binding at some site more remote from this tryptophan, will require further experiments. The first alternative is very attractive in light of the recent isolation of an albumin tryptic peptide with the sequence Lys-Val-Ala-Val-Ala-Arg (37). Efforts to locate the steroid binding site(s), have recently resulted in the isolation from a peptic digest of albumin, a tyrosine-rich peptide of molecular weight 10,050 which retains the capacity to bind progesterone, testosterone, and 17β-estradiol (27).

The carboline derivative (SLC, IV), exhibits several properties which make it an attractive probe molecule. First, the nitroxide ring is coplanar with the adjacent ring system so that the complete molecule is flat, closely resembling the structure of indole. Fatty acids bearing the doxyl function do not so closely mimic the parent molecules since the plane of the nitroxide ring lies perpendicular rather than parallel to the long axis of the molecule. Second, in the oxidized form, the molecule is fluorescent. In favorable cases where the system of interest does not contain a fluorophore which absorbs (emits) in the region of 280 (360) nm (e.g. tryptophan), the paramagnetic and diamagnetic forms of this molecule may be used to probe the same binding site by both paramagnetic resonance and fluorescence methods.

In the presence of albumin, SLC (IV) gives a mixed spectrum containing a broad component with a splitting of 68.0 G, indicating that the bound label has almost no motion independent of the albumin molecule. Kumetsov et al. (17) have obtained similar results using a similar molecule which also contains the indole nucleus. It is likely that SLC binds at the single site where indole, skatole, acetyl-L-tryptophan, tryptophan, and indole-propionate bind (38). This site is thought to be flexible and adaptable since several different small affinity labels are bound. Our results suggest that once this label is bound, it is rigidly held in the binding site. Due to absorption of SLC in the same region as albumin, the observed quenching constant is of no value in estimating the distance from this bound ligand to the perturbed tryptophan. However, the 2.5-nm blue shift which SLC induces demonstrates the ability of this molecule to decrease the polarity near the sensitive tryptophan. Further experiments to determine whether the site(s) at which SLFA (I), SLA (III), and SLC (IV) bind are identical, equivalent, or different are currently in progress.

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