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Synthesis of Homologous Fluorescent Carboxylates and Their Application to the Study of Bovine Serum Albumin[1]

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The synthesis of four fluorescent carboxylate probes with spacer groups comprising 1 to 12 atoms between the dansyl and carboxylate functions is described. These probes bind specifically to bovine serum albumin with changes in fluorescence properties. Dansyl glycine shows significantly different behavior in comparison with its longer homologs. Probe binding to protein is diminished in the presence of fatty acids. Evaluation of binding stoichiometries, dissociation constants, and limiting fluorescence values suggests that dansyl glycine binds at a site distinct from the binding site for the longer homologs.

INTRODUCTION

The recognition of a large number of binding sites on bovine serum albumin (BSA)[2] for hydrophobic organic anions has led to several investigations (*1–3*). Fluorescence spectroscopy is a sensitive and convenient method for the study of protein–ligand interactions (*4*). The high affinity of BSA for fatty acids suggested that fluorescent alkyl carboxylic acids, which bear a formal structural analogy to the fatty acids, could be advantageously employed to probe binding sites on the protein. Studies with dansyl glycine, dansyl sarcosine (*5*), and other dansylated derivatives (*6*) have been reported. These studies employed probes that possess only a single carbon atom separating the anionic group and the dansyl fluorophore. However, separate sites for long- and short-chain fatty acids have been postulated. It would therefore be desirable to study the interaction of BSA with fluorescent carboxylates having different lengths of the bridging alkyl chain. This paper presents the synthesis of a series of homologous fluorescent carboxylates and their application to the study of BSA. The structures are illustrated in Fig. 1. The coupling reactions of dansyl chloride with amino acid methyl esters described here have been carried out in organic solvents, using nearly equal proportions of the two components. Earlier methods for the dansylation of amino acids involved the use of excess dansyl chloride and were carried out in aqueous media (32). The method described here is convenient for large-scale preparations.

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[2] The abbreviations used are BSA, bovine serum albumin; HSA, human serum albumin; GABA, γ-aminobutyric acid; DCHA, dicyclohexylammonium; FFA, free fatty acid; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DCC, dicyclohexyl carbodiimide.

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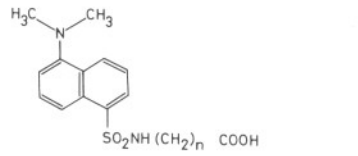
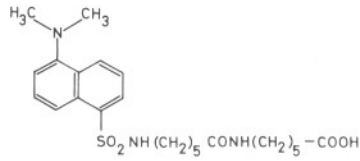


n=1, CI
n=3, CIII
n=5, CV



CXII

FIG. 1. Structures of fluorescent probes.

MATERIALS AND METHODS

Synthetic procedures. Dansyl chloride was prepared as described by Mendel (*7*). The amino acid methyl ester hydrochlorides of glycine, γ-aminobutyric acid (GABA), and ϵ-aminocaproic acid were prepared by the method of Brenner and Huber (*8*).

N-(5-Dimethylaminonaphthalene-1-sulfonamido)glycine dicyclohexylammonium salt (*dansyl glycine,* ***CI***). A mixture of 125 mg (1 mmole) of glycine methyl ester hydrochloride and 0.14 ml (1 mmole) of triethylamine in 10 ml absolute methanol was stirred for 10 min at room temperature. A second lot of 0.14 ml of triethylamine was added followed by a solution of 269 mg (1 mmole) of dansyl chloride in 20 ml of absolute methanol over a period of 1 hr. Evaporation of the solvent, after an additional 2 hr of stirring, gave an oil which was taken up in ethyl acetate and washed with water and 1 *N* $NaHCO_3$. The organic layer was dried over Na_2SO_4 and evaporated to give the methyl ester of dansyl glycine as an oil. Saponification to the free acid was effected by the addition of 3 ml of 2 *N* NaOH to the ester in 15 ml of methanol. After standing for 12 hr at room temperature, the solvent was evaporated to give a residue which was taken up in water. Traces of unreacted ester were removed by washing the aqueous layer with ethyl acetate. This layer was subsequently acidified to pH 2 and extracted with ethyl acetate. Drying and evaporation of the solvent gave dansyl glycine as an oil. This oil was dissolved in the minimum amount of acetone and 1 ml (5 mmole) of dicyclohexylamine (DCHA) was added to it. Cooling of the solution in ice resulted in the deposition of crystals of the DCHA salt of dansyl glycine. The crystals were filtered and washed with cold acetone and ether. Yield 200 mg (43%); mp 173–178°C.

N-(5-Dimethylaminonaphthalene-1-sulfonamido)-γ-aminobutyric acid dicyclohexylammonium salt (*dansyl GABA,* ***CIII***). This compound was prepared by the

procedure employed for **CI** from 600 mg (3.9 mmole) of GABA methyl ester hydrochloride, 2 × 400 mg (2 × 3.9 mmole) of triethylamine, and 800 mg (3 mmole) of dansyl chloride in methanol. Saponification to the free acid and conversion to the DCHA salt were effected as described for **CI**. Yield 350 mg (56%); mp 177°C.

N-(5-Dimethylaminonaphthalene-1-sulfonamido)-ϵ-aminocaproic acid dicyclohexylammonium salt (***CV***). This compound was prepared from 360 mg (2 mmole) of ϵ-aminocaproic acid methyl ester hydrochloride, 2 × 225 mg (2 × 2 mmole) of triethylamine, and 540 mg (2 mmole) of dansyl chloride in absolute methanol followed by hydrolysis to the free acid. The procedure followed was similar to the one described for **CI**. Four-hundred milligrams of the free acid was converted to the DCHA salt. Yield 560 mg (54%); mp 100°C.

N - (5 - Dimethylaminonaphthalene - 1 - sulfonamido) - ϵ - aminocaproyl - ϵ - aminocaproic acid dicyclohexylammonium salt (***CXII***). One gram of ϵ-aminocaproic acid methyl ester hydrochloride was dissolved in 10 ml of a saturated $NaHCO_3$ solution. Extraction with 3 × 15 ml of chloroform followed by drying and evaporation of the solvent gave 600 mg of the free base. Dansyl ϵ-aminocaproic acid (450 mg, 1.24 mmole) in 10 ml of methylene chloride was cooled in ice. ϵ-Aminocaproic acid methyl ester (225 mg, 1.55 mmole) in 5 ml of methylene chloride was added to it followed by the addition of a solution of 260 mg (1.25 mmole) of dicyclohexyl carbodiimide (DCC) in 10 ml of methylene chloride over a period of 2 hr. Filtration of the precipitated dicyclohexylurea was followed by washing of the organic layer with 1 *N* HCl, water, and 1 *N* $NaHCO_3$. Evaporation of the solvent gave 800 mg of a solid which was a mixture of two fluorescent compounds. These were separated by column chromatography (40 g silica gel, 60–120 mesh). Elution with a 1:2 mixture of petroleum ether and chloroform gave the methyl ester of **CXII** which was saponified and converted to the DCHA salt by the procedure described for **CIII**. Yield 410 mg (50%); mp 125°C.

The purity of the compounds was established by thin-layer chromatography on silica gel and from uv and 60- and 270-MHz ^1H NMR spectroscopic data.

Preparation of samples. BSA, fatty-acid-free BSA, ovalbumin, lysozyme, α-chymotrypsin, caprylic acid and lauric acid were obtained from Sigma Chemical Company. Palmitic acid was obtained from Reanal. All solutions were prepared in 10 m*M* Tris–HCl buffer (pH 8). Fatty acid solutions were prepared by adding concentrated methanol solutions to a vigorously stirred solution of buffer. The volume of methanol did not exceed 0.4% in the final solution.

Fluorescence measurements. Fluorescence measurements were carried out on a manual Perkin–Elmer spectrofluorimeter (Model 203) and are uncorrected. Matched 1-cm cuvettes were used. The excitation wavelength was 340 nm. For energy transfer measurements, the excitation wavelength was 290 nm.

RESULTS

Figure 2a shows the effect of the addition of BSA on the fluorescence spectrum of **CI**. A large-intensity enhancement and a 50-nm blue shift in the wavelength of

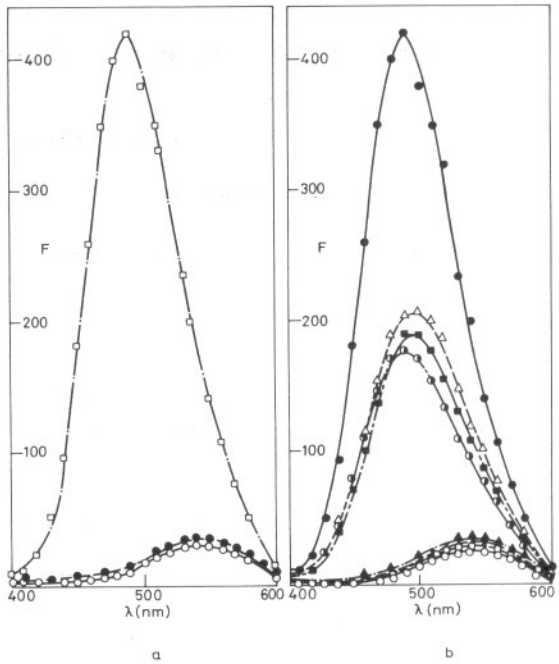


FIG. 2. (a) Fluorescence spectra of **CI** in the presence of various proteins. Excitation 340 nm; probe concentration 10 μM; protein concentration 200 μg/ml. All solutions in 10 mM Tris–HCl, pH 8.0 (○) buffer; (●) ovalbumin; (□) BSA. The spectra in the presence of lysozyme and α-chymotrypsin coincide with (○). (b) Fluorescence spectra of the carboxylate probes in the presence of BSA. Excitation 340 nm; probe concentration 10 μM; protein concentration 200 μg/ml. All solutions in 10 mM Tris–HCl, pH 8.0 (□) **CI** in buffers; (●) **CI** + BSA; (○) **CIII** in buffer; (◑) **CIII** + BSA; (X) **CV** in buffer; (■) **CV** + BSA; (▲) **CXII** in buffer; (△) **CXII** + BSA.

maximum emission (λ_{max}) are observed, indicative of the strong interaction of **CI** with a hydrophobic site on BSA. The specificity of this interaction is established by the absence of changes in the fluorescence parameters in the presence of similar concentrations of ovalbumin, lysozyme, and α-chymotrypsin (Fig. 2a). A comparison of the effects of BSA addition on the fluorescence spectra of **CI**, **CIII**, **CV**, and **CXII** (Fig. 2b) shows that the intensity enhancements follow the sequence **CI** $\gg$ **CIII** > **CV** > **CXII**. The blue shift of the emission maximum is, however, identical for the four probes. Figure 3 shows the energy transfer spectra of **CI** in the presence of BSA. Probe addition leads to quenching of protein tryptophan fluorescence at 335 nm with a concomitant increase in bound probe emission at 490 nm, resulting from fluorescence energy transfer from proximate tryptophan residues. The extent of energy transfer to **CIII** from BSA tryptophan residues is significantly lower (Fig. 4). Fluorescence titrations varying probe and protein concentrations were carried out in order to quantitate the probe–protein interaction (*9*). Double reciprocal plots of fluorescence intensity versus protein concentration (Fig. 5 inset), extrapolated to infinite protein concentration, yield limiting probe fluorescence values (F_{max}) which reflect bound probe quantum

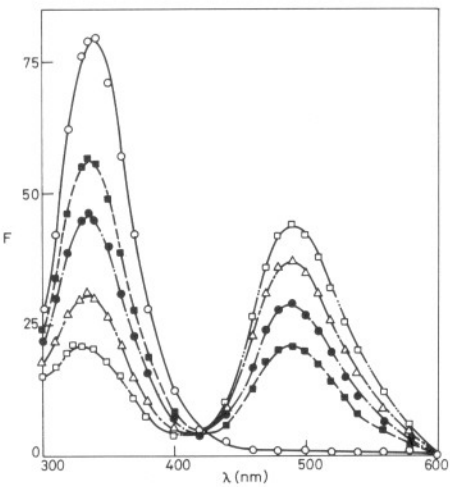


FIG. 3. The effect of **CI** addition on BSA–tryptophan fluorescence. Excitation 290 nm; protein concentration 180 μg/ml. All solutions were prepared in 10 m*M* Tris–HCl, pH 8.0. Probe concentrations: (○) 0 μM; (■) 3 μM; (●) 6 μM; (△) 13 μM; (□) 26 μM.

yields. The F_{max} value obtained for **CI** differs substantially from those of **CIII**, **CV**, and **CXII**. This suggests that the sites probed by **CI** and those occupied by **CIII**, **CV**, and **CXII** are different. Scatchard plots (*9*) of the binding data are shown in Fig. 5. The data for the four probes can be fitted to one straight line, indicative of a single binding site for each probe. The limiting fluorescence values (F_{max}), dissociation constants (K_D), and binding stoichiometries (n) obtained from these plots are listed in Table 1.

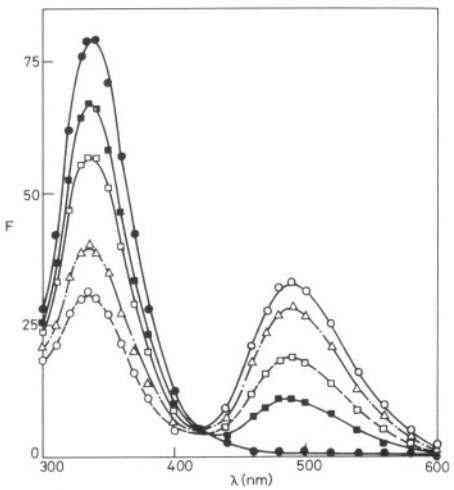


FIG. 4. The effect of **CIII** addition on BSA tryptophan fluorescence. Excitation 290 nm; protein concentration 180 μg/ml. All solutions were prepared in 10 m*M* Tris–HCl, pH 8.0. Probe concentrations (●) 0 μM; (■) 3 μM; (□) 6 μM; (△) 13 μM; (○) 26 μM.

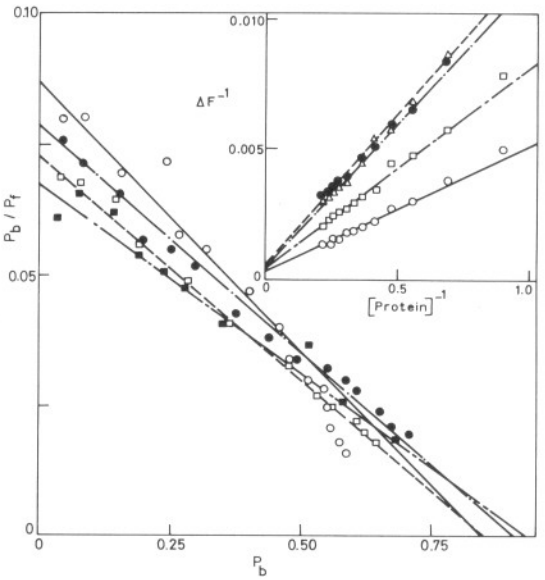


FIG. 5. Scatchard plots for the binding of **CI**, **CIII**, **CV**, and **CXII** to BSA. Protein concentration 200 μg/ml; probe concentrations 0–40 μM. All solutions in 10 mM Tris–HCl, pH 8.0. P_b and P_f are the concentrations of bound and free probe, respectively. (○) **CI**; (●) **CIII**; (■) **CV**; (□) **CXII**. (Inset) Double reciprocal plots of changes in fluorescence intensity (ΔF, arbitrary units) versus BSA concentration. Excitation 340 nm, emission 500 nm; probe concentration 10 μM; protein concentrations 0–5 μM. All solutions in 10 mM Tris–HCl, pH 8.0. (○) **CI**; (□) **CIII**; (△) **CV**; (●) **CXII**.

The effects of the addition of caprylic, lauric, and palmitic acids on the spectrum of **CI** in the presence of BSA are shown in Fig. 6. The addition of free fatty acid (FFA) causes quenching of bound probe fluorescence, with the effect

TABLE 1

BINDING PARAMETERS FOR THE INTERACTION OF THE CARBOXYLATE PROBES WITH BSA[a]

Probe	F_{max} (arbitrary fluorescence units)	n (binding sites/mole protein)	K_D (M)
CI	320	0.85	9.77×10^{-6}
CIII	200	0.91	1.15×10^{-5}
CV	200	0.93	1.28×10^{-5}
CXII	200	0.85	1.25×10^{-5}

[a] The limiting fluorescence values, F_{max}, are the reciprocal values of the intercepts in the double reciprocal plots of protein concentration versus fluorescence intensity shown in the inset of Fig. 5. The intercepts on the ordinate axis of the Scatchard plots in Fig. 5 give the values of binding stoichiometry, n. The intercepts on the abscissa represent n/K_D, from which the dissociation constant K_D can be calculated.

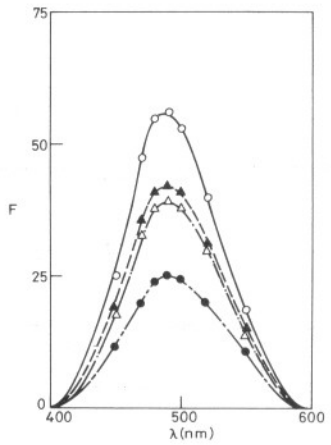


FIG. 6. Effect of fatty acids on the fluorescence spectrum of **CI** in the presence of BSA. Excitation 340 nm; probe concentration 2 μM; protein concentration 200 μg/ml; fatty acid concentration 5.4 μM. All solutions in 10 mM Tris–HCl, pH 8.0. (○) **CI** + BSA; (▲) **CI** + BSA + caprylic acid; (△) **CI** + BSA + palmitic acid; (●) **CI** + BSA + lauric acid.

being the largest for lauric acid. At the 1 : 1.85 ratio of BSA : FFA, the emission wavelength of the bound probe remains unaltered. However, the emission due to the displaced probe is not evident at 540 nm. This is not surprising in view of the low concentration used in this experiment and the large difference in the free and bound probe quantum yields. Similar results have been obtained for **CIII**, **CV**, and **CXII**. The percentage fluorescence decreases for the four probes are summarized in Table 2.

TABLE 2

PERCENTAGE DECREASES IN THE FLUORESCENCE INTENSITY OF THE CARBOXYLATE–BSA COMPLEXES ON FATTY ACID ADDITION[a]

Fatty acid	**CI**	**CIII**	**CV**	**CXII**
Caprylic acid (3.7 μM)	16.3	5.1	7.4	25.0
Caprylic acid (5.4 μM)	17.5	9.2	8.6	27.6
Lauric acid (3.7 μM)	28.4	13.8	26.6	31.2
Lauric acid (5.4 μM)	38.3	28.7	40.5	44.3
Palmitic acid (3.7 μM)	16.7	7.7	26.9	24.0
Palmitic acid (5.4 μM)	21.8	16.8	33.8	46.0

[a] Probe concentration = 2 μM; protein concentration = 200 μg/ml; excitation wavelength = 340 nm.

DISCUSSION

The large fluorescence intensity enhancement observed in the **CI** spectrum on the addition of BSA (Fig. 2a) has been reported earlier, as part of a study on the fluorescence properties of dansylated amino acids in the presence of organic solvents and proteins (*10*). The report also noted negligible changes in the fluorescence parameters of **CI** in the presence of ovalbumin, lysozyme, and α-chymotrypsin similar to the results obtained in this study (Fig. 2a). The fluorescence intensity enhancements for the four probes on interaction with BSA follow the sequence **CI** $\gg$ **CIII** $>$ **CV** $>$ **CXII**. This behavior must be contrasted with earlier reports on the binding of organic anions which exhibit greater affinity for BSA with increasing hydrophobicity. The strength of binding of the alkyl sulfate detergents (*11*) and saturated fatty acids containing 6–18 carbon atoms (*2*) increases with increasing chain length. The binding of methyl orange, azasulfathiazole, and amaranth shows a direct relation to the hydrophobicity of the molecule (*12, 13*). We have earlier reported a similar trend for the binding of positively charged cholinergic fluorescent probes to BSA (*14*). Models of the organic anion binding sites of the albumins postulate the existence of a pocket lined with nonpolar amino acid residues, with cationic groups at or near the surface of the pocket. ^{19}F NMR studies of the binding of trifluoroalkyl sulfates suggest that the strongest hydrophobic interactions occur along the length of the alkyl chain and not at the point of deepest penetration (*15*). The fluorescent carboxylates could be expected to orient at such sites with the dansyl groups and the polymethylene spacer arms interacting with the hydrophobic residues. Increasing chain length should, therefore, lead to deeper penetration and consequently greater fluorescence intensities for the longer homologs. However, the opposite trend is seen for **CI**, **CIII**, **CV**, and **CXII** (Fig. 2b).

The binding stoichiometry for the four probes is approximately 1, suggesting the existence of only a single binding site (Table 1). BSA is known to bind 10–16 anions at equivalent, noninteracting sites (*16*). For example, 12,12,12-trifluorododecyl sulfate (F_3DS^-) binds to 15 magnetically equivalent sites (*15*). The C-2 to C-8 fatty acids bind to two classes of sites with $n_1 \simeq 4$ and $n_2 \simeq 27$–31 (*17*), while three classes with $n_1 = 3$, $n_2 = 3$, and $n_3 = 63$ have been observed for palmitate (*2*). Over the pH range 5–10, five molecules of the anionic fluorescent probe, ANS, are bound to BSA (*18*). However, **CI** has been shown to bind to HSA with $n = 1$ and $K_D = 2.17 \times 10^{-6}$ M (*19*). Another study reported values of $n = 2$ with K_D values of 1.7×10^{-6} and 20×10^{-6} M for the **CI**–HSA interaction, while values of $n = 1$ and $K_D = 6 \times 10^{-6}$ M have been obtained for dansyl sarcosine (*20*). Thus it can be seen that the binding stoichiometries for the fluorescent carboxylates are significantly lower than the values generally obtained for other organic anions.

It is likely that **CI**, **CIII**, **CV**, and **CXII** bind at sites on BSA which are distinct from those available for other organic anions. The differences in the fluorescence intensities of the four probes can be attributed to one or more of the following factors: (i) differences in the number of molecules bound, n; (ii) altered quantum yields on binding leading to different values of F_{max}; and (iii) changes in the

dissociation constants, K_D. Table 1 shows that the values of n and K_D for the four probes are nearly the same. However, **CI** exhibits one value of F_{max} while the data for **CIII**, **CV**, and **CXII** show a second value of F_{max}. It is likely therefore that while **CI** binds at a site of low polarity or solvent accessibility on the protein, **CIII**, **CV**, and **CXII** interact with a comparatively more polar site. It is significant that these probes bind to only one site each on a molecule as complex as BSA. This phenomenon argues for a high degree of specificity in probe binding. Earlier studies (*21, 22*) have suggested that anion binding to BSA is sensitive to the nature of the anionic group. With the exception of the fatty acids, the dyes, detergents, and ANS studied earlier (*12, 13, 18*) are all either sulfates or sulfonates. Differences in the interaction of the probes and these molecules may well be due to the presence of different anionic functions. The lower n values observed for the probes **CI** to **CXII**, in comparison with the fatty acids, which are also carboxylates, may be the result of steric constraints imposed on the probe molecules by the bulky dansyl moiety. While a single strong binding site for these probes is established from these studies, it is possible that experiments at much higher probe concentrations may yield data on low-affinity sites. Self-quenching of probe fluorescence, however, precludes experiments at higher concentrations.

The data in Fig. 6 and Table 2 show that probe fluorescence is quenched by FFA addition. This may involve either direct displacement of the probe from its binding site on the protein or an indirect allosteric mechanism. The quenching efficiency follows the order lauric > palmitic ≥ caprylic, a sequence similar to the one observed for the ANS–BSA system (*23*). Affinity constants have been reported for the binding of six consecutive fatty acids ranging from propionic (C_3) to caprylic (C_8) acids (*24*). The standard free energy change, ΔG^0, deduced from these affinity constants, is not a linear function of chain length, but reaches a plateau at valeric acid and increases further only at heptanoic acid. A mean increase in ΔG^0 of 820 cal per methylene group was observed. This increment amounts to 1600 cal at the steepest part of the curve and 200 cal at the plateau. If **CIII** and **CV** are assumed to bind at the short-chain fatty acid site, a similar increase in ΔG^0 may be expected. For an addition of two methylene groups from **CIII** to **CV**, the mean increment $\Delta(\Delta G^0)$ would be 1640 cal,

$$\Delta(\Delta G^0) = \Delta G_{III}{}^0 - \Delta G_V{}^0 = RT \ln K_{III}/K_V,$$

where K_{III} and K_V are the affinity constants for **CIII** and **CV**, respectively. Substituting a value of 1640 cal for $\Delta(\Delta G^0)$, $K_{III}/K_V = 15.95$. However, the values of K_D for **CIII** and **CV** are 1.15×10^{-5} and 1.28×10^{-5} M, respectively. Even the minimum increment of 400 cal for two methylene groups should give a value of $K_{III}/K_V \simeq 2$. Therefore, it is unlikely that **CIII** and **CV** bind at sites on BSA occupied by fatty acids of comparative length and hydrophobicity.

Figures 3 and 4 present evidence for fluorescence energy transfer to bound probe molecules from proximate tryptophan residues. BSA contains two tryptophan residues at positions 134 and 212 (*31*). One of these residues is exposed on the surface while the other lies deep inside the globular structure (*25*). Perturbation of tryptophan fluorescence by the addition of fatty acids (*26*), SDS (*27*), steroids (*28*), and drugs (*29*) has been reported. A heptapeptide sequence

containing the lone tryptophan residue of HSA (Trp 214) has been sequenced (*30*). This fragment Lys–Ala–Trp–Ala–Val–Ala–Arg has been suggested to constitute an anion binding site. In BSA, the amino acid sequence near Trp 212 is Lys–Ala–Trp–Ser–Val–Ala–Arg (*31*). An examination of this sequence shows a cluster of nonpolar residues flanked by two cationic amino acids. While it is tempting to speculate that Trp 212 is close to the probe binding site, definite conclusions cannot be drawn from the present data.

In conclusion, it may be noted that the high degree of binding specificity and the similarity of the probe structures to those of fatty acids strongly suggest that these molecules are likely to be useful in more detailed studies of serum albumin–ligand interactions.

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