Long Chain Fatty Acid Binding to Human Plasma Albumin*

J. Douglas Ashbrook, Arthur A. Spector, Elsa C. Santos, and John F. Fletcher

From the Laboratory of Applied Studies, Division of Computer Research and Technology, National Institutes of Health, Bethesda, Maryland 20014 and the Departments of Biochemistry and Medicine, University of Iowa, Iowa City, Iowa 52242

SUMMARY

The binding of six physiologically important long chain fatty acids to defatted human plasma albumin was measured at 37° in a calcium-free Krebs-Ringer phosphate buffer, pH 7.4. The data were analyzed in terms of multiple stepwise equilibria. With the saturated acids, the magnitude of the equilibrium (association) constants, Kᵢ, increased as the chain length increased: laurate < myristate < palmitate < stearate. Oleate was bound more tightly than stearate; by contrast, linoleate was bound less tightly than stearate. The equilibrium (association) constants, Kᵢ, increased as the chain length increased: laurate < myristate < palmitate < stearate. Oleate was bound more tightly than stearate; by contrast, linoleate was bound less tightly than stearate. The equilibrium constants, Kᵢ through Kᵢ₃, ranged from 2.4 x 10⁴ - 3.5 x 10³ M⁻¹ for laurate to 2.6 x 10⁵ - 3.9 x 10³ M⁻¹ for oleate. Successive values of Kᵢ decrease for each of the acids, indicating that major cooperative binding effects do not occur over the physiological range of fatty acid concentrations. In no case could the Kᵢ be segregated into distinct classes, suggesting that any grouping of albumin binding sites is somewhat arbitrary. The results were inconclusive concerning whether premicellar association of unbound fatty acid occurs. Although corrections for premicellar association produced very little change in the Kᵢ values for myristate, they raised the Kᵢ for palmitate and stearate by 300 to 700%. A sigmoidal relationship was obtained when the logarithm of Kᵢ was plotted against chain length for the saturated fatty acids containing 6 to 18 carbon atoms, indicating that the binding energy is not simply a statistical process dependent only on the fatty acid chain length. This selectivity that albumin contributes to the binding process may be due to varying degrees of configurational adaptability of its binding sites as the fatty acid increases in length.

One of the most important functions of plasma albumin is to transport free fatty acids through the blood. Under the usual conditions, the free fatty acids present in the plasma are predominantly of the long chain variety, containing from 12 to 20 carbon atoms (1). The mechanism of fatty acid binding to plasma albumin has been studied in considerable detail (2-12). In spite of the potential clinical importance, however, there has been only one comprehensive quantitative study of long chain fatty acid binding to human plasma albumin (13). Goodman employed the equilibrium partition method for measuring fatty acid binding (13, 14) and the Scatchard method for analysis of the data (15). Subsequent to the completion of this work considerable improvements have been made in the techniques for the extraction of inherent fatty acids from albumin (16) and in the analysis of the partition of fatty acids between an organic solvent and an aqueous solution (17-19). Moreover, the severe limitations of the Scatchard analysis when a protein contains multiple binding sites recently have become apparent (20) and it now is recognized that such binding data should be analyzed according to stepwise stoichiometric equilibria (20-22). Computerized techniques for fitting complex binding data to the stepwise equilibrium model recently have been developed (20, 23). The binding of medium chain fatty acids to human plasma albumin already has been analyzed by these procedures (24). In the present study, we have extended this work to the physiologically important long chain fatty acids that contain from 12 to 18 carbon atoms.

EXPERIMENTAL PROCEDURES

Crystalline human plasma albumin was obtained from Miles Laboratories (Kankakee, Ill.). The protein was defatted with activated charcoal (16) and then dialyzed (25). The final solution contained 0.1 mM albumin, 122 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, and 16 mM NaHPO₄, and it was adjusted to pH 7.4 with 0.1 N HCl. Protein concentration was determined by the biuret method (26).

Unlabeled fatty acids were purchased from the Hormel Institute (Austin, Minn.). The purity of these acids was greater than 99% as measured by gas-liquid chromatography of the fatty acid methyl esters (27). Radioactive fatty acids were supplied by New England Nuclear Corp. (Boston, Mass.) and Amersham-Searle Corp. (Arlington Heights, Ill.). Each radioactive fatty acid was dissolved in n-heptane and then extracted into alkaline ethanol. The ethanol was acidified, and the fatty acids re-extracted into fresh heptane. Heptane (50 ml) containing the radioactive compound then was extracted with an equal volume of 0.01 N H₂SO₄, followed by five extractions with 50 ml of 0.05 N NaHPO₄, pH 7.0, to ensure removal of any radioactive short chain fatty acid impurities. Following purification of the labeled fatty acid, unlabeled carrier fatty acid (5 to 20 μg/ml) was added to the heptane solution. Analysis of the purified compounds by thin layer chromatography (28) indicated that more than 99% of the radioactivity migrated with the free fatty acid standard. Moreover, analysis of palmitate, myristate, and laurate by gas-liquid chromatography indicated that from 96.5 to 97% of the radioactivity actually was present in that particular acid. When samples of these labeled acids collected from the column effluent were rechromatographed, the radioactive purity was found to be only 97.5%. Therefore, we...
believe that the actual purity of these labeled fatty acids was 99% or greater.

Fatty acid binding to albumin was measured by the equilibrium partition method (13, 14). Incubations in specially constructed glass vials were performed as described previously (25). The concentration of human plasma albumin in the 1-ml aqueous phase was 0.1 mg, and the 1-ml heptane phase contained initially 0.01 to 20 mM fatty acid. When unsaturated fatty acids were used, the flasks were gassed with nitrogen; with saturated fatty acids, air served as the gas phase. Incubations with shaking were performed in a constant temperature water bath at 37°C. Preliminary experiments indicated that equilibrium was reached in this system within 17 hours, and all incubations were carried out for 18 to 24 hours. After incubation, aliquots of the heptane and aqueous phases from each flask were obtained (25) and added to 15 ml of a toluene-2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyl-14]benzene. A refrigerated Packard Tri-Carb 2145 liquid scintillation spectrometer was used for measurements of radioactivity. Corrections for quenching were made by monitoring with the external standard and, in a few cases, by adding an internal standard. In the case of 1-14C-decanoic acid, binding also was measured by equilibrium dialysis as described previously (24).

Preliminary studies with the equilibrium partition binding method revealed that the percentage standard error for eight samples obtained from the heptane phase of eight identically prepared incubation flasks was 2.24%. The percentage standard error for the eight samples of the aqueous phases containing albumin was 0.26%.

The total concentration of fatty acid present in both the heptane and the aqueous phases was calculated from the radioactivity measurements. Knowing the concentration in the heptane phase (C0), the molar concentration of unbound fatty acid (c) in the aqueous phases was determined from Equation 1:

\[
c = \frac{C_0}{P.R.} = \frac{2C_0}{\frac{P.C.}{10^{12} M^{-1}} (1 + \sqrt{1 + 8D_cC_0})}
\]  

(1)

where P.R. is the partition ratio, P.C. is the partition coefficient of the monomeric acid, and Dc is the dimerization constant in the organic phase (13, 14, 19). Table I lists the values of P.C. /1012 M-1 and Dc employed in this equation. The apparent pK of ionization was taken as 4.5 for each of the fatty acids (19). Except in the case of decanoate, the maximum change in the pK of the aqueous phase containing albumin was -0.07 unit and, consequently, a value of 7.4 was employed. For decanoate, the measured pH value in the aqueous phase of each vial was utilized in Equation 1. Next, the concentration of bound fatty acid in the aqueous phase was calculated by subtracting the unbound from the total concentration. Finally, the average number of moles of fatty acid bound per mol of albumin (\(K_i\)) was determined. Corrections for electrostatic interactions were not made in the binding analysis (29), and concentrations were substituted for activities in all of the calculations.

The data for each acid, consisting of values of \(v^2\) as a function of \(c\), were fitted to the stepwise equilibrium binding model shown in Equation 2 (20, 23).

\[
v^2 = \frac{K_0 + 2K_1c^2 + \cdots + nK_n c^n}{1 + K_0 + K_1c^2 + \cdots + K_n c^n}
\]  

(2)

In this model, the unknown parameters \(K_0, K_1, \ldots, K_n\) represent the equilibrium constants for the binding of the fatty acid to albumin. The analysis was done on an IBM System/370 computer using a nonlinear least squares model-fitting procedure called MODELDAIDE (30). A Tektronix 4010 graphic display terminal was employed to access this interactive PL/I program under IBM’s Time Sharing Option.

RESULTS

The association constants, \(K_i\), calculated by the stepwise equilibrium method for lauric, myristic, palmitic, oleic, and linoleic acid binding to human plasma albumin are listed in Table II. For myristate, palmitate, and stearate, the acids for which reliable aqueous phase association constants are available (18, 19), the constants calculated both in the assumed presence and absence of aqueous phase fatty acid association are presented. In the case of myristate, the values of \(K_i\) through \(K_4\) are only 17 to 25% larger when fatty acid association is assumed to occur. By contrast, these values are 313 to 340% larger in the case of palmitate and 510 to 675% larger in the case of stearate when association is taken into account. In general, the magnitude of these differences increased with successive equilibrium constants; i.e., for stearate, the increase in \(K_4\) assuming the presence of association was 510% and that in \(K_6\) was 675%.

Fig. 1 illustrates the experimental data points and the best fitting binding isotherm for myristic acid. These data are plotted with \(v^2/c\) as the ordinate and \(v\) as the abscissa as described by Scatchard (15). The shape of the binding isotherms observed in these Scatchard plots is consistent with the downward progression in the myristate \(K_i\) values. The Scatchard plots for each of the other acids also had a shape consistent with the downward progression in \(K_i\) values. Slightly stronger binding of myristate was obtained when the corrections for unbound fatty acid association were included (upper curve), but the difference is quite small as suggested by the values of \(K_i\) listed in Table II. In similar plots for palmitate and stearate, however, the intercept on the \(v/c\) axis was increased appreciably when fatty acid association was included.

For each individual \(K_i\), the magnitude of the value increased as the chain length of the fatty acid increased, i.e. stearate > palmitate > myristate > laurate. Fig. 2 shows the relationship between the \(K_i\) values for these saturated fatty acids and those for hexanoate, octanoate, and decanoate obtained previously (24). The values are plotted for \(K_i\) through \(K_4\), the physiologically important range of fatty acid binding to human albumin. For each of the \(K_i\), a sigmoidal rather than linear relationship is obtained when log \(K_i\) is plotted against the number of carbon atoms contained in the fatty acid. Use of the \(K_i\) values obtained assuming the presence of unbound fatty acid association for myristate, palmitate, and stearate tends to straighten these curves, but the over-all shape remains sigmoidal. The greatest change in the slope of these log \(K_i\) curves occurs between decanoate and laurate. Since the measurements for the three shorter acids, hexanoate, octanoate, and decanoate, were obtained by equilibrium dialysis (24) whereas those for the longer acids were obtained by equilibrium partition, it was feasible that the sigmoidal nature of the relationship in Fig. 2 was an artifact due to differences in methodology. In order to investigate this, we measured decanoate binding by both equilibrium partition and dialysis. As shown in Fig. 3, both of these procedures gave very similar results, at least over the physiologically important fatty acid...
<table>
<thead>
<tr>
<th>Equilibrium Constant</th>
<th>Laurate</th>
<th>Myristate</th>
<th>Myristate (^a)</th>
<th>Palmitate</th>
<th>Palmitate (^a)</th>
<th>Stearate</th>
<th>Stearate (^a)</th>
<th>Oleate</th>
<th>Linoleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_1)</td>
<td>(2.44 \times 10^5)</td>
<td>(2.11 \times 10^7)</td>
<td>(2.46 \times 10^7)</td>
<td>(6.15 \times 10^7)</td>
<td>(2.55 \times 10^6)</td>
<td>(1.49 \times 10^6)</td>
<td>(9.14 \times 10^6)</td>
<td>(2.56 \times 10^6)</td>
<td>(7.92 \times 10^7)</td>
</tr>
<tr>
<td>(K_2)</td>
<td>(1.12 \times 10^6)</td>
<td>(6.42 \times 10^6)</td>
<td>(7.57 \times 10^6)</td>
<td>(2.34 \times 10^7)</td>
<td>(9.77 \times 10^7)</td>
<td>(5.33 \times 10^7)</td>
<td>(3.56 \times 10^6)</td>
<td>(9.36 \times 10^7)</td>
<td>(8.71 \times 10^6)</td>
</tr>
<tr>
<td>(K_3)</td>
<td>(4.91 \times 10^5)</td>
<td>(2.66 \times 10^6)</td>
<td>(3.23 \times 10^6)</td>
<td>(1.19 \times 10^7)</td>
<td>(5.39 \times 10^7)</td>
<td>(1.89 \times 10^7)</td>
<td>(1.34 \times 10^6)</td>
<td>(2.94 \times 10^7)</td>
<td>(5.10 \times 10^6)</td>
</tr>
<tr>
<td>(K_4)</td>
<td>(2.52 \times 10^5)</td>
<td>(1.01 \times 10^6)</td>
<td>(1.26 \times 10^6)</td>
<td>(3.10 \times 10^6)</td>
<td>(1.36 \times 10^7)</td>
<td>(5.56 \times 10^6)</td>
<td>(4.31 \times 10^7)</td>
<td>(2.21 \times 10^7)</td>
<td>(3.06 \times 10^6)</td>
</tr>
<tr>
<td>(K_5)</td>
<td>(1.88 \times 10^5)</td>
<td>(8.08 \times 10^5)</td>
<td>(1.01 \times 10^6)</td>
<td>(1.48 \times 10^6)</td>
<td>(7.36 \times 10^6)</td>
<td>(4.45 \times 10^6)</td>
<td>(3.45 \times 10^7)</td>
<td>(1.12 \times 10^7)</td>
<td>(6.77 \times 10^5)</td>
</tr>
<tr>
<td>(K_6)</td>
<td>(6.23 \times 10^4)</td>
<td>(5.59 \times 10^5)</td>
<td>(7.59 \times 10^6)</td>
<td>(9.56 \times 10^5)</td>
<td>(4.43 \times 10^6)</td>
<td>(3.71 \times 10^6)</td>
<td>(2.27 \times 10^3)</td>
<td>(2.35 \times 10^6)</td>
<td>(3.35 \times 10^5)</td>
</tr>
<tr>
<td>(K_7)</td>
<td>(2.14 \times 10^4)</td>
<td>(1.17 \times 10^5)</td>
<td>(1.56 \times 10^5)</td>
<td>(4.38 \times 10^5)</td>
<td>(2.09 \times 10^6)</td>
<td>(3.18 \times 10^6)</td>
<td>(2.46 \times 10^7)</td>
<td>(2.01 \times 10^8)</td>
<td>(1.07 \times 10^5)</td>
</tr>
<tr>
<td>(K_8)</td>
<td>(1.07 \times 10^4)</td>
<td>(1.03 \times 10^5)</td>
<td>(1.36 \times 10^5)</td>
<td>(4.80 \times 10^5)</td>
<td>(1.83 \times 10^6)</td>
<td>(2.75 \times 10^6)</td>
<td>(2.16 \times 10^7)</td>
<td>(1.64 \times 10^6)</td>
<td>(9.35 \times 10^4)</td>
</tr>
<tr>
<td>(K_9)</td>
<td>(1.66 \times 10^4)</td>
<td>(9.12 \times 10^4)</td>
<td>(1.21 \times 10^5)</td>
<td>(3.38 \times 10^5)</td>
<td>(1.63 \times 10^6)</td>
<td>(2.47 \times 10^6)</td>
<td>(1.92 \times 10^7)</td>
<td>(1.11 \times 10^6)</td>
<td>(8.31 \times 10^4)</td>
</tr>
<tr>
<td>(K_{10})</td>
<td>(6.15 \times 10^3)</td>
<td>(5.66 \times 10^4)</td>
<td>(7.66 \times 10^4)</td>
<td>(3.04 \times 10^5)</td>
<td>(1.47 \times 10^6)</td>
<td>(4.64 \times 10^6)</td>
<td>(7.48 \times 10^4)</td>
<td>(4.22 \times 10^5)</td>
<td>(6.80 \times 10^4)</td>
</tr>
<tr>
<td>(K_{11})</td>
<td>(3.86 \times 10^3)</td>
<td>(5.14 \times 10^4)</td>
<td>(6.96 \times 10^4)</td>
<td>(2.77 \times 10^5)</td>
<td>(1.33 \times 10^6)</td>
<td>(4.22 \times 10^5)</td>
<td>(6.80 \times 10^4)</td>
<td>(4.22 \times 10^5)</td>
<td>(6.80 \times 10^4)</td>
</tr>
<tr>
<td>(K_{12})</td>
<td>(3.54 \times 10^3)</td>
<td>(4.71 \times 10^4)</td>
<td>(6.38 \times 10^4)</td>
<td>(2.54 \times 10^5)</td>
<td>(1.22 \times 10^6)</td>
<td>(3.87 \times 10^5)</td>
<td>(1.16 \times 10^6)</td>
<td>(4.22 \times 10^5)</td>
<td>(6.80 \times 10^4)</td>
</tr>
</tbody>
</table>

Nu. of data points: 76, 133, 133, 115, 115, 36, 36, 56, 53

Root-mean-square error: 0.574, 0.616, 0.619, 0.372, 0.371, 0.190, 0.194, 0.394, 0.290

\(^a\)Calculated using corrections for unbound fatty acid association in the albumin solution.
Fig. 1. Binding of [1-14C]myristate to human plasma albumin at 37°C. The lower data set assumes that aqueous phase association does not occur; the upper data set was calculated assuming the existence of aqueous phase association of unbound myristate.

Fig. 2. Relationship between fatty acid chain length and the equilibrium constants $K_1$ through $K_4$ for binding of saturated fatty acids to human plasma albumin. The points on the left side were calculated assuming no aqueous phase association; those on the right side assume that aqueous phase association of unbound fatty acid occurs.

Fig. 3. Comparison [1-14C]decanoate binding to human plasma albumin at 37°C as measured by equilibrium partition and equilibrium dialysis.

Fig. 4. Effect of acidification on [1-14C]palmitate binding to human plasma albumin. The data points plotted as open symbols were calculated assuming no aqueous phase association; those plotted as solid symbols assume that aqueous phase association of unbound palmitate occurs.

Palmitate binding was compared at two different pH values, 7.4 and 6.3. As seen in Fig. 4, there was actually little difference in the binding data obtained at the two pH values when the palmitate partition data were corrected for the differences in pH (19). These results are in agreement with those obtained previously for decanoate and octanoate (24) as well as for alkyl sulfates (33, 34), which indicate that there is little change in the binding of organic anions to plasma albumin over this pH range. With palmitate, the relationship between the pH 6.3 and 7.4 data was not changed appreciably when corrections for palmitate association in the aqueous phase were included in the calculations (solid symbols in Fig. 4).

DISCUSSION

In the present analysis of long chain fatty acid binding to human plasma albumin, the stepwise equilibrium constants occur in descending order for each of the acids. This suggests that major cooperative binding interactions do not occur over the physiologically important range of fatty acid-albumin molar ratios. A similar conclusion has been reached for medium chain fatty acid binding to human albumin (24) and long chain fatty acid binding...
to bovine albumin (35). This is consistent with data obtained with long chain alkyl sulfates indicating that detergent anions cause gross disorganization of the albumin structure only when more than 10 mol are bound (36).

The currently available association constants for long chain fatty acid binding to human albumin were obtained according to the Scatchard method of analysis (13). Although they are not directly comparable to the equilibrium constants determined using the stepwise binding model, some general comparisons between the two sets of data can be made. Both are similar in several respects. For example, the limiting value of $D/C$ obtained by Goodman for oleate was $2.4 \times 10^4$ (13) whereas our data, assuming that fatty acid association does not occur, give a value of $2.6 \times 10^6$. The strength of binding to the strong albumin sites ($K_1$ through $K_4$) according to our data, assuming no premicellar association, is: oleate $>$ stearate $>$ linoleate $\cong$ palmitate $>$ myristate $>$ laurate. Goodman obtained a similar pattern with the exception that linoleate was much less tightly bound than palmitate and, at higher values of $F$, stearate also was bound less firmly than palmitate (13). Our studies, however, do not support the widely accepted binding model in which albumin contains 2 strong binding sites, 4 to 5 intermediate sites, and 20 or more weak sites (13). As seen in Table II, there is no distinct separation of the binding constants into well defined classes. For most of the acids, $K_1$ is 2 to 3 times larger than $K_2$, and $K_1$ is 2 to 3 times larger than $K_2$. Likewise, there is no sharp demarcation between $K_2$ or $K_3$ and the remaining $K_i$. This suggests that the separation of the binding sites into strong, intermediate, and weak classes is somewhat arbitrary. In this context, it should be noted that uniform Scatchard models have been employed to fit the binding data obtained for a series of fatty acids primarily to facilitate comparisons between the various acids (13, 25). Better fits of the data for each individual acid always can be obtained when separate Scatchard models, in which the sites were grouped differently for each acid, are used (13, 25). This points out one of the main practical advantages of the stepwise equilibrium method of analysis. Since the equilibrium constants for the binding of each mole of ligand are determined separately, the binding of several different ligands, such as palmitate, stearate, and oleate, can be compared on a mole for mole basis without having to assume any uniformity in the manner in which the ligands are bound.

In the cases of palmitate and stearate, much larger $K_i$ values were obtained when unbound fatty acid association was taken into account, even for $K_1$ and $K_2$. This result differs markedly from our previous conclusions with bovine albumin, where dimerization of fatty acid was thought to have little effect on palmitate and stearate binding in the low $F$ range (35). The apparent discrepancy can be accounted for in part by the fact that we have used larger corrections for fatty acid association, based on the recent work of Smith and Tanford (18), as well as our own studies on fatty acid partition (19). It was hoped that certain of the binding data, particularly the pH study shown in Fig. 4, might aid in resolving the question of whether fatty acid premicellar association occurs in the physiological concentration range. These studies, however, do not answer this question definitively. Several inconsistencies in the partition data obtained with palmitate and stearate are resolved by the assumption that dimerization occurs in the aqueous phase (17, 18). On the other hand, a number of serious objections can be raised with the dimerization hypothesis in its present form (19). Until a more convincing case for fatty acid premicellar association is made, we think it best to continue to use the monomer data for biological applications. It is quite possible that the premicellar association hypothesis ultimately will prove to be correct and, therefore, we have included the data analyzed according to this assumption for possible future reference.

Previous studies demonstrated that fatty acid binding to plasma albumin involves electrostatic interactions between the carboxylate anion of the fatty acid and positively charged side chains of the protein, together with nonpolar interactions between the hydrocarbon chain of the acid and hydrophobic side chains (2, 37-39). Since $K_i$ increases greatly as the fatty acid chain length increases, most of the binding energy is thought to be derived from the nonpolar interactions (13, 29, 40). As shown in Fig. 2, the relationship between $\log K_i$ and fatty acid chain length for the saturated acids containing 6 to 18 carbon atoms is sigmoidal, not linear. Therefore, the binding energy is not simply a statistical process dependent entirely on chain length. The nonpolar binding sites of plasma albumin are able to interact with the hydrocarbon chains of laureate and myristate to a greater extent than would be predicted by their content of hydrocarbon groups. This probably is due to the configurational adaptability of the albumin binding site, described originally by Karush (41, 42). The binding sites, however, apparently cannot adapt sufficiently for maximal nonpolar interactions with the 16 and 18 carbon atom saturated acids, as indicated by the deviation from linearity in the upper portion of the curves in Fig. 2. Although corrections for unbound fatty acid association produce some straightening of the upper segments of these curves, the relationship still remains sigmoidal. The fact that the over-all shape of the curves is similar for each of the first four $K_i$'s supports our view that these higher energy binding sites of albumin cannot be separated into distinct classes, as was suggested by previous studies (6, 13, 25).

REFERENCES

41. KARUSH, F. (1950) J. Am. Chem. Soc. 72, 2705–2713
42. KARUSH, F. (1950) J. Am. Chem. Soc. 72, 2714–2718
Long chain fatty acid binding to human plasma albumin.
J D Ashbrook, A A Spector, E C Santos and J E Fletcher


Access the most updated version of this article at http://www.jbc.org/content/250/6/2333

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/6/2333.full.html#ref-list-1