Myristic Acid Binding to Human Serum Albumin Investigated by Dialytic Exchange Rate*

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Dialysis rate determinations of several fatty acids in the absence of albumin revealed that the myristate anion, like that of laurate, in aqueous solution, pH 7.5, is present as a monomer anion when the concentration is below 25 μM. Palmitate and oleate solutions, on the other hand, show a tendency to aggregation even at concentrations below 0.5 μM.

Multiple binding of myristate to human serum albumin in phosphate buffer, at pH 7.5, 37°C, was investigated by exchange of 14C-labeled myristate across a dialysis membrane under conditions of binding equilibrium. A binding isotherm was established by least squares fitting of the stoichiometric binding constants in the stepwise binding equation to the experimental data. The best-fit solution was supplemented with 30 acceptable solutions within a probability limit of 0.95. A concept of one or two distinct high-affinity sites for binding of fatty acids could not be verified; the observations allow a variety of binding mechanisms ranging from cooperativity of the first two myristates to a model with four equal and independent sites.

Serum albumin comprises about 60% of the plasma proteins and is capable of binding a broad spectrum of ligands (1). These include some inorganic ions and a multitude of metabolites, hormones, and drugs (2). Albumin is the major carrier of fatty acids in plasma (3). There are indications of a specific function of myristate regarding co-translational covalent modification of certain mammalian proteins (4), but the bulk of myristate, as well as the other naturally occurring fatty acids, serves as fuel for energy metabolism. Myristic acid constitutes 2–4% of the nonesterified fatty acids in plasma (5).

A dialysis method allows determination of the concentration of unbound fatty acid anion in the presence of albumin, by measuring the rate of exchange of 14C-labeled fatty acid across a dialysis membrane with an identical sample on the other side (6). In the present paper we apply this method to myristic acid binding equilibria with human serum albumin. We have further attempted to use the same procedure for palmitate and oleate binding studies.

The experimental results were analyzed according to a stepwise binding model yielding stoichiometric binding constants. By incorporating the stochastic variation of the data in a variety of acceptable solutions to the stoichiometric binding equation, patterns of binding interaction were elucidated.

EXPERIMENTAL PROCEDURES

Chemicals—Human serum albumin (AB Kabi, Sweden) was defatted with charcoal after acidification with H2SO4 to pH 3 at 0°C, dialyzed against pure water at 5°C, and lyophilized. 60 μM solutions of defatted albumin, and 180 μM in some experiments, were prepared by weighing and dissolution in a 132 mM sodium phosphate buffer, final pH 7.5, and were used on the same day. In experiments with dialysis times longer than 4 h, gentamicin sulfate, 20 mg/liter, was added to prevent bacterial growth.

Myristic acid (n-tetradecanoic acid; purest grade >99.5%) was from Fluka AG, Switzerland. A stock solution of 500 μM myristate was prepared in 57 mg of myristic acid in 0.5 liter NaOH, 2 mM, under gentle stirring at 45°C for 2–3 h. This solution, stored at 5°C, could be used for 1 week. Diluted myristate solutions, prepared from the stock solution, were used on the same day.

[1-14C]Myristic acid, 31 Ci/mmol, solution 100 μCi/ml, was from Amer sham International, England. Aqueous solutions were prepared by removal of the toluene under a stream of nitrogen and, when redisolving with 2 mM NaOH, the concentration of myristate was 50 μM.

Dialysis Exchange Rate Determination—The dialytic method, measuring the equilibrium concentration of unbound fatty acid anion by the rate of exchange of radiolabeled ligand with an identical fatty acid-albumin mixture, as described in our previous work (6). Membranes were cut from cellophane dialysis tubing, Union Carbide Corp., type 36/32, nominal thickness 0.0008 inch. In the absence of albumin, equilibration of 14C label was achieved in 3 h at 37°C when 25 μM solutions of myristate in sodium phosphate buffer, pH 7.5, with and without radiolabel, were placed on either side of the membrane.

Radiochemical Purity of [14C]Myristate—On each side of the dialysis membrane was injected 1.00 ml of albumin solution (range of concentration, 24–384 μM) in 66 mM sodium phosphate, pH 7.4. On the left side was added 50 μl of [14C]myristate, 50 μM, and on the right side a similar volume of 50 μM myristate. Dialysis, at 37°C, was allowed for 19 h. Linear extrapolation of dialysis rate to infinite concentration of albumin yields a non-zero residual value corresponding to approximately 0.1% nonbinding radioactive material in the [14C]myristate. According to the manufacturer, the radiochemical purity of the [14C]myristate sample was 98%. The difference between these two estimates must be due to components binding to albumin, possibly other fatty acids. This degree of radiochemical purity is considered sufficient for the present investigation, since, as shown by Honore (7), dialysis rate determination is less sensitive to impurities than conventional equilibrium dialysis.

Rate Constant for Dialysis of Myristate without Albumin—On each side of the dialysis membrane was injected 1.00 ml of myristate (concentration range, 0.4–40 μM) in 66 mM sodium phosphate, final pH 7.5. On the left side was added 10 μl of [14C]myristate, 50 μM, and on the right side a similar volume of 50 μM myristate. Dialysis, at 37°C, was allowed for periods from 5 to 60 min. In experiments in which the concentration of myristate was 25 μM or lower, the dialytic equilibration of radioactivity proceeded in accordance with first-order kinetics, and the rate constant, k, could be calculated.

Binding Isotherm for Myristate—On each side of the dialysis membrane, 500 μl of myristate (concentration range, 1–500 μM) and 500 μl of albumin, 60 μM, in 132 mM sodium phosphate, final pH 7.5, was injected. On the left side was added 10 μl of [14C]myristate, 50 μM, and on the right side a similar volume of 50 μM myristate. The time

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of dialysis was varied from 240 min for the highest concentrations of myristate to 1080 min for the lowest concentrations, in order to obtain convenient degrees of diffusion, considerably short of equilibration. The concentration of unbound myristate and the molar ratio of bound myristate to albumin were calculated as described previously (6).

Experiments with a higher albumin concentration, 180 µM, same buffer, and myristate concentration range of 140-500 µM, were also undertaken.

In experiments where the expected concentration of unbound myristate was higher than 25 µM, reproducibility was poor and solutions could appear cloudy. We have removed such results from the data.

Data Analysis—Multiple binding equilibria between myristate and albumin were analyzed in terms of stepwise binding according to the stoichiometric equation (8).

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Q = K_0 + 2K_0K_1c + \ldots + NK_0K_1 \ldots K_Nc^N \]

where \( Q \) is the average number of myristate bound per albumin molecule, \( c \) is the equilibrium concentration of myristate, and \( K_i \) is the stoichiometric binding constant for step \( i \). In the statistical analysis, as developed by Brodersen et al. (9), Equation 1 was fitted to the experimental data, iterating \( K \) values until minimum of the sum of weighted and squared deviations. This best-fit solution was supplemented by a number of other solutions acceptable within a chosen probability limit, as judged by an \( F \)-test.

RESULTS

Dialysis of Fatty Acids in the Absence of Albumin—The first-order rate constant for exchange of \(^{14}C\)-labeled fatty acid across the dialysis membrane was determined graphically. Measurements were carried out with four fatty acid anions, laurate, myristate, palmitate, and oleate.

As described in our previous paper (6), dialysis of laurate strictly follows first-order kinetics with a reproducible rate constant, \( k = 0.034 \text{ min}^{-1} \), in the concentration range from 0.1 to 500 µM.

Dialysis of myristate follows a similar pattern in a concentration range from 0.4 to 25 µM. The rate constant was 0.027 ± 0.001 min\(^{-1}\) (n = 7). When the concentration of myristate exceeded 25 µM, the solutions appeared unstable, as indicated by the scattering of experimental points in Fig. 1. These solutions of myristate are supersaturated with a tendency to aggregation.

Palmitate solutions appeared unstable in concentrations as low as 0.4 µM, as indicated in Fig. 1. Furthermore, recovery of radioactivity, 25%, was much lower than with laurate and myristate, 90%. We conclude that determination of a rate constant for dialysis of palmitate is not possible with the present equipment or method. The same was found for oleate.

Dialysis of Fatty Acids in the Presence of Albumin—When the rate constant for dialysis of myristate in the absence of albumin is known, measurement of dialysis rate of fatty acids in the presence of albumin allows determination of the unbound fatty acid concentration, \( c \), and the molar ratio of bound fatty acid to albumin, \( r \). The present binding data for myristate and the previous findings for laurate have been pictured together in Fig. 2, to facilitate the conclusion that albumin binds myristate tighter than laurate.

The measurements presented in Fig. 2 were all carried out at a constant concentration of albumin, 30 µM. Binding of myristate was also investigated at an albumin concentration of 90 µM. The latter measurements resulted in points on the same binding curve, proving that reversible albumin-to-albumin interaction does not interfere with the myristate binding equilibria.

Binding Constants—A best-fit approximation to the experimental data was obtained by iteration of the stoichiometric binding constants in Equation 1 until minimum of the sum of weighted and squared deviations. The integer \( N \), the formally maximal number of moles of myristate bound to 1 mol of albumin, was taken as the least value compatible with optimal fitting. This was 9 in the present study and was equal to the integer value of the highest observed number of bound myristate plus one. The stoichiometric constants giving this best-fit approximation are shown in Table I.

Binding Interaction—The binding constants are plotted as a Klotz affinity profile in Fig. 3. In this diagram (8), homogeneous binding of ligand to a carrier with nine equal and independent sites would appear as a straight line from point

![Table 1: Stoichiometric binding constants for myristate-human serum albumin.](image)

![Fig. 2. Fatty acid anion binding. Semilogarithmic plot of concentration of bound versus unbound fatty acid. □, myristate; ●, laurate.](image)
Binding of Myristate to Albumin

**FIG. 3.** Klotz affinity profiles for binding of myristate. Two sets of $K$ values plotted as $iK$ versus $i$. Full line, best-fit approximation indicating cooperative binding of two molecules of myristate; dotted line, another approximation among 30, this one indicating homogeneous binding of four molecules of myristate.

**FIG. 4.** Calculated isotherms for binding of myristate. Full line, binding curve derived from the best-fit approximation; dotted line, binding curve derived from the binding constants, reported by Ashbrook et al. (14).

**FIG. 5.** Calculated Scatchard plots for binding of myristate. Binding curves derived from 30 acceptable sets of binding constants. For clarity, data for $r > 5$ have been omitted.

(1, $K_i$) to point (10,0). On the other hand, if the line connecting two consecutive steps shows a more positive or a more negative slope, then positive or negative cooperativity between these two steps is indicated. Cooperativity of the first two myristate is apparently present, as concluded from the best-fit binding constants.

In addition to the best-fit solution we generated 30 acceptable sets of binding constants to the probability limit 0.95 (9). The range of these constants are given in Table I. Several of these sets did not show cooperativity of the first two steps. One of these is tabulated in Table I and pictured in Fig. 3. If we consider the first four steps alone it is seen that approximately homogeneous binding is indicated by this particular set of constants. The observed data can thus be satisfied by a variety of different binding patterns, ranging from a mechanism with cooperative binding of the two first myristate to homogeneous binding to four equal and independent sites.

**DISCUSSION**

**Fatty Acids in Aqueous Solution**—Quantitative analysis of fatty acid binding to albumin by the present method depends upon a knowledge of the dialysis rate constant. In addition this parameters appears informative on the physical state of fatty acids in aqueous solution, being related to the molar mass of the migrating particle. In the case of laurate (6), we have reported an unchanged rate constant over a concentration range from 0.1 to 500 $\mu$M, showing unchanged molar mass within the same interval. We concluded that the migrating particle is a monomer laurate anion. This is in accordance with the results of Eagland and Franks (10), who found agreement between pH versus conductivity measurements in laurate solutions with the Debye-Hückel limiting law for a uni-univalent electrolyte.

By comparing the dialysis rate of myristate with that of laurate, we note a constant and slightly lower dialysis rate over a concentration range from 0.4 to 25 $\mu$M, showing a higher molar mass. We conclude that the migrating particle is a monomer myristate anion. However, when the concentration of myristate exceeds approximately 25 $\mu$M, the observations of dialysis rate lost reproducibility, as shown in Fig. 1. We assume that this is due to time-dependent aggregation.

With palmitate and oleate we were not able to get reproducible results at all. Even very dilute aqueous solutions of palmitate are heterogeneous with respect to state of aggregation. Establishment of a binding isotherm is not possible with the present method or equipment.

The above conclusions do not agree with solubility data in the literature (11, 12), where significantly higher values are reported. The latter could possibly be explained by the presence of soluble aggregates.

**Binding of Myristate to Albumin**—Goodman in 1958 (13) reported binding isotherms for several fatty acids including myristic acid to human serum albumin. It is noteworthy that Goodman’s curve, bound versus log free ligand, shows the
same peculiar shape as seen in our Fig. 4. A comparison of numerical results is, however, not possible due to differences of experimental temperatures.

Ashbrook et al. (14) obviated the difficulties incurred with site models by introducing the stoichiometric description for the study of fatty acid binding. They obtained a set of stoichiometric binding constants for myristate-human serum albumin under similar experimental conditions as in our studies. For comparison, we have recalculated their binding curve from the reported constants, and a plot is included in Fig. 4. This curve appear similar in shape to ours, when less than five molecules are bound, but is displaced toward higher free concentrations, indicating weaker binding. The deviations between the two binding isotherms might be explained by methodological differences since Ashbrook et al. (14), using the phase distribution technique with n-heptane, have worked in an aqueous solution saturated with n-heptane. It is an advantage to use a method in which organic solvents are absent, and which discriminates between monomer and aggregates of the fatty acid anion, and is rather insensitive to errors caused by radioactive impurities. The dialytic exchange rate technique fulfils these criteria.

We finally note that a level of saturation could not be reached although we carried the experiments to the upper concentration limit for obtaining reproducible results, 25 \( \mu \text{M} \) unbound myristate. It is a general feature of albumin binding isotherms that saturation cannot be obtained (15).

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REFERENCES