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The rate of access to the organic ligand-binding region of serum albumin is entropy controlled

(kinetic/reaction rate/activation/dielectric)

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ABSTRACT **The technique of real time dielectric relaxation measurement coupled with a conventional stopped-flow device has made it possible to measure the rates of association and dissociation of the complex of human serum albumin with its most prevalent ligands, the long-chain fatty acids. This association was previously shown to proceed in two steps: a fast, probably diffusion-controlled, nonspecific association, followed by a slower ($\approx 3\ sec^{-1}$) rearrangement of the intermediate protein–ligand configuration, whose kinetics is first order. By use of the Arrhenius relation and standard theory of rate processes, it is determined that there is virtually no activation enthalpy in the forward binding reaction and that the rate of access to the interior hydrophobic binding region of serum albumin is controlled by a negative entropy of activation, reflecting a high degree of ordering in the transition state. A complete thermodynamic and kinetic profile of the association reaction is given.**

Long-chain fatty acids (FA), which in higher animals are the source of about half the normal energy requirements of the organism, are transported in the serum by means of reversible association of high affinity (10^7–$10^8\ M^{-1}$) with serum albumin. Aside from its physiological importance, this binding reaction shares a number of unique properties with a limited class of other strongly hydrophobic ligands. For these reasons the present investigation was aimed at developing an understanding of the mechanism of this association. There is reason to believe that it will be possible, and useful, ultimately to compare the characterization of the fatty acid complexation with that of other organic ligands of serum albumin to attempt to define more precisely the classes of ligand and the categories of binding site.

Serum albumin, a globular protein of molecular weight 67,000, performs multiple functions, of which the most extensively studied is the transport function. Some 200 distinct organic species, including metabolites, hormones, drugs, and waste products, are bound with affinities ranging from 10^4 to $10^8\ M^{-1}$. Albumin serves as solubilizer, reservoir, controlled release mechanism, and detoxifier. Thirty to 70 mol of FA can be bound to each mol of human serum albumin (HSA), but the physiological range is normally between 1 and 5 mol/mol. The first 2 or 3 mol are bound very tightly ($K_{eq} \approx 10^7$–$10^8\ M^{-1}$) (1). The equilibrium properties of the HSA–FA association and the widespread and complex interactions of this association with the binding affinities of many other compounds have been extensively studied (1–12).

In spite of the long history of this problem, the mechanism of such binding is not well understood. The work of Foster *et al.* (13) on the acid transition suggested that HSA contains a hydrophobic portion of its surface which is normally interior to the protein and which forms the primary binding region for a limited number of strongly hydrophobic ligands, including the first 2–5 mol of FA, bilirubin, and various indol compounds such as tryptophan. More recently, with the determination of the complete amino acid sequence of HSA (14–18), this view has gained support from proposed models of secondary structure showing how a hydrophobic surface might arise from the juxtaposition of several segments of helix, each with a high asymmetric density of hydrophobic side chains on one side.

One of the important sources of information about the mechanism of a chemical reaction is the measurement of the reaction *rates*, both forward and reverse, and of the temperature dependence of these rates. From such data one can deduce the activation energy, which provides a measure of the thermal chemical disruption, or bond cleavage, required to form the activated state from which the reactants return to either the reacted or the unreacted form. Such measurements also permit calculation of activation *entropy*. For reactions involving highly ordered molecules such as proteins, activation entropy, either positive or negative, can be an important factor in determining reaction rates. Ordering, or loss of order, in protein reactions can come about either by change in the configurational probability or by change in the degree of order of the solvent environment.

Equilibrium constants (1) and an earlier determination of the dissociation rate of a HSA–FA complex (19) suggested that the association would turn out to be limited by an activation process. However, attempts to study the kinetics of this reaction by stopped-flow methods (20) with conventional optical monitoring techniques yielded either null results or only marginally useful data. Ott's measurements (21) were at pH 11.5, at which the protein is far from its physiological conformation. Other efforts to take advantage of a mildly binding-sensitive fluorescent tryptophan (residue 134) (22) in bovine serum albumin (BSA) produced a null result. This residue is, in any case, absent in HSA. Until very recently (23) there were no sufficiently sensitive optical probes of FA binding to serum albumin for stopped-flow studies. I therefore developed a different kind of probe for the present investigation.

METHODS AND MATERIALS

In this study, the kinetics of the association of oleic acid with HSA were measured by a method (24) that couples repeatable rapid dielectric measurements with stopped-flow mixing. A diagram of the measurement system is shown in Fig. 1. This method monitors the progress of association by taking advantage of the strong dependence of dielectric increment of serum albumin on the mole ratio of bound FA (25). Moreover, by taking advantage of the sizeable quantitative difference between this dependence in HSA and in BSA, it has been possible to measure the kinetics of *dissociation*, by measuring FA exchange between HSA and BSA (26).

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Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; FA, fatty acid.

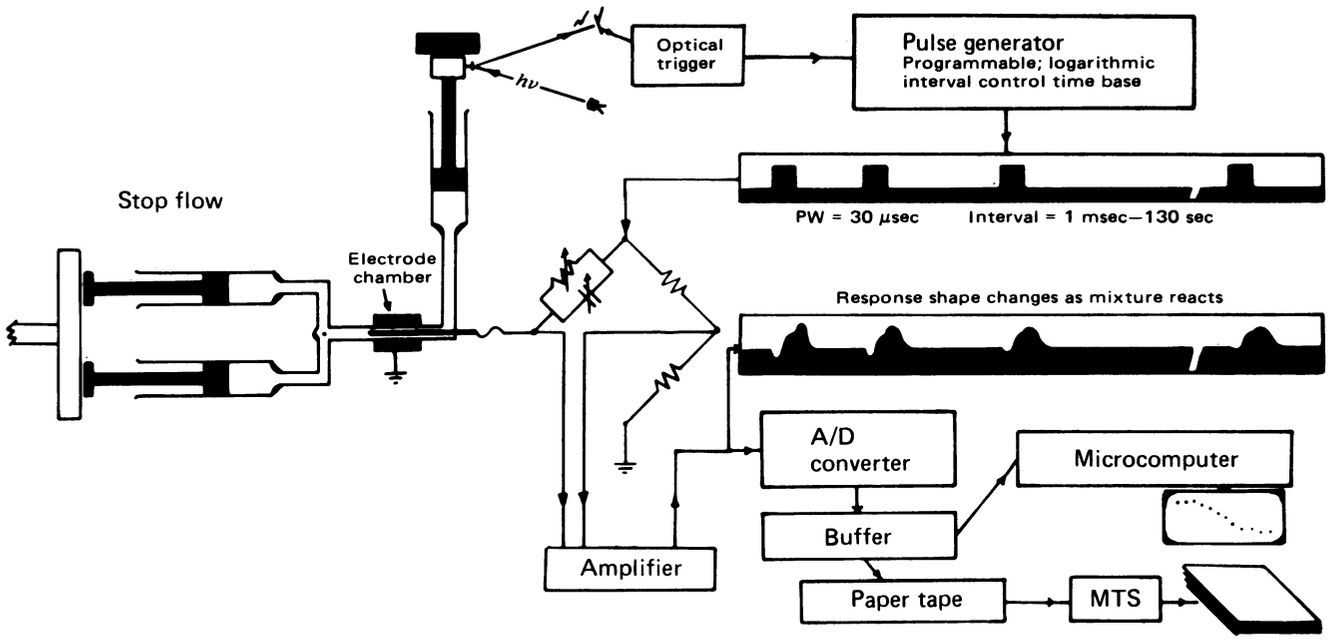

FIG. 1. Data acquisition from pulse-response rapid dielectric measurement system coupled to stopped-flow apparatus.

Reworked fraction V, batch 343, containing >97% HSA, and by our analysis <10% dimer, was obtained from the Blood Fractionation Laboratory (Michigan Department of Public Health, Lansing, MI). The powder was dissolved to 5% concentration in distilled water at <5°C. After 12 hr, the solution was filtered through a 0.45-μm Millipore filter. The native HSA contained 1.4 mol/mol fatty acid, as determined by the Dole titration (27). This material was defatted by the column method of Scheider and Fuller (28). This method was modified to decrease the time that the protein remained acidified to under 2.5 hr. The protein solution was acidified in the cold by adding 0.2 M HCl to a pH of 3.1 in a stirred container and was immediately applied to the top of a 60-cm column of XAD-2 (Mallinckrodt) hydrophobic surface area resin. The protein solution, acidified in small aliquots, was passed over the resin at a rate of 1/2 column volume per hr and directly into a mixed-bed (H^+ and OH^-) ion-exchange column, which restored the solution to its isoionic pH. Any defatted preparation whose measured pH in deionized solution at this point was not between 5.15 and 5.25 was discarded. The residual FA levels of these preparations were typically between 0.1 and 0.2 mol/mol. Preparations were stored in CO_2-free containers and were used within 10 days. Fatty acid-free serum albumin is known to be labile.

Two other types of preparation were used as controls. In the first, a sample of the same albumin was defatted by a different method by use of charcoal (29). In the second, a sample was prepared from a batch of HSA fractionated by S. Allerton at Harvard University. Both control preparations were indistinguishable from the standard preparation in the experiments of this study.

Sodium oleate stock solution, 20 mM in distilled water, was prepared by mixing equimolar quantities of oleic acid (lot R2009, chromatographically pure, Mann Research Labs, New York, NY) and reagent-grade sodium hydroxide. The stock solutions were kept under nitrogen in sealed vials and diluted just prior to use in experiments. The sodium oleate solutions were used both as reactant in stopped-flow, and for relipidation of defatted HSA for the ligand-exchange experiments. In relipidation, sodium oleate was added directly to solutions of defatted protein. The mixture was, where necessary, reconcentrated in an Amicon concentrator and finally deionized by passage over a mixed-bed ion-exchange resin.

Defatted BSA was prepared in similar fashion, from an albumin fraction, batch 3, prepared by T. Thompson at Harvard University.

For determination of association rates, a salt-free solution, 0.3–0.4 mM in defatted HSA and a solution of sodium oleate of a concentration approximately 75% of that of the protein were loaded into the two syringes of the stopped-flow mixing section by CO_2-free methods. The two solutions begin reacting within 1 msec of reaching the stopped-flow mixer, and the mixture is then passed to the monitoring cell (Fig. 1), where rapid dielectric measurements are automatically initiated.

For *dissociation* rate determination by ligand exchange, the two syringes are filled with HSA relipidated to a level of <1 mol/mol with oleic acid and with defatted BSA.

A control for the integrity of the relipidated protein was conducted with HSA that had never been defatted and whose ligand therefore consisted of native-bound FA. A second, critical, control consisted of exchanging lipid in the opposite direction, from relipidated BSA to defatted HSA; as required, the direction of the change in dielectric increment was opposite to that in the original ligand-exchange experiment.

Dielectric increment was calculated for each measurement point by iterative convergence of a single relaxation component approximation to the data, as described in ref. 24. From a sequence of such points, an association time constant was determined by least-squares fitting of a single exponential in the reaction time coordinate in the interval between 5 msec and 2 sec after the defatted protein was mixed with oleate. A second component of decay of dielectric increment in the interval (2–900 sec) is believed to result from ligand redistribution between protein that initially bound two or more ligand molecules and remaining apoprotein; these components can be adequately separated provided initial protein and oleate concentrations are below 0.3 mM.

In a previous article (24) I identified two steps in the association of FA with HSA. The first step is complete during the dead time of mixing (1 msec), and the second step follows first-order kinetics. Thus, the reaction rates reported here correspond to those of the second step of association. Since ligand exchange rates are about 1000 times slower than association, it is clear that ligand exchange is rate limited by the dis-

sociation of FA from the protein. Thus, dissociation rates are obtained directly by fitting the ligand exchange data (26).

RESULTS

By means of the methods described, it has been possible to obtain a complete thermodynamic and kinetic profile of the reaction,

$$\text{HSA} + \text{oleate} \underset{\text{1st step}}{\rightleftharpoons} \text{HSA-----oleate} \underset{\text{2nd step}}{\rightleftharpoons} \text{HSA-oleate} \qquad [1]$$

Arrhenius plots of the rate constant of the second step of association and of the rate constant of dissociation are shown in Fig. 2. From the slopes of these plots, forward and reverse activation *enthalpies* were calculated from the Arrhenius equation; activation *entropies* were calculated from equations related to the intercept at $1/T \rightarrow 0$ (30).

The most striking feature of these results is the virtual absence of temperature dependence in the second step of association, implying that this step occurs without an enthalpy barrier. The enthalpy barrier differs from the total energy of activation, which appears in the Arrhenius relation only by the quantity RT, the molar thermal energy term, or approximately 0.6 kcal/mol (1 cal = 4.184 joules). Thus, in a conventional activation barrier profile diagram, which does not include entropy terms, there would be no indication that the association of FA with HSA is rate limited by an activation process.

Consideration of the activation *entropy* shows that it is this kinetic variable which figures in the association rate, limiting the opportunity for the ligand to reach its final binding site to about once in 300 msec, irrespective of temperature.

In Table 1 are presented the results of these experiments, as well as preliminary results from recent calorimetric measurements (unpublished data) and other quantities deduced from equilibrium constants measured by Spector and coworkers (9). This table lists 13 thermodynamic and kinetic quantities involved in a complete characterization of a two-step reaction whose first step is approximated by a diffusion-limited process. Listed below the table are seven independent identities which relate these 13 quantities; thus, only six of the 13 are independent. Since eight experimental determinations are included, it is clear that two are redundant, providing two checks of consistency.

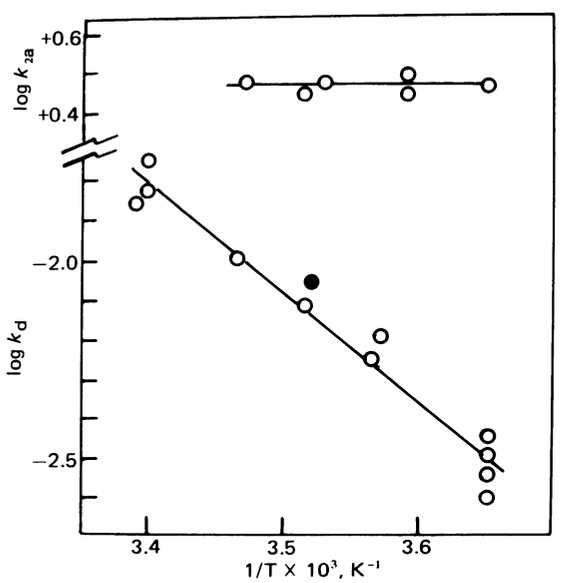

FIG. 2. Arrhenius plots of the second step of association (k_{2a}) and of dissociation (k_d) of the complex of oleate and HSA. In the plot of k_d: O, ligand exchange using defatted HSA to which oleate was subsequently added; ●, ligand exchange using HSA that had not been defatted and in which the ligand consisted of physiologically bound FA.

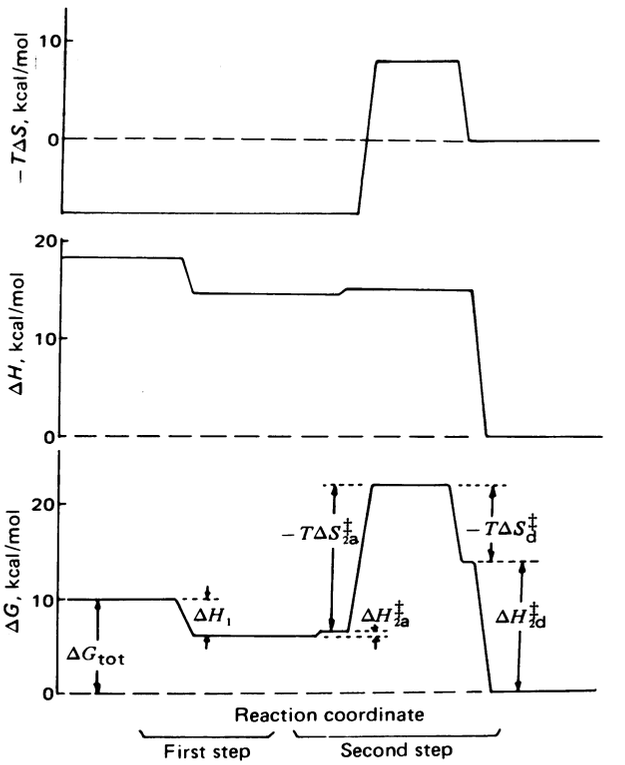

FIG. 3. Thermodynamic/kinetic profile of HSA-oleate binding.

The change in enthalpy during the first binding step, calculated by using identity 2 with two calorimetric determinations, is consistent with the hypothesis that the first step is equivalent to FA binding at one of the "tertiary" binding sites. These are sites of lowest binding affinity, and they become permanently filled only after about six primary and secondary sites are occupied. At these sites, FA competes with other ligands that do not compete for the primary sites on the hydrophobic interior of the protein. It is thus reasonable to propose that the first step of binding consists of a loose, nonspecific association of the ligand with the exterior surface of the protein. My interpretation of the second step is based on this hypothesis. The quantities in Table 1 are presented graphically in the profile representation of Fig. 3.

DISCUSSION AND CONCLUSION

The evidence presented suggests the following characteristics of the association of long-chain FA with HSA:

(*i*) Association occurs in two steps. The first step is diffusion controlled, or nearly so, and consists of attachment of FA in a nonspecific manner to one of the tertiary binding sites of low affinity on the external surface of the protein.

(*ii*) The second step of association is rate limited by a negative *entropy* of activation; the *enthalpy* of activation is nearly zero. Thus, minimal bond disruption is required in the association, but a particular configuration of the protein or its solvent environment (or both), having a relatively high degree of order, is required.

Table 1. Thermodynamic and kinetic specification of two-step binding of oleate to HSA

Symbol	Name of quantity	Value, kcal/mol	Method	Data used	Source of data
ΔG_{tot}	Total free energy of binding	−10	$\Delta G = -RT \ln K_{eq}$	Equilibrium binding constants	Ref. 9
ΔG_1	Free energy of 1st step of binding	− 4 ± 2	$\Delta G = -RT \ln K_{eq}$	Tertiary site equilibrium binding constants	Ref. 9
ΔG_2	Free energy of 2nd step of binding	− 6 ± 2	Identity 1		
ΔH_{tot}	Total enthalpy of binding	−18.5 ± 3 −17.7	Microcalorimetry van't Hoff's equation	Heat of reaction Equilibrium binding constants	Unpublished Ref. 9
ΔH_1	Enthalpy of 1st step of binding	− 3.5 ± 3	Identity 2		
ΔH_2	Enthalpy of 2nd step of binding	−15 ± 3	Stopped-flow calorimetry	Heat of second peak	Unpublished
$\Delta H_{2a}^{\ddagger}$	2nd step association activation enthalpy	0 ± 3	Arrhenius plot of k_{2a}	Stopped-flow dielectric	This work
$\Delta H_{2d}^{\ddagger}$	Dissociation activation enthalpy	14 ± 3	Arrhenius plot of k_{2d}	Stopped-flow dielectric with ligand exchange	This work
$-T\Delta S_{tot}$	Total entropy of binding	8	Identity 6		
$-T\Delta S_1$	Entropy of 1st step of binding	0 ± 4	Identity 7		
$-T\Delta S_2$	Entropy of 2nd step of binding	8 ± 3	Identity 5		
$-T\Delta S_{2a}^{\ddagger}$	2nd step association activation entropy	16 ± 2	Arrhenius "A," k_{2a}	Stopped-flow dielectric	This work
$-T\Delta S_{2d}^{\ddagger}$	Dissociation activation entropy	8 ± 2	Arrhenius "A," k_{2d}	Stopped-flow dielectric with ligand exchange	This work

Identities:
1. $\Delta G_{tot} = \Delta G_1 + \Delta G_2$
2. $\Delta H_{tot} = \Delta H_1 + \Delta H_2$
3. $\Delta S_{tot} = \Delta S_1 + \Delta S_2$
4. $\Delta H_2 = \Delta H_{2a}^{\ddagger} - \Delta H_{2d}^{\ddagger}$
5. $\Delta S_2 = \Delta S_{2a}^{\ddagger} - \Delta S_{2d}^{\ddagger}$
6. $\Delta G_{tot} = \Delta H_{tot} - T\Delta S_{tot}$
7. $\Delta G_1 = \Delta H_1 - T\Delta S_1$

(*iii*) The second step of association involves a rearrangement of the protein–FA conformation, as shown by the change in dipole vector. During this step, the ligand gains access to the hydrophobic interior surface of the protein where the primary binding region is located.

(*iv*) The transient activated state of the second step is identical, or closely related, to the opening up of the tertiary structure of the serum albumin molecule which is thought to occur in the acid transition.

(*v*) Dissociation activation requires enthalpy sufficient to break the hydrophobic bonds that hold the ligand to the primary binding region as well as a negative entropy change sufficient to bring the protein into the more highly ordered activated state described for step two of association above.

There are aspects of this characterization that remain to be determined. Among these is the extent to which the ligand plays a role in the activation configuration. One can visualize two possible extremes: (*i*) the ligand plays no role in activation—i.e., the protein periodically, and on a purely probabilistic basis, assumes the activated configuration and, when it does and a ligand is present on the external surface, the ligand is permitted to pass to its most stable binding configuration inside the protein; or (*ii*) the ligand is closely involved in activation—i.e., the ligand's movement on the exterior surface occasionally brings it to the mouth of the hydrophobic cleft, facilitating the formation of the activated configuration. These alternative views are subject to experimental differentiation.

Two possible alternatives to the mechanism from which the activated state derives its highly ordered quality also remain to be explored. Ordering may derive from a particularly improbable configuration of the protein; alternatively, ordering may be a hydrophobic effect (31) primarily localized in solvent water that is exposed to additional hydrophobic interface in the temporarily accessible but normally interior surface of the protein. These two factors may both contribute to the negative entropy of activation.

The view proposed here may have more general implications for the characterization of the forces that maintain the tertiary structure of serum albumin and of other proteins as well. The process that permits FA to gain access to the interior of the albumin molecule is postulated to be a conformational change in the protein; this process is shown here to be rate limited by activation entropy. This suggests that protein conformation generally may involve some stabilization by entropy maximization, perhaps through the mechanism of minimization of hydration at hydrophobic residues.

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