Binding of arachidonate and oleate to bovine serum albumin

Inge N. Bojesen[1] **and Eigil Bojesen**

Department of Medical Biochemistry and Genetics, Laboratory of Medical Biochemistry B, University of Copenhagen, The Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark

Abstract Using the "ghost method" previously applied to study the binding of palmitate to bovine serum albumin (BSA) (Bojesen, I. N., and E. Bojesen. 1992. *J. Lipid Res.* **33:** 1327–1334) we have measured the water-phase concentrations of arachidonate (AR) and oleate (OL) in 165 mM KCl and in 165 mM NaCl, 2 mM phosphate buffer at pH 7.3 in equilibrium with AR and OL bound to BSA (about 30 μM) inside resealed human red cell ghosts at low molar ratio (ν). Data were obtained at 0°C, 10°C, 23°C, and 38°C for ν between 0.012 and 1.5. Regression analyses of the data suggest that BSA has three equivalent binding sites for AR and OL at the four temperatures. The equilibrium dissociation constants (K_d) were the same in potassium and in sodium buffers. They were calculated for AR and OL on basis of three equivalent binding sites per mol BSA. K_d values increase with temperature and the AR values are, on average, approximately 5-fold greater than those of OL within the investigated temperature range. At 23°C, K_d values for three equivalent sites are 15.60 ± 0.73 nM and 2.89 ± 0.14 nM corresponding to −44.2 ± 0.15 kJ/mol and −48.3 ± 0.1 kJ/mol in free energies of binding AR and OL, respectively. The difference, 4.1 kJ/mol, fits fairly well with the theoretical difference in hydrophobic effects of the two aliphatic chains, 3.7 kJ/mol. The large enthalpy contributions to the free energies, −35.4 kJ/mol and −29.7 kJ/mol of AR and OL bindings, respectively, are understood as the binding energies required for the access of the ligands to the lipophilic domains with corresponding decreased probability of the BSA conformation. **—Bojesen, I. N., and E. Bojesen.** Binding of arachidonate and oleate to bovine serum albumin. *J. Lipid Res.* 1994. **35:** 770–778.

Supplementary key words erythrocyte ghosts • equilibrium constant of arachidonate-albumin complex • long-chain fatty acids • equilibrium constant of oleate-albumin complex • arachidonate monomer concentration • oleate monomer concentration

A clue to the understanding of the physiology of long-chain fatty acids (FA) in the body is knowledge of their binding to serum albumin. The transfer of FA between albumin, lipoproteins, and cellular membranes is mediated by a water-phase (W-FA) shuttle of monomers, as the albumin receptor theory has been shown to be unfounded (1, 2). A large turnover in the body of FA is normally ensured by the large transport capacity of serum albumin and the strong binding of FA to this protein. This strong binding also minimizes undesirable detergent effects that depend strongly on the water-phase concentrations below the critical micelle concentrations (3). Therefore, it is expected that low levels of FA in the water-phase are important and physiologically controlled. The W-FA concentration is usually, but erroneously, considered as a direct determinant of the transport between serum and cells, although it must be more or less offset by a great flow of the FA, dependent on rate constants of dissociation (4–6). However, it is important as a limiting value, representing the chemical activity of FA in serum at any time.

Measurements by the phase partition method (7) of the W-FA concentrations have encountered great difficulties, both technical and theoretical, due to dimerization and/or formation of higher aggregates in the two phases (8). The theoretically questionable validity of the results has prompted research of alternatives such as dialysis techniques. Within hours, diffusion is observed through acetylcellulose membranes of FA to buffer containing bovine serum albumin (BSA), whereas to pure buffer the diffusion is very slow or nonexistent (9, 10). However, diffusion to pure buffer of protonated palmitic acid (PA) has been observed through polyethylene membranes (11).

Recently we have presented a new method based upon the use of albumin-filled red cell ghosts as a dispersed biological "reference-binder" instead of a liquid binder, heptane (12). Compared with the method of Goodman (7), the procedure is easy and equilibria are very rapidly established. It has provided information on the equilibrium constants of PA binding to BSA in molar ratio of PA to BSA (ν) within the physiologically relevant range. Impor-

Abbreviations: PA, palmitic acid; AR, arachidonic acid; OL, oleic acid; BSA, bovine serum albumin; FA, fatty acids or the corresponding anions with 16 or more carbon atoms; B-FA, FA bound to albumin or ghost membranes; W-FA, water-phase FA in equilibrium with B-FA; DB-FA, dissolved B-FA; ν, equal to [albumin bound FA]/[BSA] ≈ ([albumin bound FA]+[W-FA])/[BSA].

[1]To whom correspondence should be addressed.

tant is the observation that the estimated equilibrium dissociation constants (K_d) are ν independent for $\nu \leq 1.5$ and considerably smaller than previously thought. Furthermore, the van't Hoff plot of K_d for binding of PA to BSA between 0°C and 38°C is linear, which supports the contention that the method provides information on a true equilibrium concerning monomeric PA in water.

In the present study we have addressed the albumin binding of the polyunsaturated arachidonate and the monounsaturated oleate and compared the information obtained with our previously published results on PA.

In contrast to the two other FA, arachidonic acid (AR) constitutes only a small part of total FA in serum. The normal molar ratio (ν) of arachidonate to serum albumin is approximately 0.02 (13). However, AR is particularly interesting as it is the unique precursor of the physiologically important compounds prostaglandins and leucotrienes. As the only known control of these compounds is on the substrate level rather than on the activity of metabolizing enzyme present, the equilibrium water-phase concentration [W-FA] of AR in serum is important.

Oleic acid (OL) is, as PA, one of the major FA in serum and both have a ν value of approximately 0.2 (13). OL is also used extensively in in vitro studies on cellular uptake of FA.

The new information of main interest is probably that BSA at pH 7.3 and $\nu \leq 1.5$ has three equivalent binding sites for OL and AR at temperatures from 0°C to 38°C and that the OL affinity is an order of magnitude greater than that suggested by the Goodman method (5, 14, 15).

MATERIALS AND METHODS

[(5,6,8,9,11,12,14,15)-^{3}H]arachidonic acid, sp act 209 Ci/mmol, and [(9,10)-^{3}H]oleic acid, sp act 4.2 Ci/mmol, were obtained from Amersham International plc, Amersham, England. Unlabeled acids were obtained from Sigma. Unlabeled as well as labeled acids were purified every month. The purity was controlled by an elution pattern of a single compound in chromatography on a silicic acid column using 0.05% ethyl acetate and 0.05% acetic acid in benzene as eluant. The scintillation fluid Ultima Gold was purchased from Packard Instrument Company, Inc. (Downers Grove, IL). Bovine serum albumin (BSA) (Behring Institute, Germany) was defatted according to the method of Chen (16). Polycarbonate tubes were used for the analyses.

Preparation of ghosts

Preparation of albumin-filled ghosts was carried out by adding BSA to the hemolyzing solution as described previously (12). The BSA concentration in the intracellular phase of resealed ghosts was checked with ^{125}I-labeled BSA to be 93.8% of the 30 μM in the lysate (17). The ghosts were prepared from human red cells from five donors. The resealed ghosts were isolated from the hemolysate by centrifugation and washed twice at 0°C with 7 vol of 165 mM KCl, 2 mM phosphate buffer, pH 7.3, containing 0.02 mM EDTA-EGTA 1:1. A third washing was carried out with the same buffer containing 0.2% BSA. This BSA-containing buffer was also used for storage of ghosts and as medium for labeling the ghosts (charge-buffer). In experiments with NaCl, all K^+ was replaced by Na^+.

The charge-buffers contained about 5 μCi/ml of the radioactive FA and suitable amounts of unlabeled FA giving the final molar ratios of acids to BSA (ν) from 0.012 to 1.77.

BSA-filled ghosts were packed by centrifugation for 7 min at 22,200 rpm (36,470 *g*) in a Cryofuge 6-4 (Heraeus Christ). Complete equilibrium of FA between internal and external BSA was obtained by incubating 1 part of packed ghosts with 1.5 part of charge-buffer for 50 min at 0°C, for 30 min at 10°C or 17°C, and for 10 min at 23°C or 38°C. Charge buffers were removed and the ghosts were washed 4 times with 50 volumes of the buffer at 4°C without BSA. Ghosts (0.5 ml) were then suspended in buffer to a total volume of 3 ml and this suspension was distributed into four tubes according to the following scheme: *1)* 1.25 ml ghost suspension to 1.75 ml buffer; *2)* 0.75 ml to 2.25 ml buffer; *3)* 0.5 ml to 2.5 ml buffer; and *4)* 0.25 ml to 2.75 ml buffer. The four suspensions were gently shaken in a water bath at the appropriate temperature for 50 min at 0°C, 30 min at 10°C or 17°C, and 10 min at 23°C or 38°C. After this complete equilibration, ghosts were sedimented by centrifugation for 10 min at 36,470 *g* and 2 × 200 μl ghost-free supernatants were pipeted right away from the top of each tube for scintillation counting. For measurement of radioactivity, the use of a glass pipette, of disposal pipette tips, or of the same pipette several times did not make any difference.

Scintillation counting

The counter was a MINAXI TRI-CARB 4000 series (United Technologies, Packard, IL) calibrated to give dpm as output according to the spectral index of the external standard (SIE), the efficiency was 67% for ^{3}H in unquenched samples. Counting rates of samples were determined, after the addition of 3.9 ml Ultima Gold scintillation fluid, to a probable error smaller than 1%. Twenty five μl of the charge-buffers before and after use were taken for counting and the counting rates were determined as C_b and C_a (dpm/ml), respectively. Counting rates of duplicates of ghost-free supernatants were C_s (dpm/ml) and the concentrations of FA were calculated from these counting rates and the specific activities S (dpm/nmol) as C_s/S. These concentrations were subjected to a regression analysis with the reciprocal dilution

factor (ghost concentrations) as an independent variable (Fig. 1 and (12)). The ghost-free supernatants also contained, besides W-FA, a small amount of dissolved B-FA, the concentration of which decreased in proportion to the dilution of the ghost suspensions in contrast to the constant W-FA (Fig. 1 and (12)). Therefore, extrapolation to infinite dilution of this DB-FA gave [W-FA] as the intercept. [W-FA] of the charge-buffers constituted much less than one-thousandth of [B-FA]. Thus C_a represents the counting rate of [B-FA] and ν is calculated as:

$$\nu = C_a/(S30),\ 0.2\%\ \text{BSA equals 30 nmol/ml} \qquad Eq.\ 1)$$

K_d determinations

K_d values and the number of equivalent binding sites of BSA (N) (to be distinguished from the number of determinations n, see Table 1 and 2) were estimated from the equation:

$$K_d = ([\text{W-FA}](N - \nu))/\nu \qquad Eq.\ 2)$$

using the non-linear regression procedure given by STATGRAPHICS version 5 to determine the best fit of ν (calculated by Eq. 1) and [W-FA] to Eq. 2. K_d was also estimated by regression analyses of Eq. 2 linearized according to Wilkinson (18).

$$[\text{W-FA}]/\nu = (1/N)[\text{W-FA}] + K_d/N \qquad Eq.\ 3)$$

In this plot the slope (the reciprocal number of equivalent binding sites) defines N and the intercept (K_d/N) provides a corresponding value of K_d. K_d values of Table 2 were calculated by Eq. 2 using the fixed value 3 for N.

Statistics

Regression lines of Wilkinson plots and the statistics of slopes and intercepts were estimated by standard methods (19). Standard errors (SE) of estimated parameters N and K_d values were calculated according to the general function given by Armitage (19), neglecting the unknown contribution of covariance. Weighted means of K_d values were calculated by giving single estimates the weights of the reciprocal variances of estimations (19).

Analytical procedure in theory

The method is based upon the following premises. Possible failures are discussed later.

Equilibrium between the charge-buffer and the ghosts containing BSA implies that [W-FA] is the same everywhere and in equilibrium with FA bound to BSA inside and outside the ghosts as well as to the ghost membrane. The binding capacities and high affinities of FA to BSA and to the membrane ensure that B-FA per ml ghost and [W-FA] of the ghost suspension remain essentially unchanged after the complete removal of BSA outside the ghosts by the described washing procedure and by the final four different dilutions of the washed ghost suspension.

A small fraction of the ghosts was damaged by the washing procedure involving packing and resuspension of the cells. This resulted in the production of dissolved binding capacities originating from the membranes and/or released BSA. **Fig. 1A** illustrates the theoretical relations between supernatant tracer concentrations and the four volume fractions of two washed ghost suspensions initially equilibrated with the same charge-buffer, but with and

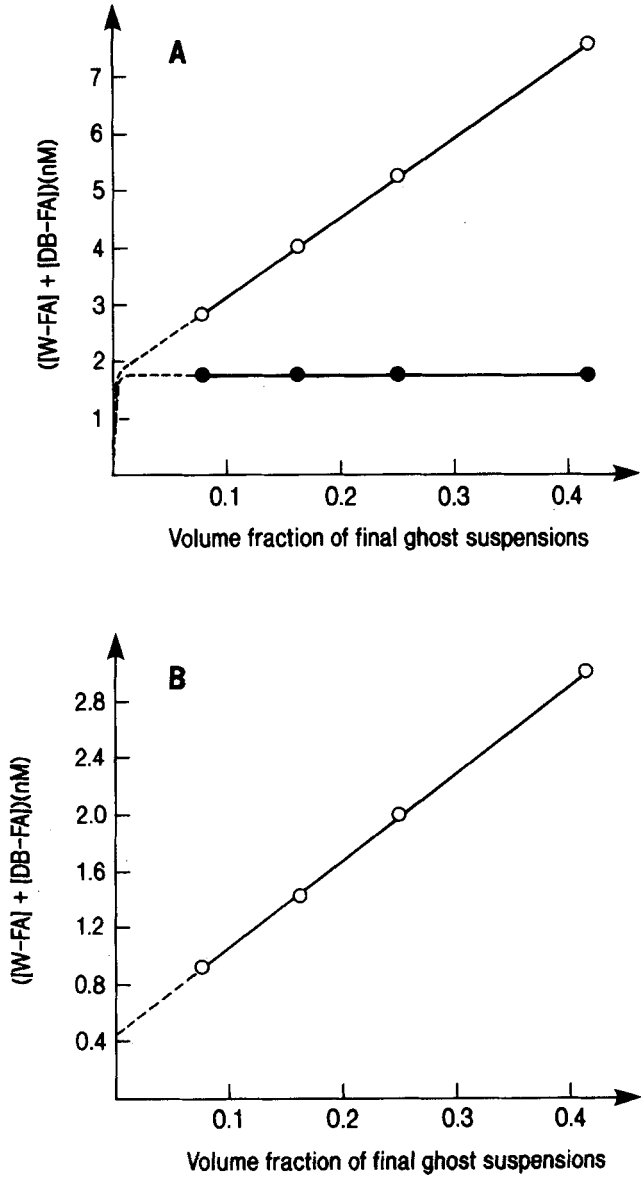

Fig. 1. A: Regression lines of the theoretical dilution effects on equilibrium supernatants FA concentrations from ghost suspensions with (○) and without (●) dissolved binding capacity originating from damaged ghosts. B: Regression line of experimental data (○). Analysis of [W-FA] of OL at 0°C ν = 0.77. Constant dilution volume 3 ml.

without the presence of DB-FA. In the extreme case of ghost fractions approaching zero, both DB-FA and [W-FA] also approach zero. Supposing the greatest dilution depletes the ghosts significantly of B-FA, i.e., the ν value within the cells is diminished, then the four points do not form a straight line because the point signifying [W-FA] of the most diluted sample would be below the line defined by less diluted suspensions. The validity of this control is of course most important when [DB-FA] and [W-FA] are of similar magnitudes.

RESULTS

Fig. 1B shows an example of supernatant concentrations at 0°C of the four volume fractions of a washed ghost suspension initially in equilibrium with a charge-buffer containing BSA-bound oleate with ν = 0.77. The regression line has the equation y (nM) = 5.97 (±0.16) x (volume fraction) + 0.44 (±0.04). In this series of dilutions the slope of the straight line demonstrates, as previously (12), the presence of a component in the supernatant of the washed ghost suspension, the concentration of which increases in proportion to the volume fraction besides an invariant concentration indicated by the ordinate intercept (0.44 nM). Thus the data conform with the expected dilution effect presented in Fig. 1A, and the intercept is the equilibrium [W-FA] corresponding to B-FA of the washed ghosts. [W-FA] is the same in the charge-buffer, provided the ghosts are not depleted significantly by washings and by the final dilutions. That no such depletion of ghost B-FA takes place to a significant extent is demonstrated in Fig. 1B. It shows that [W-FA] + [DB-FA] of the supernatant in the most diluted suspension is not different from that expected by the three other measurements. This is in accordance with the following calculation. At ν = 0.77, the 28 μM BSA-filled ghosts contain 21.6 nmol OL per ml plus OL bound to the membranes. Removal of OL by washing is 0.44 pmol × 4 × 50 equal to 88 pmol which amounts to 0.38%. However, the real percentage is much less because the ghosts contain more than BSA-bound OL and the washings are carried out rapidly at low temperature, which means that the equilibrium [W-FA] (0.44 nM) is not reached within the time of washing. In the case of AR, the percentage removal is less than 1.7%. Thus the final ν and [W-FA] are indistinguishable from the initial values.

The [W-FA] values obtained in NaCl buffers and in KCl buffers are pooled. In only one case, OL at 38°C (shown in Table 1), an apparent discrepancy was observed in the regression analysis. However the NaCl data were not significantly different from KCl data ($P < 0.2$), therefore all 28 values are pooled for the calculation of K_d in Table 2.

Analyses of data obtained for AR and OL at the different temperatures were carried out after linearization of the relations between the two parameters (ν and [W-FA]) according to Wilkinson (equation 3). **Fig. 2** shows examples of such plots and the estimated number of binding sites and corresponding K_d values together with the levels of significance (P), and the coefficient of correlations (r) of such regression analyses are given in **Table 1.**

Three equivalent binding sites are suggested according to these analyses for AR as well as for OL on average for all temperatures. A considerable deviation from three of the estimated N values is N = 2.44 ± 0.47 for AR at 0°C. However, assuming the presence of a well-defined small number of equivalent binding sites of BSA, we have also evaluated data by non-linear regression analyses (see Methods). According to this curve-fitting method we obtained the fitted curve of **Fig. 3** for AR at 0°C and the parameter values K_d = 3.62 nM ± 0.69 nM and N =

TABLE 1. Estimation by regression analyses according to Wilkinson (18) of the number of binding sites (N) on bovine serum albumin (BSA) of arachidonic acid and oleic acid with equilibrium dissociation constants (K_d) of acid-BSA complexes at different temperatures

	Temperature					
Variable	0°C	10°C	17°C	23°C	38°C	
Arachidonic acid						
N	2.44 ± 0.46	3.4 ± 0.9		3.2 ± 0.9	3.5 ± 0.6	
K_d	3.70 ± 0.77	8.94 ± 2.50		17.6 ± 5.3	29.27 ± 5.08	
n	19	16		16	28(4)	
r	0.78	0.69		0.67	0.76	
P	<0.001	<0.005		<0.005	<<0.001	
Oleic acid						
N	3.26 ± 0.50	2.4 ± 0.6	2.53 ± 0.40	3.28 ± 0.57	2.5 ± 0.7	2.6 ± 0.5
K_d	1.4 ± 0.3	1.6 ± 0.4	1.67 ± 0.35	3.19 ± 0.61	3.9 ± 1.1	6.4 ± 1.5
n	11	11	(10)	13(4)	13	(15)
r	0.87	0.82	0.91	0.87	0.67	0.81
P	<0.001	<0.005	<0.001	<0.001	<0.01	<0.001

Abbreviations: r, coefficients of correlation; P, levels of significance; n, number of determinations. The numbers in parentheses are estimates made in NaCl buffers.

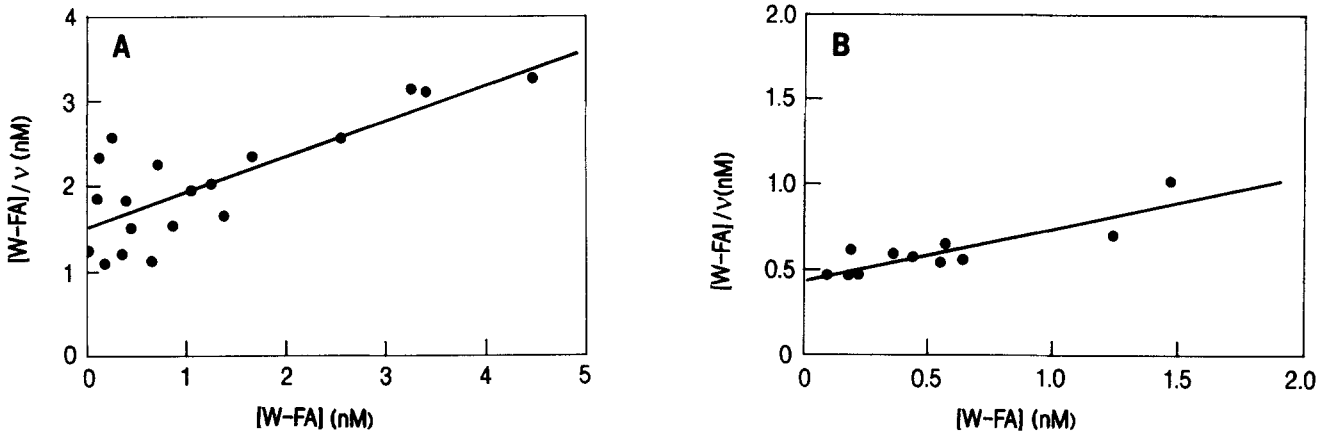

Fig. 2. The relationship between fatty acid water-phase concentrations [W-FA] and molar ratios of fatty acids to BSA (ν) presented according to equation 3. A: Arachidonic acid data (Table 1) at 0°C, pH 7.3. The regression line is Y = 0.415 (±0.080)X + 1.52(±0.14). B: Oleic acid data (Table 1) at 0°C, pH 7.3. The regression line is Y = 0.308(± 0.058)X + 0.435(±0.040).

TABLE 2. Equilibrium dissociation constants (K_d) of arachidonic acid and oleic acid calculated according to equation 2 with three equivalent binding sites (N = 3) of bovine serum albumin (BSA) and corresponding coefficients of variation at four and five different temperatures, respectively, and at different ranges of molar ratio of fatty acids to BSA (ν)

		Dissociation Constants (nM)				
Temp.	Interval ν	Mean	Weighted Mean	± SE	n[a]	Coeff. of Var.
Arachidonic acid						
0°C	0.012–1.37	4.85	4.51	± 0.30	19	0.28
0°C	0.012–0.32	4.99	4.50	± 0.53	9	0.33
0°C	0.32–1.37	4.73	4.96	± 0.34	10	0.21
10°C	0.055–1.37	7.58	7.36	± 0.39	16	0.20
10°C	0.055–0.35	8.38	7.36	± 0.45	5	0.12
10°C	0.35–1.37	7.22	7.32	± 0.50	11	0.21
23°C	0.05–1.37	16.46	15.60	± 0.73	16	0.18
23°C	0.05–0.30	16.67	15.62	± 0.46	11	0.09
23°C	0.30–1.37	16.01	13.16	± 2.29	5	0.35
38°C	0.012–1.46	24.14	27.94	± 0.83	28	0.15
38°C	0.012–0.30	25.83	27.98	± 1.22	13	0.15
38°C	0.300–1.46	22.68	20.14	± 1.02	15	0.19
Oleic acid						
0°C	0.20–1.77	1.22	1.21	± 0.07	11	0.18
0°C	0.20–0.47	1.33	1.21	± 0.11	4	0.15
0°C	0.47–1.77	1.23	1.23	± 0.09	7	0.19
10°C	0.10–1.37	2.15	2.32	± 0.19	11	0.26
10°C	0.10–0.46	2.29	2.35	± 0.34	5	0.29
10°C	0.46–1.37	2.03	1.68	± 0.22	6	0.29
17°C	0.65–1.48	3.45	2.40	± 0.21	10	0.19
23°C	0.09–1.47	2.59	2.89	± 0.14	13	0.19
23°C	0.09–0.45	2.58	2.75	± 0.15	5	0.11
23°C	0.45–1.47	2.97	2.98	± 0.21	8	0.19
38°C	0.09–1.34	5.19	6.60	± 0.41	28	0.41
38°C	0.09–0.45	5.09	6.18	± 0.69	9	0.38
38°C	0.45–1.34	6.59	6.78	± 0.52	19	0.34

[a]n, number of determinations.

2.43 ± 0.25, which agree with the values obtained by equation 3 (Table 1).

In **Table 2** simple means as well as weighted means (see Methods) are given because the standard errors of estimated [W-FA] vary considerably. However, on the other hand, the weighting may incidentally be misleading as the standard errors are based upon two degrees of freedom only. Notable is that the K_d values are not different for the low and high range of ν. It confirms that the three sites are equivalent.

The weighted mean values of K_d (Table 2) are used to illustrate the K_d dependence on temperature in terms of the van't Hoff isochors: $(d(\ln K_d/d(1/T) = -\Delta H/R)$ (**Fig. 4**). The identity of NaCl and KCl data is illustrated by the fit of the 17°C K_d calculated on the basis of ten experiments with OL and NaCl buffer. From such a plot we get information on the thermodynamics of the BSA binding of the two FA. In the case of AR, the isochor is clearly linear within the standard errors and defines a constant binding enthalpy (Δ H) of −35.4 kJ/mol between 0°C and 38°C. Although the points are less well defined in the isochor of OL, they fit a straight line. The slope corresponds to a binding enthalpy of −29.7 kJ/mol in the range of 0°C to 38°C.

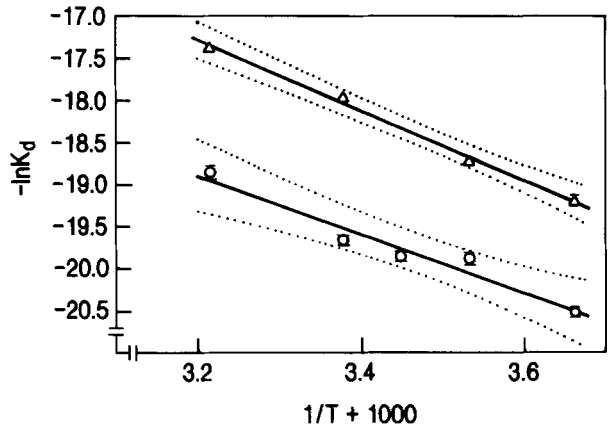

Fig. 4. Van't Hoff plot of the temperature effects on the K_d values of arachidonate (△) and of oleate (○) calculated by equation 2 on basis of three equivalent binding sites of the acids of BSA (Table 2). Each value is given with ± standard error. The confidence limits (95%) are presented by (...).

DISCUSSION

Possible failures of the premises

The equilibrium time periods used in this work were the same at 0°C, but at 10°C, 23°C, and at 38°C were twice as long as the periods used before in similar PA binding studies (12). Using the information obtained by efflux studies on PA (17), the equilibration times were calculated to ensure more than 99% equilibrium between charge-buffers and ghosts with enclosed 28 μM BSA and they were ample periods in studies with "empty" ghosts. Thus the observation that the two ghost preparations provide indistinguishable results (12) verifies that the theoretical equilibrium times are sufficient. Similar calculations from efflux data with regard to AR (20) show that the equilibrium in this case is obtained at least tenfold faster than for PA. On the other hand, the same equilibrium time periods are required in the case of OL because the major determinant, the rate constants of dissociation of FA-BSA complexes, are similar for OL (21) and PA (17).

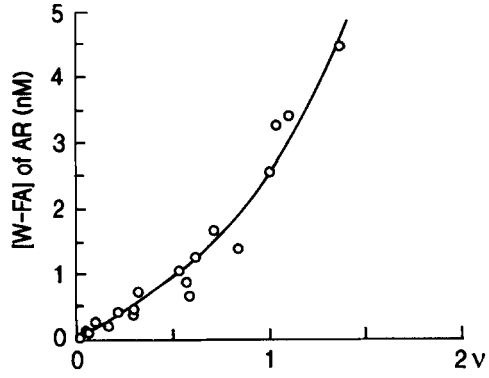

Fig. 3. The relationship between arachidonate water-phase concentrations [W-FA] of AR at 0°C, pH 7.3, and molar ratios of arachidonate to BSA (ν) ($[W\text{-}FA] = K_d\,\nu/(N-\nu)$). The curve is the best fit of our data to this equation according to non-linear regression (STATGRAPHIC 5). The parameters estimated are K_d = 3.62 nM (±0.69) and N = 2.43 (±0.25).

The question of albumin leak is also answered by the concordant results of modification I and II (12). Only a part of the DB-FA is due to FA bound to albumin originating from a few damaged ghosts.

The conclusion that the depletion of ghosts by washings and dilutions (see calculations in results) is negligible has been confirmed by comparing the initial uptakes on ghosts of OL and AR with direct measurement of B-FA in washed ghosts.

Possible significant adsorptions of W-FA and DB-FA to the tube walls were not discussed previously, because physical adsorptions of W-FA and DB-FA, which are present in so very low concentrations in the ghost suspensions, are presumably proportional to the concentrations and, in the washings, contribute very little to the insignificant depletions. As B-FA remains essentially unchanged, the adsorption of W-FA in the final four dilutions is negligible and the equilibrium [W-FA] is not changed. On the other hand, adsorption of DB-FA in proportion to the concentration only diminishes the slope of the dilution effect on supernatants ([W-FA] + [DB-FA]), Fig. 1B. The expected constant fractional adsorption of supernatants was confirmed by transferring supernatants of the final four dilutions to new tubes. The adsorbed fractions, some 30%, were indistinguishable in the

four samples of AR as well as OL analyses. Furthermore, the adsorption by Corex glass tubes is not demonstrable, but the analytical results [W-FA] were the same as in polycarbonate tubes.

Analytical use in general of ghost preparations

Essential features of the analysis were discussed previously (12) but require further comment. When a charge-buffer can be quantitatively removed from ghost preparations without any significant depletion of B-FA, then the final [W-FA] necessarily is the same as in the charge-buffer. In other words, the ghost preparation carries the information of the [W-FA] in the charge-buffer and is therefore called the reference-binder. The property of the ghosts as semipermeable bags is used merely to get a large high affinity binding capacity when the membrane binding alone is insufficient to ensure a constant B-FA. Such ghost preparations are used in the present work, because the OL binding of the membrane was not well known and the membrane binding of AR was found to be insufficient. This was revealed by non-linearity of the relation of supernatant concentrations versus the volume fractions in the final dilution series (see Fig. 1B). Thus we have acquiesced in the lower precision of the version with BSA-filled ghosts (12). In general, when a ghost preparation can be used as a reference-binder the analytical procedure is quite simple, but if an enclosed protein-ligand complex is somewhat ligand-depleted by the washing procedure, then the ghosts may still be used as dialysis bags. However, the remaining ligand fraction must be estimated and the relation of membrane binding and water-phase concentration must be known.

It may be of interest to note that if ghosts contain a protein with sufficiently high affinity to a ligand, then such preparation is useful as a reference-binder to estimate the ligand water-phase concentration in a charge-buffer with the ligand bound to another protein with lower affinity.

Why investigations at $\nu \leq 1.7$?

Recently the three-domain model of BSA (22) for binding of FA was substantiated by investigations by NMR spectroscopy of ^{13}C-enriched FA-BSA complexes (23–25). The spectra are assigned to at least two different binding sites at low ν (0.5 to 1) and three with ν up to 3. The structures of these three sites of similar high affinities are described as binding channels with slightly different positively charged amino acid residues. For myristate and laurate, the thermodynamics of BSA binding are very different in the low ν and the high ν ranges (26). In the high affinity range, the binding is largely enthalpic as it is for PA (12), in contrast to the temperature-independent entropic low affinity binding at high ν values. Thus, complete occupancy of the specific domains enables the complex to bind weakly an "unlimited" number of FA. It is readily computed by subsequent binding simulations that about 12% of the BSA molecules are saturated already when the average ν is 1.5, if the three specific sites are equivalent. The low affinity of a small fraction of molecules with three sites fully occupied has no appreciable effect on the average affinity, but to be sure of investigating only the physiologically relevant high affinity binding sites, we have confined our studies to ν not exceeding 1.7. In this way the secondary binding property of the saturated complex limits the precision by which the number of high affinity sites is estimated.

Number of equivalent high affinity binding sites on BSA

We have no reason to believe that the number of equivalent sites is temperature-dependent and the analytical results of the data (Table 1) are therefore in this respect expected, just as the ν independent K_d values calculated on basis of three equivalent sites (Table 2). In the case of PA (12) two equivalent sites were considered less likely than three, in view of the coefficients of variations of the calculated K_d values. This criterion may however be misleading, as regression analyses of the most reliable data indicate two equivalents for the binding of PA (data not shown). The question is, however, whether there is support for an exceptional position of PA. A hint may be the great enthalpy contribution to the free energy of binding. As discussed later, this is probably the energy required to accommodate a lipophilic interior of a specific domain to ligand binding. Accordingly, it is conceivable that one of the three domains is not sufficiently pliable to accommodate to PA binding. The question may be accessible to independent investigations.

It may be useful to know the correct number of equivalent sites when one wants to estimate [W-FA] of more than one kind of FA bound to BSA. Thus according to Eq. 2 [W-FA] of OL at 38°C is expected to be simply 2.57 nM = $6.6 \times 0.7/(3 - 0.7 - 0.5)$ in a solution of OL and AR bound to BSA in the molar ratios 0.7 and 0.5, respectively, and K_d = 6.6 for OL. However, the expectancy remains to be verified.

Comparison of the present method with the phase partition method (the Goodman method)

The BSA affinities of PA and OL have previously been investigated by the Goodman method (14). [W-FA] of PA is measured some threefold lower with the ghost method (12) in the low range of ν values but under otherwise similar conditions. In **Table 3** we show that the discrepancy is much greater in the case of OL. The two methods have one feature in common. Both of them use reference-binders, ghosts and heptane, respectively. By the ghost method the relevant [W-FA] is measured directly, whereas in the Goodman method it is calculated from the uptake in heptane and the heptane-water partitions in separate experiments. Revealed by the ghost method, the thermo-

TABLE 3. Reported association equilibrium constants (K_a) for the binding of oleate to high affinity binding sites of bovine serum albumin (BSA), pH 7.3, calculated for N equivalent sites

$K_a \times 10^{-8}$ M^{-1}	Temp. °C	N	Method[a]	Ref.
6.35	38	1	(1) $\nu \leq 0.5$	[b]
0.17	37	1	(2) $\nu \leq 6$	15
0.19	37	1	(1) $\nu \leq 0.5$	5[c]
1.27	37	1	(2) $\nu \leq 6$	28
2.75	38	2	(1) $\nu \leq 1.34$	[b]
0.08	37	2	(1) $\nu \leq 2$	5[c]
1.52	38	3	(1) $\nu \leq 1.34$	[b]
0.04	37	3	(1)	14
0.05	37	3	(1) $\nu \leq 2$	5[c]

[a]Calculations: (1) According to a Scatchard model, which groups binding sites into distinct classes, each containing a number of individual sites that have nearly the same affinity. K_a values are calculated for sites in the first class. (2) According to a stepwise equilibrium model in which the binding is described by progressively decreasing affinity constant. K_a is here the first constant of highest affinity.

[b]The present study: K_a calculated according to equation 2.

[c]K_a calculated according to equation 2.

dynamics of the binding as well as the consistent account of the bindings in terms of three equivalent binding sites strongly suggest that the binding is a simple bimolecular reaction involving W-FA in the monomer state. In contrast, FAs are all dissolved in heptane as stable dimers (7). By assuming only monomers in water, it has been possible to account for the variation with concentrations of water partitions of laurate and myristate, whereas this was not possible for FA. In fact, it was impossible to account consistently for the partition data even by assuming dimers also in water (27). Curiously, a significant effect of the predictable 10^{-5} M heptane in water has never been suggested. Anyhow, the failure of understanding the basic phase partitions of FA implies, as pointed out (7, 8), that the Goodman method is expected to provide reliable information only for laurate and myristate. The absence of an alternative method explains why it nevertheless was used also for the physiologically important FA.

With the dispersed physiological reference-binder the W-FA is in the physiological molecular state and the same as in the charge-buffer. Thus the ghost method is as much superior in theory as it is easier to work with.

Recently a new kind of reference-binder has been suggested (28). The fluorescence of a high affinity probe is quenched when binding FA. Therefore, the quench gauges [W-FA] in solutions of albumin-bound FA provided the quench response to [W-FA] is known. In the case of PA and OL bound to BSA at 37°C, the [W-FA] values at physiological ν levels are clearly close to the limit of the method. This appears from hardly detectable increases of [W-FA] for ν values up to 3 or more (28, Fig. 1). The binding constants of interest are therefore obtained by extrapolations, applying an unlinear algorithm up to $\nu = 6$.

Thermodynamics of the FA binding

The low solubilities of aliphatic hydrocarbons in water has been called "the hydrophobic effect" (29). The increase in free energy by transferring such compounds from liquid hydrocarbons to water is almost entirely entropic and is, in saturated chains, a linear function of the number of methylene groups, each of which contributes with about 3.7 kJ/mol at 23°C. Introduction of a double bond is equivalent to removing a methylene group from a fully saturated chain, therefore AR and PA are equally hydrophobic and OL is more hydrophobic corresponding to 3.7 kJ/mol. According to the ghost method the free energies of AR, PA, and OL bindings at 38°C are calculated to −44.9 kJ/mol, −44.4 kJ/mol, and −48.7 kJ/mol, respectively. They are related as expected of hydrophobic binding of hydrocarbon chains. That this "fit" is not fortuitous is emphasized by the binding data of laurate and myristate (14), measured by the Goodman method, which in this case provide theoretically reliable information (7). The reported free energies of bindings calculated for three binding sites at 37°C are −31 kJ/mol and −38 kJ/mol, respectively. Together with our PA value the methylene group equivalence for this series is on average 3.4 kJ/mol. In contrast, the free energies of PA and OL bindings determined by the phase partition method are about −40 kJ/mol and therefore theoretically improbable. The enthalpy contributions to the free energies of AR and OL bindings are large but different. This apparently contradicts the notion of hydrophobic bindings but the explanation is probably that the lipophilic regions of the binding domains are shielded from water in the most probable conformations (higher entropy). In the case of AR the gain in entropy by transfer from water to the lipophilic regions is greatly diminished by the decreased probability of the adapted conformation and the net effect is mainly the enthalpy change involved in the adaptation. According to this picture the access of AR and, to an even larger extent, the access of OL require smaller conformational changes and smaller enthalpy changes than in the case of PA binding. In other words, similar hydrophobic chains with different structures may fit more or less to the most probable conformation of the binding domains.

Conclusion

The water-phase concentrations of solutions of AR and OL bound to BSA in molar ratios up to 1.7 are measured by a procedure that uses resealed red cell membranes containing 28 μM BSA as reference-binder. Independent of temperatures between 0°C and 38°C, the bindings are accounted for by simple bimolecular reactions between water-phase monomers and three equivalent binding sites of BSA. The affinity of OL is so much higher than the affinities of AR and PA, as it is expected to be according to its greater hydrophobicity, and is an order of magnitude greater than suggested previously by using heptane

as reference-binder. The different affinities of FA correspond to different hydrophobicities of the hydrocarbon chains, as expected of binding to lipophilic regions of BSA. The large enthalpy contribution to the free binding energy suggests that the high affinity lipophilic unoccupied domains in question are shielded from water. Independent of the nature of albumin binding, the informations on [W-FA] dependence of ν are directly biologically relevant since they are measured in a purely biological system.

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