Binding of anandamide to bovine serum albumin

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Abstract The endocannabinoid anandamide is of lipid nature and may thus bind to albumin in the vascular system, as do fatty acids. The knowledge of the free water-phase concentration of anandamide is essential for the investigations of its transfer from the binding protein to cellular membranes, because a water-phase shuttle of monomers mediates such transfers. We have used our method based upon the use of albumin-filled red cell ghosts as a dispersed biological "reference binder" to measure the water-phase concentrations of anandamide. These concentrations were measured in buffer (pH 7.3) in equilibrium with anandamide bound to BSA inside resealed human red cell membranes at low molar ratios below one. Data were obtained at 0°C, 10°C, 23°C, and 37°C. The equilibrium dissociation constant (K_d) increases with temperature from 6.87 ± 0.53 nM at 0°C to 54.92 ± 1.91 nM at 37°C. Regression analyses of the data suggest that BSA has one high-affinity binding site for anandamide at all four temperatures. The free energy of anandamide binding (ΔG^0) is calculated to −43.05 kJ mol^{-1} with a large enthalpy (ΔH^0) contribution of −42.09 kJ mol^{-1}. Anandamide has vasodilator activity, and the binding to albumin may mediate its transport in aqueous compartments.—Bojesen, I. N., and H. S. Hansen. **Binding of anandamide to bovine serum albumin.** *J. Lipid Res.* 2003. **44**: 1790–1794.

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Anandamide and other *N*-acylethanolamines can be formed in many mammalian tissues (1), and anandamide is a partial agonist for the cannnabinoid receptors (2) and for the vanilloid receptor (3). Anandamide can be formed during tissue injury (1) and it can have pharmacological effects on the vascular system (4–6). Endocannabinoids appear to have a key vasodilator role in the hypotension associated with hemorrhagic, endotoxic, and cardiogenic shock as well as late-stage cirrhosis (6).

It can be presumed that anandamide normally will occur in biological fluid in very low concentrations, and owing to its hydrophobic character, it must be transported bound to protein. This presumption is verified by Giuffrida et al. (7), who found that anandamide is bound to a plasma protein identified as albumin. The concentration of anandamide in rat and human plasma is in the nM range [0.7–8 nM (7–9) and 4 nM (10), respectively]. This concentration is regarded as being too low for anandamide to act as a circulating active compound, insofar as the K_i for displacing synthetic radiolabeled ligands from the cannabinoid receptor I is in the range of 44 to 266 nM in the presence of a fatty acid amide-hydrolase inhibitor (11).

The concentration of albumin is ∼640 μM (12) and 630 μM (13) in rat and human plasma, respectively. Thus, the molar ratio (ν) of anandamide to albumin in rat and human plasma is in the range of 1–10 × 10^{-6}. Therefore, we have chosen to study the binding of anandamide to serum albumin at low ν values using the method developed for long-chain fatty acids (14). The equilibrium binding constant of anandamide to BSA has not been determined previously, but the binding of the corresponding fatty acid, arachidonic acid, has been studied (15). At ν values lower than 3, three equivalent binding sites were found with a equilibrium dissociation constant (K_d) value of 28 nM at 37°C (15). Recently, high-resolution crystal structures of human serum albumin (HSA) complexed with arachidonic acid have been presented. At high unphysiological ν values, as many as seven different sites for arachidonic acid are described (16). *N*-oleoylethanolamine has recently been shown to bind to HSA and BSA with high affinity (17), but binding of anandamide has not been reported.

The aim of the present study is to measure binding of anandamide to BSA and measure the free water-phase concentration of anandamide (A_w) using our method of studying binding of fatty acids to BSA (14, 15).

MATERIALS AND METHODS

Materials

Radioactive anandamide, *N*-arachidonoyl-[5,6,8,9,11,12,14,15-³H]ethanolamine, spec.act., 215 Ci/mmol, was obtained from

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Perkin-Elmer Life Sciences, Inc., Boston, MA, and unlabeled anandamide was purchased from BIOMOL Research Laboratories Inc., Plymouth Meeting, PA. Both labeled and unlabeled anandamide were purified before use by chromatography on a 160 × 0.8 mm column filled with Sephadex LH-20 using dichloromethane as eluant. The scintillation fluid was Ultima Gold from Packard Instrument Co., Inc., and BSA albumin fraction V (fatty acid free) was from Boehringer Mannheim GmbH, Germany.

Preparation of erythrocyte ghosts

The preparation of uniform populations of BSA-filled and BSA-free resealed red cell membranes ("pink" ghosts) from freshly drawn human blood was carried out as described previously (18). The ghosts were isolated from the hemolysate by centrifugation and washed at 0°C with 165 nM KCl, 2 mM phosphate buffer, pH 7.3, containing 0.02 mM EDTA-EGTA (1:1, v/v) (buffer I). They were stored in the same buffer containing BSA of appropriate concentrations and used for experiments within 2 days.

Preparation of incubation buffers

[^{3}H]anandamide and unlabeled anandamide were dissolved in 50 μl benzene just enough to moisten 200 mg small glass beads (diameter 0.1 mm). The benzene was sublimated at low pressure, and incubation buffers were prepared by shaking the anandamide-loaded beads with buffer I containing BSA for 15 min at room temperature.

Determination of the dissociation equilibrium constants of anandamide binding to BSA

Human resealed red cell membranes have been used in a method originally developed for measuring the equilibrium constants of long-chain fatty acid binding to BSA (14, 15). BSA-filled ghosts were packed by centrifugation for 7 min at 30,000 g in a Sorval RC SC at the appropriate temperature and equilibrated with buffer I containing labeled as well as unlabeled anandamide bound to BSA (BSA-A) in different molar ratios of anandamide to BSA (ν). The water-phase concentrations were determined for anandamide A_w in equilibrium with BSA-A inside ghosts as a function of the ν of anandamide to total BSA as described by Bojesen and Bojesen (14, 15). In the ν values, A_w is neglected compared with the concentration of bound anandamide, because it is more than three orders of magnitude lower (see Discussion). The BSA-A inside ghosts are at equilibrium with anandamide in the membrane and with anandamide in the outer medium. Efflux data (unpublished) show that this equilibrium is obtained very quickly in accordance with the rapid free diffusion of anandamide across membranes seen by Glaser et al. (19).

Determinations were carried out at four different temperatures after equilibration for 50 min at 0°C, 30 min at 10°C, 20 min at 23°C, and 15 min at 37°C.

The definition of the K_d of anandamide dissociation from BSA is given in equation 1:

$$K_d = [A_w] \, [BSA]/[BSA\text{-}A] \qquad (Eq.\ 1)$$

According to a model in which all binding sites on BSA are equivalent and independent (20), i.e., the sites have the same affinity for anandamide and the binding is noncooperative, we get (as shown in equation 2a, b):

$$1/\nu = (1/N) \, (K_d/[A_w]) + 1/N \text{ or}$$
$$K_d = [A_w] \, (N-\nu)/\nu \qquad (Eq.\ 2a)$$

which, linearized according to Wilkinson (21), gives

$$[A_w]/\nu = (1/N) \, [A_w] + K_d/N \qquad (Eq.\ 2b)$$

From this equation, K_d values and the number of binding sites on BSA (N) were estimated by linear regression analyses.

The equilibrium association constant of anandamide to BSA (K_a) is equal to $1/K_d$.

Gibbs free energy of binding (ΔG^0) was calculated as shown in equation 3:

$$\Delta G^0 = -RT \ln K_a \qquad (Eq.\ 3)$$

where R is the gas constant and T is temperature in Kelvin. ΔH^0 for the dissociation process was obtained from the slope (Fig. 2) after linear regression analysis of the van't Hoff equation: $\ln K_d/(1/T) = \Delta H^0/R$. $T\Delta S^0$ was calculated as $\Delta G^0 - \Delta H^0$.

Scintillation counting

We used a Tri-Carb 2200CA liquid scintillation analyser from Hewlett-Packard. The efficiency was 67% for ^{3}H in unquenched samples. Counting rates were determined, after the addition of 3.9 ml Ultima Gold scintillation fluid, to a probable error smaller than 1%. Aliquots of buffers after the equilibration of charge buffers with ghosts were taken for counting, and ν values were calculated from the counting rates (C_a) (dpm/ml) and the specific activities (S) (dpm/nmol) as $C_a/(S [BSA])$. A_ws were determined from counting rates of duplicates of ghost-free supernatant (C_s) (dpm/ml) and S as C_s/S.

Statistics

Regression lines of Wilkinson plots and the statistics of slopes and intercepts were estimated by standard methods (22). Standard errors of estimated parameters of N and K_d values were calculated according to the general function given by Armitage (22), 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 (22).

RESULTS

K_ds of N number of BSA binding sites

A_ws in equilibrium with BSA have been determined. In order to be sure that no significant amount of anandamide was washed out from BSA during the procedure, we conducted a series of experiments with BSA-free as well as BSA-filled ghosts that were washed with 50 vol of BSA-free buffer I. No depletion of BSA-A inside ghosts took place.

Analyses of data obtained at 0°C, 10°C, 23°C, and 37°C were carried out after linearization of the relations between ν and A_w according to Wilkinson (equation 2). **Figure 1A** and B show examples of such plots. **Table 1** shows corresponding measurement of K_d and N for anandamide at the four temperatures. The data clearly show that there is only one binding site on BSA for anandamide independent of temperature from 0°C to 37°C. In contrast, the K_d is temperature dependent. The values in column 5 are weighted mean values of K_d calculated for N = 1. From the temperature dependence of the equilibrium dissociation constants, it is possible to calculate values for the thermodynamic functions involved in the binding and dissociation process. In the calculation of ΔG^0 according to equation 3, we have used K_a values calculated from K_d values normalized to N = 1. The free energy of anandamide
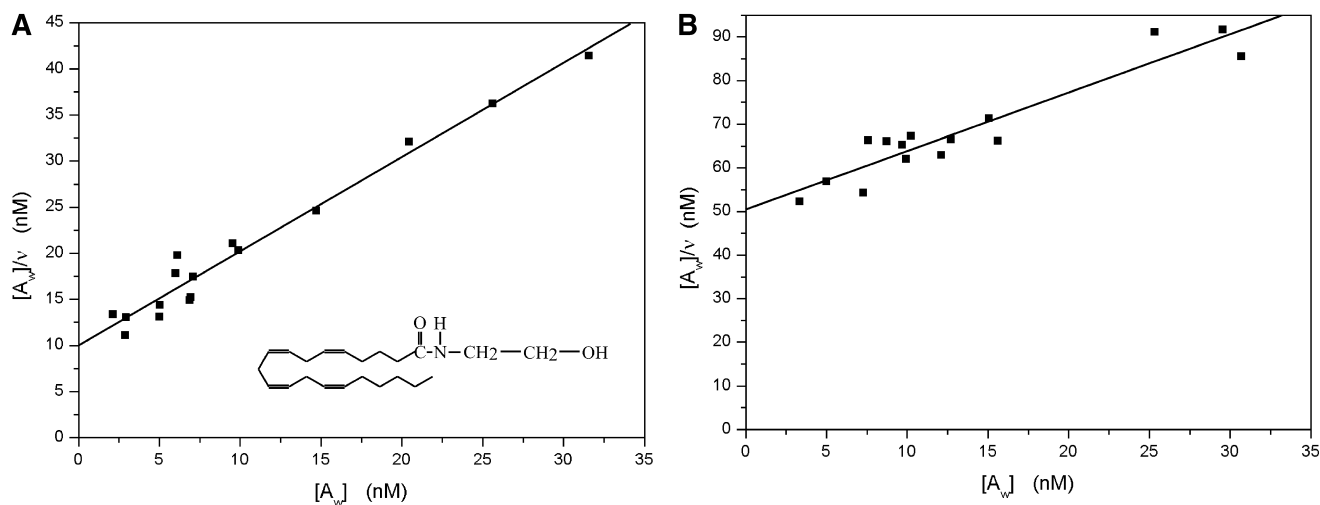
Fig. 1. Examples of plots to determine equilibrium dissociation constants (K_ds) and number of binding sites (N) according to equation 2b. A: Anandamide data at 10°C, pH 7.3. The regression line is Y = 1.02 (± 0.05) X + 9.97 (± 0.64), R = 0.99. The insert shows the structural formula of anandamide. B: Anandamide data at 37°C, pH 7.3. The regression line is Y = 1.34 (± 0.13) X + 50.28 (± 2.11), R = 0.95.

binding (ΔG^0) is calculated to −43.05 kJ mol^{-1} (range, 42.7–43.1 kJ mol^{-1}). In **Fig. 2**, data for the K_d values calculated for one binding site on BSA are plotted according to the van't Hoff equation. The linear correlation is good, and a binding enthalpy (ΔH^0) of −42.09 kJ mol^{-1} was obtained from the slope.

DISCUSSION

BSA as well as HSA is able to bind a variety of hydrophobic compounds, among others, fatty acids. The architecture of such proteins is best described by their interactions with fatty acids. The most important approach to studying such interactions is the use of NMR spectroscopy and X-ray crystallography. NMR is a powerful tool for the study of microenvironments of sites in specific domains. In fatty acid-BSA interactions, three primary binding sites are found for physiological ν values lower than three. At these sites, there are two kinds of interactions, hydrophobic and electrostatic. In the first, the alkyl chain interacts with hydrophobic amino acid residues, whereas in the second, the carboxyl group interacts with basic amino acid residues (23–26). At higher ν values, sites with lower affinity appear with less-defined interactions between the carboxyl group and basic amino acid residues.

X-ray diffraction studies are consistent with NMR data but are carried out mainly with HSA, which is very similar to BSA. The first crystallographic analyses revealed that the protein contains three homologous domains and that each domain is a product of two subdomains able to bind fatty acids (27, 28). Later, precise locations of up to seven fatty acid binding sites were reported and each site was described in detail (16, 29). However, very high unphysiological ν values were used in these studies. For HSA complexed with 12 myristic molecules, six binding sites were observed. Five of these appear to have electrostatic interactions with basic amino acid residues, and the methylene tails were accommodated within hydrophobic cavities (29). The last site has less-well-defined interactions at the carboxyl group.

TABLE 1. Estimation by regression analyses according to Wilkinson (21) of the number of binding sites (N, mean ± SEM) on BSA for anandamide, and equilibrium dissociation constants (K_ds, mean ± SEM) of anandamide-BSA complexes at different temperatures

Temperature and Number of Experiments	N	K_d (nM)	R	K_d (nM) (N = 1)
0°C (n = 18)	1.14 ± 0.15	7.82 ± 1.39	0.88	6.87 ± 0.53
10°C (n = 16)	0.98 ± 0.05	9.77 ± 0.80	0.99	11.28 ± 0.45
23°C (n = 9)	1.06 ± 0.13	30.44 ± 4.42	0.95	26.05 ± 0.76
37°C (n = 15)	0.75 ± 0.08	37.54 ± 4.42	0.95	54.92 ± 1.91
Mean	0.98 ± 0.11			

R is the correlation coefficient. Column 5 shows K_d (mean ± SEM) calculated according to equation 2a with one binding site (N = 1).
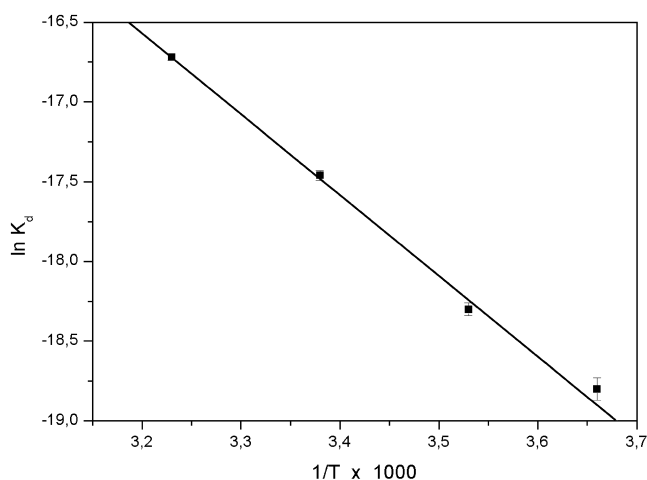

Fig. 2. Van't Hoff plot of the temperature effect on the K_d values of anandamide. Each value is given with ± standard error; linear regression gives R = −0.999.

Anandamide differs from the fatty acids insofar as it is a neutral molecule, and in the present paper, we find that for ν values lower than 1, anandamide binds to BSA in only one high-affinity binding site, with a somewhat lower binding constant than seen for arachidonic acid and the other long-chain fatty acids studied (30). The finding of only one binding site is quite interesting; we had expected three binding sites, as seen for arachidonic acid. This means that BSA must bind the neutral anandamide differently, as compared with a hydrophobic anion such as arachidonic acid. Perhaps the binding of anandamide is favored in one of the above-mentioned sites in which the electrostatic interactions are less well defined. It is also important to note that not only is anandamide a neutral molecule but it is also much larger than arachidonic acid. In this respect it is interesting that BSA has only one binding site for the very long chain fatty acid, hexacosanoic acid (26:0) (31). However, a complete and reliable assignment of the anandamide binding site on BSA is unsettled and will require further work.

Zolese et al. (17) have studied the binding of *N*-oleylethanolamine to BSA . Fluorescence data disclosed that *N*-oleylethanolamine binds not only to hydrophobic sites near tryptophan-212 in BSA but also at other binding sites affecting the environment of tryptophan-134. However, their averaged K_d (21 μM) is orders of magnitude higher than that determined for anandamide in this study. A binding site for oleic acid has also been found in the N terminal part of BSA. After proteolytic fragmentation of the BSA molecule, a single distinct NMR resonance peak is seen after the addition of 1 mol oleic acid to the fragment, which consists of amino acid residues 1–306 (25). The site is defined as a primary (high-affinity) site, but whether this site is the same as that defined by Zolese et al. is unsettled.

The thermodynamic analyses show that the binding energy is mainly enthalpic in both anandamide and arachidonic acid. Arachidonic acid has a carboxyl group, but anandamide has an amide bond as well as a hydroxyl group. Therefore anandamide is a less-hydrophobic molecule than arachidonic acid. This is confirmed by the lower entropy contribution to the free energy of binding (**Table 2**). Or, in other words, in solution, the water is much more structured around a hydrophobic molecule, which means that the transfer of arachidonic acid from water to BSA will result in an entropy contribution that is higher than in the case of anandamide. A difference of a factor of 10 is seen (Table 2). Furthermore, anandamide has more possibilities to form hydrogen bonds than does arachidonic acid, which possibly explains the more negative ΔH^0 (Table 2) for the transfer from water to BSA.

A very high percentage of plasma anandamide is bound to albumin (>99%). With a concentration of total anandamide in human plasma of 4 nM and an albumin concentration of 630 μM, the normal ν value is 0.63×10^{-5}, which gives a free water-phase concentration of 3.5×10^{-13} M (according to equation 2a) and a free-to-bound ratio of anandamide of ~0.01%. Although the aqueous concentration of anandamide is very low, a fast dissociation from albumin will allow equilibrium to binding sites on cells. A fast

TABLE 2. Thermodynamic parameters for binding of arachidonic acid and anandamide to BSA

Compound	ΔG^0 kJ mol^{-1}	ΔH^0 kJ mol^{-1}	TΔS^0 kJ mol^{-1}	K_a M^{-1} (at 310 K)
Arachidonic acid	−44.9	−35.4	9.5	3.57×10^7
Anandamide	−43.1	−42.1	1.0	1.82×10^7

ΔG^0 was calculated from equation 3 as $-RT \ln K_a$, ΔH^0 was obtained from the slope of the regression line (Fig. 2), and TΔS^0 was obtained from $\Delta H^0 - \Delta G^0$. The data for arachidonic acid are taken from (15). K_a, equilibrium association constant of anandamide.

dissociation from albumin can be deduced from the rapid uptake of anandamide by cells from albumin-bound anandamide (19).

One aspect of the importance of the results obtained in the present paper is probably that knowing the values of K_d and N, one is able to calculate the water-phase concentration of anandamide according to equation 2a at all ν lower than one. The knowledge of such concentrations is helpful for studying effects and binding of anandamide to membranes and receptors, as well as for understanding the physiological functions of anandamide.∎

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