Octanoate binding to the indole- and benzodiazepine-binding region of human serum albumin

Ulrich KRAGH-HANSEN

Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark

Binding of L-tryptophan, diazepam and octanoate to defatted human serum albumin was studied at pH 7.0 by equilibrium dialysis at low ligand/protein molar ratios. L-Tryptophan binding takes place at only one site of the protein with an association constant of $4.4 \times 10^4 \text{ M}^{-1}$. Under the present experimental conditions, binding of diazepam and octanoate could be accounted for by high-affinity binding alone with primary association constants of $3.8 \times 10^5 \text{ M}^{-1}$ and $1.6 \times 10^6 \text{ M}^{-1}$ respectively. During the simultaneous presence of L-tryptophan plus octanoate or diazepam plus octanoate, pronounced mutual reductions in binding were observed. Analysis of the data suggests that the reductions in binding represent competition for a common high-affinity binding site. Thus a region seems to exist that is capable of binding one molecule of these diverse ligands with a high affinity. The location of this region within the albumin molecule is discussed.

INTRODUCTION

The unique capability of serum albumin to bind reversibly a large number of different endogenous and exogenous compounds is usually explained by the existence of a limited number of binding regions of a very different specificity. One of these is the indole- and benzodiazepine-binding region (Sjöholm *et al.*, 1979; Fehske *et al.*, 1981; Kragh-Hansen, 1981a, 1983a,b, 1985; Peters, 1985), which is probably identical with site II in the model proposed by Sudlow *et al.* (1975, 1976). In addition to L-tryptophan and diazepam, and derivatives thereof, the region is probably also a primary binding site for other aromatic ligands. By contrast, the primary binding site(s) for ions of aliphatic fatty acids has been considered to be located in another region of the protein (Fehske *et al.*, 1981; Kragh-Hansen, 1981a; Peters, 1985). However, various types of information indicate that only ions of long-chain fatty acids, and not, for example, ions of medium-chain fatty acids, are bound to this region with a high affinity (Kragh-Hansen, 1981a,b; Spector, 1986). Instead, several authors have, on the basis of indirect evidence, suggested that the primary binding site for medium-chain fatty acid ions, such as octanoate, is placed in the same region as those for L-tryptophan and diazepam. Thus King & Spencer (1970) suggested a common primary binding site for tryptophan and octanoate, because a very high concentration of the weakly bound D-tryptophan was able to displace both L-tryptophan and octanoate bound to bovine serum albumin. Koh & Means (1979) studied the inhibitory effect of different ligands on the fast reaction between *p*-nitrophenyl acetate and human serum albumin and proposed that small fatty acid anions (C_3 – C_9) and L-tryptophan interact strongly with a binding site that possesses a readily acetylated tyrosine residue. Finally, Wanwimolruk *et al.* (1983) found, by the combined use of fluorescence measurements and equilibrium dialysis, a pronounced displacement of certain site II marker ligands by ions of medium-chain fatty acids (C_6 – C_{11}) and diazepam.

In the present work an attempt has been made to clarify whether the primary binding site for ions of medium-chain fatty acids is indeed placed in the indole- and benzodiazepine-binding region of human serum albumin. To this end direct competition experiments were performed with both the pairs L-tryptophan plus octanoate and diazepam plus octanoate.

EXPERIMENTAL

Materials

Human serum albumin (97% pure according to the manufacturer) from AB Kabi, Stockholm, Sweden, was defatted by the charcoal method of Chen (1967), freeze-dried and then stored in a desiccator at 4 °C until use. Octanoic acid was obtained from Merck, Darmstadt, Germany, and L-tryptophan was bought from Cambrian Chemicals, Croydon, Surrey, U.K. A sample of pure diazepam was donated by Dumex, Copenhagen, Denmark. [^{14}C]Octanoic acid (58 mCi/mmol) was purchased from ICN Pharmaceuticals, Irvine, CA, U.S.A.; L-[G- ^3H]tryptophan (5.8 Ci/mmol) and [*N*-methyl- ^3H]diazepam (85 Ci/mmol) were supplied by Amersham International, Amersham, Bucks., U.K. The amount of radiochemical impurities that do not bind to albumin was estimated by determining the saturation limit for binding of ligand at infinite albumin concentration (Honoré, 1987). They were found to be approx. 0% (octanoic acid), 10% (L-tryptophan) and 0.4% (diazepam) in the batches used in the present work. The predominant impurity in the batch of L-tryptophan is probably the D-isomer of the amino acid, which binds very weakly to human serum albumin (McMenamy & Oncley, 1958). In the binding experiments involving L-tryptophan, corrections for these impurities were carried out when calculating the fraction of unbound ligand. The absence of aliphatic fatty acids of different chain lengths from the batch of [^{14}C]octanoic acid was ensured as follows (Honoré & Brodersen, 1988). A small amount of the ^{14}C -labelled compound(s) was added to 2,2-dimethoxypropane in order to form methyl esters. Afterwards, the material was subjected to reverse-phase t.l.c. on KC_{18} plates from Whatman, Clifton, NJ, U.S.A. The eluent was acetonitrile/5% (v/v) aq. conc. NH_3 (19:1, v/v), and only one spot was found by autoradiography.

Equilibrium dialysis

Binding of single ligands and pairs of ligands was studied in media containing 33 mM-sodium phosphate buffer, pH 7.0, at 20 °C. The albumin concentration was 0.373 mM (2.5%, w/v). To examine octanoate binding, solutions of different concentrations of unlabelled and ^{14}C -labelled ligand, with and without albumin, were prepared. For the competition experiments four

different sets of solutions were made, namely two sets with one and two ligands respectively dissolved in buffer, and two other sets of the same ligand composition but with albumin. The ligands were L-tryptophan + L-[³H]tryptophan alone or with octanoate + [¹⁴C]octanoate and diazepam + [³H]diazepam alone or with octanoate + [¹⁴C]octanoate. The ligands were added, with magnetic stirring, to the solutions as small volumes (10–50 µl) of concentrated stock solutions consisting of both non-labelled and isotopically labelled ligand dissolved in 0.1 M-NaOH (octanoate and L-tryptophan) or ethanol (diazepam). The final solutions of octanoate and L-tryptophan changed less than 0.05 pH unit, and the solutions of diazepam contained 0.1–0.5% (v/v) ethanol. Gentamicin sulphate (donated by Essex Pharma A/S, Farum, Denmark) was added to a final concentration of 20 µg/ml in order to avoid bacterial growth.

The degree of ligand binding was determined by a Dianorm equilibrium dialyser (Dianorm Geräte, München, Germany) with half-cell volumes of 250 µl. The dialysis membranes were made from natural cellulose and had a molecular-mass cut-off of 5000 Da (Diachema dialysis membranes). Samples (200 µl) containing ligand(s) and albumin were pipetted into the left-side half-cells, and 200 µl samples with the same ligand composition, but without albumin, were injected into the right-side half-cells. As references, representing 100% unbound ligand(s), cells were prepared with the same albumin-free solution on both sides of the dialysis membrane. After being filled, the equilibrium dialysers were placed in a temperature-controlled water bath, and the cells were rotated about their axis at a speed of 12 rev./min for 17–18 h. After this period of time, the concentration of ligand(s) in the albumin-free media was determined by liquid-scintillation counting by using one or two channel settings of an LKB Wallac 1209 Rackbeta spectrometer. Binding percentages were calculated from the concentrations of ligand(s) in the right-side media of albumin-containing cells and their corresponding reference cells.

Control experiments with reference solutions on one side of the dialysis membranes and buffer on the other side showed that the membranes were fully permeable for all three ligands under the present conditions, that ligand adsorption to membranes and cell walls was negligible, and that equilibrium was established within the period of time used. As measured by the method of Lowry *et al.* (1951), protein leakage could be neglected.

RESULTS

L-Tryptophan plus octanoate

Binding of L-tryptophan to defatted human serum albumin (□), at low ligand/protein molar ratios, is shown in Fig. 1(a). In order to characterize the binding in terms of binding classes, consisting of n_i sites with the association constants K_i , the experimental data were analysed according to the following equation:

$$\bar{\nu}_L = \sum_{i=1}^j \frac{n_i \cdot K_i \cdot [L_i]}{1 + K_i \cdot [L_i]} \quad (1)$$

In this general formulation $\bar{\nu}_L$ and $[L_i]$ are the average number of molecules of ligand bound per molecule of protein and the concentration of unbound ligand respectively. Least-squares calculations showed (cf. the full curve in Fig. 1a) that L-tryptophan binding can be characterized by binding to one site with an association constant of $4.4 \times 10^4 \pm 0.5 \times 10^4 \text{ M}^{-1}$ ($\pm$ S.D., $n = 4$). The finding of only one albumin-binding site for this amino acid is in agreement with previous publications (McMenamy & Oncley, 1958; Müller & Wollert, 1975; Kober *et al.*, 1978; Bruderlein & Bernstein, 1979; Kragh-Hansen, 1983a). The

Fig. 1. Binding of L-tryptophan and octanoate to human serum albumin

(a) Binding of various concentrations of L-tryptophan (0.015–0.107 mM) to albumin (0.373 mM) in the absence (□) and presence (■) of a constant concentration of total octanoate (0.073 mM). The full curve describes binding of L-tryptophan alone and was constructed by means of eqn. (1) and $n = 1$ and $K = 4.4 \times 10^4 \text{ M}^{-1}$. The broken curve was made according to eqn. (2) assuming competition of the two ligands for a common high-affinity binding site. The dotted curve was constructed assuming that 20% of the octanoate binding takes place to secondary sites and that only high-affinity octanoate binding competes with L-tryptophan binding. (b) Data from the same competition experiments as presented (■) in (a) showing the concentration of unbound octanoate as a function of $\bar{\nu}_{\text{Trp}}$. $\bar{\nu}_{\text{Trp}}$ represents the average number of molecules of L-tryptophan bound per molecule of albumin. The subscript f stands for free ligand concentrations. The symbols represent averages $\pm$ S.D. of four duplicate experiments.

present association constant for L-tryptophan binding is similar to, or somewhat higher than, those given by the authors referred to.

Fig. 1(a) also shows binding of L-tryptophan to albumin in the presence of a constant concentration of total octanoate (■). As seen, addition of the fatty acid anion causes a pronounced reduction in binding of the aromatic amino acid. From Fig. 1(b) it is clear that reduction in binding is a mutual phenomenon, since the concentration of unbound octanoate concomitantly increases with a rise in $\bar{\nu}_{\text{Trp}}$. The possibility that the diminished L-tryptophan binding is the result of competitive inhibition by octanoate was analysed by use of the following equation:

$$\bar{\nu}_A = \frac{K_A \cdot [A_i]}{1 + K_A \cdot [A_i] + K_B \cdot [B_i]} \quad (2)$$

where $\bar{\nu}_A$ is the average number of molecules of ligand A bound per molecule of protein, K_A and K_B are the association constants for individual binding of ligand A and B respectively, and $[A_i]$ and $[B_i]$ are the concentrations of the unbound forms of the ligands. In the present context, A and B stand for L-tryptophan and octanoate respectively. The magnitude of K_A is known ($4.4 \times 10^4 \text{ M}^{-1}$) together with the simultaneously determined values for $[A_i]$ (Fig. 1a) and $[B_i]$ (Fig. 1b). Therefore, if K_B was also known, a theoretical binding curve representing competitive binding could be constructed for L-tryptophan and compared with the experimental results (■).

In order to determine K_B , binding of low concentrations of octanoate to albumin in the absence of L-tryptophan was studied (Fig. 2). Calculations, based on eqn. (1), revealed that the results could be adequately described as ligand binding to one high-affinity site with an association constant of $1.6 \times 10^6 \pm 0.2 \times 10^6 \text{ M}^{-1}$ ($n = 8$) (cf. the curve in Fig. 2). High-affinity binding of octanoate to only one site has previously been proposed (Meisner *et al.*, 1980; Lee & McMenamy, 1980; Heaney-Kieras

Fig. 2. Binding of octanoate to human serum albumin

The concentration of octanoate varied from 0.010 mM to 0.073 mM, whereas that of the protein was kept constant at 0.373 mM. The binding curve was constructed by means of eqn. (1) and $n = 1$ and $K = 1.6 \times 10^6 \text{ M}^{-1}$. $\bar{v}_{\text{Oct}}$ and $[\text{Octanoate}]_f$ represent the average number of molecules of octanoate bound per molecule of albumin and the concentration of free octanoate respectively. The symbols represent averages $\pm$ S.D. of eight duplicate experiments.

& King, 1977; Kragh-Hansen, 1981b). However, the present K value is somewhat higher than the primary association constants given by Meisner *et al.* (1980) and Lee & McMenamy (1980), who reported K_1 values of $8.1 \times 10^5 \text{ M}^{-1}$ and approx. $3 \times 10^5 \text{ M}^{-1}$ respectively. Furthermore, it is more than one order of magnitude higher than that published by Heaney-Kieras & King (1977) and Kragh-Hansen (1981b) reporting $K_1 = 8.3 \times 10^4 \text{ M}^{-1}$. The high association constant found in the present study can probably be explained by the fact (cf. the Experimental section) that the batch of ^{14}C -labelled octanoate was very pure, leading to lower experimental values for the concentration of unbound octanoate. If the radioactive preparation of the fatty acid had contained, for example, 1% or 2% impurities that do not bind to albumin, the K value would have been calculated as $4.7 \times 10^5 \pm 0.3 \times 10^5 \text{ M}^{-1}$ and $2.7 \times 10^5 \pm 0.2 \times 10^5 \text{ M}^{-1}$ respectively.

With this information on the magnitude of K for octanoate binding it is now possible to examine whether L-tryptophan and octanoate compete for a common high-affinity binding site. As seen in Fig. 1(a), the theoretical (broken) binding curve fits the experimental points (■) pretty well, supporting the proposal of a common primary binding site for L-tryptophan and octanoate.

Diazepam plus octanoate

Binding of diazepam to albumin (Δ) is shown in Fig. 3(a). Analyses, based on eqn. (1), showed that, under the present experimental conditions, binding of this ligand also takes place to one high-affinity site. The association constant was calculated to be $3.8 \times 10^5 \pm 0.3 \times 10^5 \text{ M}^{-1}$ ($n = 4$) (cf. the full curve in Fig. 3a), a value comparable with those previously published (Kragh-Hansen, 1983a, 1985).

The effect on diazepam binding of adding a constant concentration of octanoate is also illustrated in Fig. 3(a). As seen ($\blacktriangle$), the presence of the fatty acid anion resulted in a very pronounced reduction in diazepam binding. From Fig. 3(b) it is apparent that the decrease in binding is mutual. The possibility that the reduction in binding reflects competition of the two ligands for a common primary site was tested by means of eqn. (2) as outlined above. It is seen in Fig. 3(a) that the theoretical (broken) binding curve is in full agreement with the experimental results ($\blacktriangle$). Thus the data show that high-affinity binding of diazepam and octanoate to albumin takes place according to a competitive scheme.

Fig. 3. Binding of diazepam and octanoate to human serum albumin

(a) Binding of various concentrations of diazepam (0.011–0.073 mM) to albumin (0.373 mM) in the absence (Δ) and presence ($\blacktriangle$) of a constant concentration of total octanoate (0.073 mM). The full curve describes binding of diazepam alone and was constructed by means of eqn. (1) and $n = 1$ and $K = 3.8 \times 10^5 \text{ M}^{-1}$. The broken curve was constructed according to eqn. (2) assuming competition of the two ligands for a common high-affinity binding site. The dotted curve was constructed assuming that 20% of the octanoate binding takes place to secondary sites and that only high-affinity octanoate binding competes with diazepam binding. (b) Data from the same competition experiments as presented ($\blacktriangle$) in (a) showing the concentration of unbound octanoate as a function of $\bar{v}_{\text{Dia}}$. $\bar{v}_{\text{Dia}}$ represents the average number of molecules of diazepam bound per molecule of albumin. The subscript f stands for free ligand concentrations. The symbols represent averages $\pm$ S.D. of four duplicate experiments.

DISCUSSION

In this study, competitive binding experiments were used to investigate the relation between high-affinity binding of L-tryptophan, diazepam and octanoate. Such an approach presupposes that the ligands, under the conditions used, only bind to one, or predominantly to one, site of the protein. In the case of L-tryptophan binding this requirement is fully met, because it is generally accepted that this amino acid binds to only one site in the human serum albumin molecule (McMenamy & Oncley, 1958; Müller & Wollert, 1975; Kober *et al.*, 1978; Bruderlein & Bernstein, 1979; Kragh-Hansen, 1983a). By contrast, diazepam and octanoate bind to a number of different sites. However, at low $\bar{v}$, binding of diazepam takes place predominantly to one site, since the association constant for this site is more than two orders of magnitude higher than that calculated for the secondarily bound drug molecules ($n_2 = 2$). The K_1 value is 4×10^5 – $5 \times 10^5 \text{ M}^{-1}$ (Fig. 3a; Kragh-Hansen, 1983a, 1985), whereas K_2 is only about $2 \times 10^3 \text{ M}^{-1}$ (Kragh-Hansen, 1983a, 1985).

For octanoate binding K_1 has also been reported to be much higher than K_2 . For example, Lee & McMenamy (1980) published a K_1 value of about $3 \times 10^5 \text{ M}^{-1}$, whereas K_2 was determined to be as low as $3 \times 10^2 \text{ M}^{-1}$ ($n_2 = 10$). Furthermore, in the studies by Heaney-Kieras & King (1977) and Kragh-Hansen (1981b) K_1 was found to be almost two orders of magnitude higher than K_2 [$8.3 \times 10^4 \text{ M}^{-1}$ versus $1 \times 10^3 \text{ M}^{-1}$ ($n_2 = 6$)]. However, Meisner *et al.* (1980) calculated K_1 and K_2 to be less different, namely $8.1 \times 10^5 \text{ M}^{-1}$ and $3.4 \times 10^4 \text{ M}^{-1}$ ($n_2 = 7$) respectively. If this relation between the K values and the number of secondary binding sites is correct, it would imply that at the present $\bar{v}$ values 20–30% of the octanoate binding would take place to secondary sites. In order to test the plausibility of this possibility, K_1 for octanoate binding ($1.60 \times 10^6 \text{ M}^{-1}$) was recalculated from the data shown in Fig. 2 assuming 20% ligand binding to weaker sites. Afterwards, the new K_1 ($1.15 \times 10^6 \text{ M}^{-1}$) was used to re-analyse the results of the competition experiments. It is

apparent from the Figures that the ensuing (dotted) binding curves are not consistent with the experimental data but that they overestimate the binding of both L-tryptophan (Fig. 1a) and diazepam (Fig. 3a). These findings show that the difference between K_1 on one hand and K_2 and $n_2 \times K_2$ on the other must be substantially larger than proposed by Meisner *et al.* (1980). Other binding schemes such as to assume binding of octanoate in dimeric form could not account for the data either (results not shown).

The large differences between the primary and the secondary binding constants not only for diazepam but also for octanoate imply that the respective K_1 values can be determined to a high degree of accuracy at low $\bar{\nu}$, because under this condition the contribution of secondary binding to total binding is very small.

These large differences between the binding constants for diazepam and octanoate also imply that a simple competition scheme could in all cases be used to evaluate the binding data under the present experimental conditions. The results showed mutual competitive displacements between the pairs L-tryptophan plus octanoate (Fig. 1) and diazepam plus octanoate (Fig. 3). These findings render the existence of a common region for high-affinity binding of the ligands very probable. The existence of such a region is also supported by results from previous studies showing competitive high-affinity binding of L-tryptophan and diazepam (Kragh-Hansen, 1983a,b).

Where in the albumin molecule is the binding region for L-tryptophan, diazepam and octanoate placed? A detailed answer to this question cannot be given at present, but some hints can be found from other reports. Bos *et al.* (1988) studied the binding of diazepam both to intact human serum albumin and to large tryptic and peptic fragments by circular dichroism and equilibrium dialysis. They concluded that at least the main part of the high-affinity binding site is located in domain III of the protein. The work of Means and co-workers is also relevant for this discussion. Means & Wu (1979) observed high reactivity of diisopropyl phosphorofluoridate and *p*-nitrophenyl acetate with the same tyrosine residue of human serum albumin. This residue is probably number 411 in the complete protein sequence (Means & Wu, 1979; Moravek *et al.*, 1979). Since these fast reactions were stoichiometrically inhibited by medium-chain fatty acid anions (Means & Wu, 1979; Koh & Means, 1979) and L-tryptophan (Koh & Means, 1979), Tyr-411, which is placed in domain III, could be part of the region.

The binding sites are apparently located within a crevice in the human serum albumin molecule. Oida (1986) investigated the binding of ions of both medium-chain and long-chain fatty acids by ^1H -n.m.r. spectroscopy and proposed that the ligands are primarily bound within such structures in the protein. On the basis of a series of fluorescence studies, Wanwimolruk *et al.* (1983) proposed that site II, which was suggested to be able to bind for example octanoate and diazepam, constitutes a hydro-

phobic cleft with a cationic group located near the surface of the protein molecule.

In summary, a predominantly hydrophobic crevice seems to exist in human serum albumin that mainly involves domain III of the molecule. It has the capacity to bind with high affinity different aromatic ligands (L-tryptophan and diazepam) or aliphatic ligands of a relatively small size such as octanoate and possibly also ions of other medium-chain fatty acids.

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