Effects of aliphatic fatty acids on the binding of Phenol Red to human serum albumin

Ulrich KRAGH-HANSEN

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

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Binding of Phenol Red to human serum albumin at pH 7.0 was studied by ultrafiltration ($n_1 = 1$, $K_1 = 3.9 \times 10^4 \text{ M}^{-1}$, $n_2 = 5$, $K_2 = 9.6 \times 10^2 \text{ M}^{-1}$). The presence of 1 mol of octanoate or decanoate per mol of albumin caused a decrease in dye binding (dye/protein molar ratio 1:1), which, in contrast with additional fatty acid, was very pronounced: 1–8 mol of palmitate or stearate resulted in a small, and apparently linear, displacement of Phenol Red. The displacement effect of 1–5 mol of oleate, linoleate or linolenate per mol of albumin was comparable with that of the equimolar concentrations of palmitate or stearate. At higher molar ratios the unsaturated acids caused a drastic decrease in dye binding. The different Phenol Red-displacement effects of low molar ratios of medium-chain and long-chain fatty acids indicate that these acids have different high-affinity binding sites. In accordance with this proposal, low concentrations of stearate had only a small effect on the Phenol Red-displacement effect of octanoate. Phenol Red-binding curves in the presence of 1 mol of octanoate, 8 mol of stearate and 6 or 7 mol of linolenate per mol of albumin respectively indicated that the dye and the fatty acids do not compete for a common primary binding site. In contrast, a secondary Phenol Red-binding site could be identical with the primary octanoate-binding site. Furthermore, the primary Phenol Red-binding site could be the same as a secondary linolenate-binding site. Assignment of the different primary binding sites for Phenol Red and for medium-chain and long-chain fatty acids to a model of the secondary structure of albumin is attempted.

Albumin is the most abundant protein in blood plasma, and is able to bind, and thereby transport, various compounds such as fatty acids, bilirubin, tryptophan, steroids and a great number of different drugs (Peters, 1975; Kragh-Hansen, 1981). The binding capability of albumin is probably based on the existence of different regions on the protein molecule to which ligands can be bound with a high affinity (Peters, 1975; Kragh-Hansen, 1981). The results of competitive binding studies have revealed that a distinct binding region for each ligand does not exist (Kragh-Hansen, 1981). However, present knowledge does not allow for a complete assignment of the different ligands to high-affinity binding regions of albumin. It is the aim of the present paper to contribute some information about the binding sites for free fatty acids of various chain lengths on human serum albumin.

In vivo, the fatty acids bound to albumin mainly are long-chain aliphatic acids such as oleate, palmitate, linoleate and stearate (Saifer & Goldman, 1961; Watson, 1965; Rosseneu-Motreff *et al.*,

1971). However, the protein is also able to bind with a relatively high affinity fatty acids with shorter chain lengths (Spector, 1975; Heaney-Kieras & King, 1977). Results reported in the literature indicate that fatty acids with long chain length and fatty acids with medium chain length do not have the same high-affinity binding sites: Cunningham *et al.* (1975) have studied the binding of L-tryptophan to bovine serum albumin and human serum albumin in the presence of various fatty acids and observed that tryptophan binding was diminished more by the presence of low concentrations of dodecanoate than by equimolar amounts of palmitate or oleate. These authors suggested that the primary binding class for medium-chain fatty acids corresponds to a site in the secondary class, but is distinct from the primary class, for long fatty acids. Soltys & Hsia (1978) have investigated the displacement effect of various fatty acids on the binding of a spin-labelled ligand bound to the same site as bilirubin on human serum albumin. On the basis of the differences in the displacement effect of the fatty acids, the existence

of two separate sets of high-affinity fatty acid-binding sites was suggested, namely one for fatty acids of chain length less than 10 carbon atoms and one for fatty acids with more than 10 carbon atoms. Finally, Koh & Means (1979) have investigated the inhibitory effect of fatty acids on the interaction of *p*-nitrophenyl acetate and human serum albumin. These authors suggested that fatty acids larger than decanoate interact with high-affinity sites other than those for fatty acids with shorter chain lengths.

Investigation of the possibility of separate high-affinity binding sites for fatty acids having less than and more than 10–12 carbon atoms by studies of simultaneous binding of the fatty acids themselves is impeded by the strong tendency of long-chain fatty acids to self-assemble in aqueous solutions (Spector, 1975). In the present study the question of different binding sites has been approached by investigating the influence of nine different fatty acids on the binding of the relatively weakly bound indicator dye Phenol Red. Great differences in the displacement effect of the fatty acids on the Phenol Red–human serum albumin association were observed. The results indicate pronounced differences in the binding of medium-chain fatty acid ions (e.g. octanoate), long-chain saturated fatty acid ions (e.g. stearate) and long-chain unsaturated fatty acid ions (e.g. linolenate) to albumin.

Experimental

Materials

Human serum albumin (98% pure) was obtained from AB Kabi, Stockholm, Sweden. Phenol Red was a product of Merck A.G., Darmstadt, Germany. The following fatty acids (puriss grade) were all from Fluka A.G., Buchs, Switzerland: octanoic acid, decanoic acid, dodecanoic acid, myristic acid, palmitic acid, stearic acid and oleic acid. α -Linoleic acid and α -linolenic acid (approx. 99% pure) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. n -[1- 14 C]Octanoic acid (sodium salt) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of A.R. grade.

Albumin was defatted by using the charcoal method described by Chen (1967). The aqueous solutions of defatted albumin were freeze-dried, and the resulting powder was stored in a desiccator at 4°C before use. The fatty acid content of albumin, before and after the defatting procedure, was determined titrimetrically in accordance with the principles of Dole (1956).

Oleic acid was stored under N_2 at 4°C. Linoleic acid and linolenic acid were stored as small lots under N_2 in sealed ampoules at –20°C.

Solutions

All binding experiments were conducted with media containing 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. The concentration of Phenol Red varied between 0.033 mM and 8.475 mM, whereas that of albumin (molecular weight 66 300) was kept constant at 0.38 mM (2.5%, w/v). Samples of volume 10 ml containing Phenol Red alone and albumin plus Phenol Red were prepared. When the influence of a fatty acid on the binding of Phenol Red was investigated, 25–200 μ l of a heated stock solution of the fatty acid in question dissolved in 0.15 M-NaOH was added with magnetic stirring to the albumin-containing samples by using pre-heated pipettes. Control experiments indicated that no fatty acid stock solution was lost during transfer to the albumin containing solutions. With the unsaturated fatty acids a new stock solution was prepared in a broken ampoule for each binding experiment.

Binding of octanoate (0.18–1.45 mM) to human serum albumin (0.38 mM) was also studied. Samples were prepared by adding, with stirring, 25–200 μ l of an alkaline stock solution of octanoate plus [14 C]-octanoate to 10 ml of phosphate buffer alone and to buffer containing albumin.

In some experiments the percentage binding of both octanoate and Phenol Red in the solutions containing albumin was determined simultaneously. In these experiments the following three kinds of samples were used: Phenol Red dissolved in phosphate buffer, octanoate (unlabelled and 14 C-labelled) dissolved in buffer and solutions containing all three reactants.

The pH of the solutions after addition of fatty acids was measured with a PHM 64 Research pH-meter equipped with a GK 2320 C Combined Electrode, both from Radiometer, Copenhagen, Denmark, but not corrected. Increases of pH of 0.00–0.03 unit were observed.

Ultrafiltration

Protein and reference solutions (4 ml) were enclosed in cellophan bags (Visking; 18 mm diameter) and placed in plastic tubes consisting of two parts screwed together (Kragh-Hansen *et al.*, 1972). Reference solutions, representing 100% free ligand, were the solutions without albumin. Ultrafiltration was performed by centrifuging the tubes in a Christ IV KS refrigerating centrifuge for 1 h at 320 *g* at 20°C. The percentage binding of the ligands was calculated from the differences between the concentration of the ligand in the ultrafiltrates of the albumin solutions and those of the corresponding reference solutions (Kragh-Hansen *et al.*, 1972). The concentration of Phenol Red was determined spectrophotometrically. A portion (typically 200 μ l) of the ultrafiltrate was diluted with a suitable volume of 10 mM-NaOH, and the absorbance was read at

559nm on a Zeiss PM2 DL Spektralphotometer. With octanoate the concentration was determined by liquid-scintillation counting of radioactivity. A 150 μ l portion of ultrafiltrate was added to 10ml of Luma Gel (Lumac Systems A.G., Basel, Switzerland) and its radioactivity was counted in a Packard Tri-Carb spectrometer; 33 mM-phosphate buffer was used for counting of the background radioactivity.

Equilibrium dialysis

Cellophan bags, containing 4ml solution of Phenol Red and/or octanoate with or without albumin, were placed in 4ml of 33 mM-sodium phosphate buffer, pH 7.0, in test tubes (diameter 2.5cm). The tubes were closed with Parafilm (American Can Co., Greenwich, CT, U.S.A.) and shaken gently for 20–24h at room temperature (approx. 20°C). Determination of the concentrations of free Phenol Red and of octanoate in the solutions outside the bags after the dialysis and the calculation of percentage binding were performed as mentioned above.

Control experiments

No dye binding to the membranes could be detected. Ultrafiltrability of Phenol Red was not influenced by the presence of octanoate, and vice versa. No interference was observed in determining the concentration of Phenol Red in the presence of octanoate. Counting of [14 C]octanoate radioactivity was affected by Phenol Red only at the highest concentrations of free dye used in the present study (>0.1 mM). Where quenching was suspected the appropriate corrections were made.

Determination of n_i and K_i

The number of binding sites (n_i) and the corresponding association constants (K_i) in the i th binding class of Phenol Red and octanoate binding were calculated by using the double-reciprocal plot described by Klotz (1946). In order to check the quality of the calculations binding curves were drawn from the following equation:

$$\bar{v}_L = \sum_{i=1}^J \frac{n_i K_i [L_f]}{1 + K_i [L_f]} \quad (1)$$

where $\bar{v}_L$ and $[L_f]$ are the average number of mol of ligand bound per mol of albumin and the concentration of free ligand respectively. If the experimental binding data and the theoretical binding curve deviated appreciably, one or more of the K_i values were modified in order to obtain the best possible fit.

Results

Binding of Phenol Red at pH 7.0 to native, defatted and re-fatted human serum albumin was

studied by ultrafiltration. The binding curves showing the interaction between the dye and native albumin and defatted albumin at relatively low dye/protein ratios are shown in Fig. 1. These data indicate that Phenol Red is bound with a higher affinity to defatted albumin than to the native protein. However, the two sets of binding data show similar patterns: a steep increase at $\bar{v}_{PR}$ (average number of mol of Phenol Red bound per mol of albumin) below unity followed by a less-pronounced increase at higher $\bar{v}_{PR}$. These results presented in Fig. 1, and those obtained at higher dye/protein ratios,

Fig. 1. Binding of Phenol Red and octanoate to human serum albumin

The experiments were performed in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. The concentration of albumin was 0.38 mM (2.5%). The curves are calculated on the basis of the association constants and number of binding sites given in the text as described in the Experimental section. $\bar{v}_L$ and $[L_f]$ represent the average number of mol of ligand bound per mol of albumin and the concentration of free ligand respectively. ● and ○ denote binding of Phenol Red to native and defatted albumin respectively. ■ represents binding of octanoate to defatted albumin. Each point shows the average of three duplicate experiments. The bars represent standard deviations.

were analysed as described in the Experimental section. It was found that the binding results could be adequately described in terms of three binding classes (Fig. 1). When Phenol Red binds to native albumin, the first and second classes consist of one and five binding sites (n_1 and n_2) with association constants $K_1 = 3.2 \times 10^4 \text{ M}^{-1}$ and $K_2 = 8.0 \times 10^2 \text{ M}^{-1}$ respectively. The third binding class is only characterized by $n_3 K_3$ ($3.1 \times 10^2 \text{ M}^{-1}$), since, owing to uncertainty in the calculation of n_3 , only $n_3 K_3$ can be estimated. n_1 and K_1 calculated for the binding of Phenol Red to native albumin are comparable with those obtained previously with other batches of the protein (Kragh-Hansen & Møller, 1973a; Kragh-Hansen *et al.*, 1974). Defatting of albumin (fatty acid content approx. 0.1 mol/mol of protein) does not change n_1 or n_2 , but increases K_1 , K_2 and $n_3 K_3$ to $3.9 \times 10^4 \text{ M}^{-1}$, $9.6 \times 10^2 \text{ M}^{-1}$ and $3.7 \times 10^2 \text{ M}^{-1}$ respectively.

The fatty acids associated with native albumin (approx. 0.6 mol/mol) are a heterogeneous mixture of long-chain aliphatic acids (Saifer & Goldman, 1961; Watson, 1965; Rosseneu-Motreff *et al.*, 1971). In the following, the influence of different fatty acids on the binding of Phenol Red is investigated. With a dye/protein molar ratio of 1:1, the effects obtained are mainly attributable to the high-affinity binding site of Phenol Red.

The addition of 1 mol of octanoate or decanoate per mol of albumin (0.38 mM) results in a pronounced decrease in Phenol Red binding (Fig. 2): $\bar{\nu}_{\text{PR}}$ is decreased from approx. 0.89 to approx. 0.77. Increasing the amount of the two acids causes a less-pronounced, and almost linear, decrease in dye binding, to give $\bar{\nu}_{\text{PR}} = 0.60$ in the presence of 8 mol of octanoate or decanoate per mol of protein. In contrast, addition of 1 mol of palmitate or stearate per mol of albumin results in only a small displacement of Phenol Red from albumin. The effect of these acids on dye binding seems to be linear. After addition of 8 mol of palmitate or stearate per mol of albumin, binding was decreased to the same value as that in the presence of only 1 mol of octanoate or decanoate per mol of albumin ($\bar{\nu}_{\text{PR}} \sim 0.77$). The influence of dodecanoate and myristate on the binding of Phenol Red is intermediate between those of the fatty acids with shorter and longer chain lengths except at molar ratios greater than 6 (Fig. 2). The decrease in dye binding in the presence of 7 or 8 mol of dodecanoate or myristate per mol of albumin is the same, and larger than that caused by equimolar concentrations of octanoate or decanoate respectively.

Binding of Phenol Red in the presence of various amounts of long-chain unsaturated fatty acids is shown in Fig. 3. At molar ratios below 6:1 the decrease in Phenol Red binding seems to be linear and comparable with that caused by stearate, which

Fig. 2. Binding of Phenol Red to human serum albumin in the presence of various amounts of different saturated aliphatic fatty acids

The media contained 0.35 mM-Phenol Red and 0.38 mM-albumin in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C and one of the following fatty acid ions: ○, octanoate; ●, decanoate; □, dodecanoate; ◇, myristate; △, palmitate; ▲, stearate. ■ denotes Phenol Red binding in the absence of fatty acid ions. Each point represents the average of three duplicate experiments. The bars represent standard deviations.

is the corresponding saturated fatty acid. However, a drastic decrease in dye binding is observed at higher unsaturated fatty acid/albumin molar ratios. The effectiveness of Phenol Red displacement increases with the number of double bonds in the fatty acid (linolenate > linoleate > oleate). The decrease in dye binding in the presence of 8 mol of the unsaturated fatty acids per mol is larger than that caused by the same amount of octanoate (Fig. 2).

On the basis of their effect on the interaction between Phenol Red and albumin, the binding of the fatty acids can be conveniently classified into three groups; medium-chain fatty acids (octanoate and decanoate), long-chain saturated fatty acids (palmitate and stearate) and long-chain unsaturated fatty acids (oleate, linoleate and linolenate). The effects of dodecanoate and myristate are intermediate between

Fig. 3. Binding of Phenol Red to human serum albumin in the presence of various amounts of different unsaturated aliphatic fatty acids

The media contained 0.35 mM-Phenol Red and 0.38 mM-albumin in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C and one of the following fatty acid ions: ○, oleate; △, linoleate; □, linolenate. The broken curve represents binding of Phenol Red in the presence of stearate (taken from Fig. 2). ■ denotes dye binding in the absence of fatty acid ions. Each point shows the average of three duplicate experiments. The bars represent standard deviations.

Fig. 4. Binding of Phenol Red to human serum albumin in the presence of octanoate and of stearate

The media contained 0.35 mM-Phenol Red and 0.38 mM-albumin in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. ● shows $\bar{v}_{PR}$ in the presence of octanoate (0.38 mM) and stearate (0.19–3.04 mM) corrected for the decrease of $\bar{v}_{PR}$ caused by the presence of stearate alone (0.19–3.04 mM). The continuous line represents dye binding in the absence of fatty acid ions. The broken line represents $\bar{v}_{PR}$ in the presence of 1 mol of octanoate per mol of albumin (taken from Fig. 2). Each point represents the average of three duplicate experiments. The bars represent standard deviations.

those of the fatty acids representing the first two groups, indicating a gradual transition between the binding characteristics of these groups of fatty acids.

The observation that long-chain fatty acids, although bound with very high association constants (Goodman, 1958), displace Phenol Red to a much smaller extent than do medium-chain fatty acids indicates that the high-affinity binding sites for the two types of fatty acids are not identical. This possibility was examined further by studying the effect that various concentrations of stearate have on the Phenol Red-displacing effect of 1 mol of octanoate per mol of albumin (Fig. 4). It is seen that addition of 0.5–1.5 mol of stearate per mol of protein has only a small effect on the ability of octanoate to displace the dye. This observation strongly supports the proposal of separate high-affinity binding sites

for long-chain and medium-chain fatty acids. In the concentration range 1.5–3.0 mol of stearate per mol of albumin an almost linear decrease in the displacement of Phenol Red is observed. In the presence of 3.0 mol of stearate per mol of protein the inhibitory effect of octanoate on the binding of the dye diminished to about 50% of the effect in the presence of low concentrations of stearate. This finding is probably the result of displacement of octanoate from its primary binding site and caused by binding of stearate to one or more of its secondary sites. It is seen (Fig. 4) that $\bar{v}_{PR}$ is not affected further by stearate concentrations above 3 mol per mol of albumin.

The influence of one representative from each of the three groups of fatty acids mentioned above on

the binding of Phenol Red was studied in greater detail.

Binding of Phenol Red to albumin in the presence of octanoate

Binding of octanoate to albumin in the absence of Phenol Red was studied, and the binding curve is included in Fig. 1. By using equilibrium dialysis Heaney-Kieras & King (1977) studied the binding of octanoate to human serum albumin at pH 7.95. They calculated n_i and K_i to be: $n_1 = 1$, $K_1 = 8.3 \times 10^4 \text{ M}^{-1}$, $n_2 = 6$ and $K_2 = 1 \times 10^3 \text{ M}^{-1}$. As shown in Fig. 1, these binding data can also describe the octanoate–human serum albumin interaction at pH 7.0. This finding is in accordance with results reported by Ashbrook *et al.* (1972), who, by equilibrium dialysis, found that binding of octanoate to human serum albumin was relatively insensitive to pH over the range 6.0–8.2.

The decreased Phenol Red binding in the presence of 1 mol of octanoate per mol of albumin (Fig. 2) could be the result of competition between the high-affinity binding site for octanoate and a Phenol Red-binding site. With the aid of n_i and K_i of octanoate and Phenol Red binding to albumin, this possibility can be analysed.

For competitive binding of two ligands, A and B, to one site on a protein molecule, the following equations apply:

$$\bar{v}_A = \frac{K_A[A_f]}{1 + K_A[A_f] + K_B[B_f]} \quad (2)$$

$$\bar{v}_B = \frac{K_B[B_f]}{1 + K_B[B_f] + K_A[A_f]} \quad (2a)$$

where $\bar{v}_A$ and $\bar{v}_B$ are the average number of mol of A and B bound per mol of protein, K_A and K_B are the association constants of ligands A and B respectively, and $[A_f]$ and $[B_f]$ are the concentrations of the free forms of the ligands. In the present study A and B stand for Phenol Red and octanoate.

First, the possibility of competitive binding of Phenol Red and octanoate to a common high-affinity binding site on albumin is tested. In that case K_A and K_B are $3.9 \times 10^4 \text{ M}^{-1}$ and $8.3 \times 10^4 \text{ M}^{-1}$ respectively. In the following, Phenol Red binding in the presence of 1 mol of octanoate per mol of albumin is analysed (see Fig. 5). Since the total concentration of octanoate and the concentration of albumin (both 0.38 mM) are known, it is possible by the use of eqns. (2) and (2a) to calculate $\bar{v}_{PR}$ as a function of the concentration of free Phenol Red. Fig. 5 shows that the curve calculated (broken curve 1) is not in accordance with the experimental results: at low concentrations of free dye the experimentally determined values of $\bar{v}_{PR}$ are higher than those evaluated theoretically, whereas the opposite ob-

Fig. 5. *Binding of Phenol Red to human serum albumin in the presence of octanoate*

The experiments were performed in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. The concentration of albumin was 0.38 mM (2.5%). ● denotes dye binding in the presence of 1 mol of octanoate per mol of albumin. The points show the average of three duplicate experiments. The bars represent standard deviations. Broken curve 0 represents binding of Phenol Red in the absence of fatty acid (taken from Fig. 1). Broken curve 1 is the theoretically calculated dye binding assuming competitive binding of Phenol Red and octanoate to a common high-affinity binding site. Broken curve 2 is the calculated dye binding assuming competition between a secondary Phenol Red-binding site and the high-affinity binding site for octanoate.

servation occurs at higher dye concentrations. These findings indicate that Phenol Red and octanoate do not compete for a common high-affinity binding site. Assuming competition between the high-affinity octanoate-binding site and a secondary Phenol Red-binding site, then the calculations are re-made, but this time K_A is set equal to $9.6 \times 10^2 \text{ M}^{-1}$. As shown in Fig. 5 (broken curve 2), this proposal cannot describe the decreased Phenol Red binding either. All the theoretically calculated $\bar{v}_{PR}$ values are higher than those determined experimentally. As a consequence of these results, the decreased Phenol Red binding could be explained partly by com-

petition between the high-affinity binding site for octanoate and a secondary binding site for Phenol Red.

In the calculations the binding of octanoate to its secondary sites was ignored, and this means an error is introduced, but this probably cannot explain the differences found. Thus the decrease in Phenol Red binding in the presence of octanoate cannot simply be explained by competition between the primary octanoate-binding site and a Phenol Red-binding site.

In some experiments the binding of both ligands was determined simultaneously. Binding of Phenol Red was decreased as expected (cf. Figs. 2 and 5). By contrast, the binding of octanoate was unchanged or increased. The same experiments were performed with the use of equilibrium dialysis. The results obtained with this technique also showed that Phenol Red binding is decreased in the presence of octanoate. However, no significant changes in the binding of octanoate could be detected. In these experiments the molar ratio of the concentrations of octanoate and albumin was 1:1 and that of Phenol Red and the protein was less than or equal to 1:1. By choosing these concentrations, attention was focused on binding of ligands to their high-affinity binding sites.

Binding of Phenol Red to albumin in the presence of stearate

Dye binding in the presence of 8 mol of stearate per mol of albumin is shown in Fig. 6 (black circles). In this Figure, the broken curve 1 represents the calculated Phenol Red binding assuming competition of the dye and the fatty acid ion for a common high-affinity binding site on the protein molecule. The broken curve 2 shows the calculated Phenol Red binding assuming identity between the high-affinity binding site for Phenol Red and a secondary site for stearate. The calculations were performed as described for octanoate. K_1 and K_2 for binding of stearate were $8.0 \times 10^7 \text{ M}^{-1}$ and $8.0 \times 10^5 \text{ M}^{-1}$ respectively (Goodman, 1958). It is shown in Fig. 6 that the displacement of Phenol Red from albumin by addition of as much as 8 mol of stearate per mol is less than predicted by any of the two proposals. Thus competition does not exist between the high-affinity binding site for Phenol Red and a primary or a secondary site for stearate.

Binding of Phenol Red to albumin in the presence of linolenate

The influence of 6 or 7 mol of linolenate per mol of albumin on the binding of Phenol Red is illustrated in Fig. 7. The broken curves 1 and 2 show dye binding assuming competition between the high-affinity binding site for Phenol Red and a primary and a secondary binding site for linolenate res-

Fig. 6. *Binding of Phenol Red to human serum albumin in the presence of stearate*

The experiments were performed in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. The concentration of albumin was 0.38 mM (2.5%). ● denotes dye binding in the presence of 8 mol of stearate per mol of albumin. The points show the average of three duplicate experiments. The bars represent standard deviations. Broken curve 0 represents binding of Phenol Red in the absence of fatty acid (taken from Fig. 1). Broken curve 1 is the theoretically calculated dye binding assuming competitive binding of Phenol Red and stearate to a common high-affinity binding site. Broken curve 2 is the calculated dye binding assuming competition between the high-affinity binding site for Phenol Red and a secondary binding site for stearate.

pectively. The calculations were performed as mentioned above. To my knowledge, no values of n_i and K_i for the binding of linolenate to albumin have been given in the literature. Therefore the values for linoleate were used: $K_1 = 1.3 \times 10^7 \text{ M}^{-1}$ and $K_2 = 2.5 \times 10^6 \text{ M}^{-1}$ (Goodman, 1958). It is shown in Fig. 7 that displacement of Phenol Red from albumin by addition of 6 or 7 mol of linolenate per mol of protein is less than predicted by the proposal of a common high-affinity binding site. This finding is in accordance with the observation that 1 mol of linolenate per mol of albumin causes only a small decrease in Phenol Red binding (Fig. 3). Phenol Red binding in the presence of 6 mol of linolenate per mol

Fig. 7. Binding of Phenol Red to human serum albumin in the presence of linolenate

The experiments were performed in 33 mM-sodium phosphate buffer, pH 7.0, at 20°C. The concentration of albumin was 0.38 mM (2.5%). ■ and ● denote dye binding in the presence of 6 and 7 mol of linolenate per mol of albumin respectively. The points show the average of three duplicate experiments. The bars represent standard deviations. Broken curve 0 represents binding of Phenol Red in the absence of fatty acid (taken from Fig. 1). Broken curve 1 is the theoretically calculated dye binding assuming competitive binding of Phenol Red and linolenate to a common high-affinity binding site. Broken curve 2 is the calculated dye binding assuming competition between the high-affinity binding site for Phenol Red and a secondary binding site for linolenate.

of albumin is greater than expected if the high-affinity dye-binding site is the same as a secondary fatty acid-binding site. In contrast, Phenol Red binding in the presence of 7 mol of linolenate per mol is well described by the theoretical binding curve at low concentrations of free Phenol Red (below approx. 0.15 mM). At higher dye concentrations Phenol Red binding is decreased more than is predicted by the curve. Thus, if K_i for linolenate binding is not greatly different from K_i for linoleate binding, the displacement of Phenol Red in the presence of linolenate could partly be explained by competition between the primary dye-binding site and a secondary fatty acid-binding site.

Discussion

In the present investigation the effects of different aliphatic fatty acids on the binding of Phenol Red to human serum albumin was studied. Although the pattern in most cases varied, addition of any of the fatty acids resulted in a decrease in dye binding. This decrease can be caused by one or more of the following mechanisms. (1) The fatty acid anions and Phenol Red compete for one or more binding sites on the albumin molecule. (2) Binding of fatty acid ions to albumin results in changes of the conformation of the protein molecule to forms that bind the dye less firmly. (3) Interaction between fatty acid ions and albumin results in an increase of the net negative charge of the protein; this change in net charge results in an increased electrostatic repulsion between albumin and the negatively charged Phenol Red.

Theoretical considerations (Fig. 5) indicated that the pronounced decrease in Phenol Red binding caused by addition of 1 mol of octanoate per mol of albumin could not be explained by competition of the two ligands for a common high-affinity binding site on albumin. In contrast, the high-affinity octanoate-binding site could be in common with a secondary dye-binding site. However, this proposal can only partially account for the decreased dye binding, and other mechanisms as outlined above must therefore be operative as well.

In some experiments the binding of Phenol Red and octanoate to albumin was determined simultaneously. The molar concentrations of the ligands were less than or equal to that of the protein. Binding of octanoate seemed to be unaffected by the presence of the dye. This observation is in agreement with the proposal of separate high-affinity binding sites for the two ligands. However, binding of Phenol Red was decreased by the presence of the fatty acid. According to the principle of linked functions (Wyman, 1964; Weber, 1972), the mutual effects of the two ligands should be the same, i.e. if octanoate decreased the binding of Phenol Red, the association of octanoate with albumin should be diminished by the addition of Phenol Red. The deviation from this rule can perhaps be explained by the heterogeneity of the ligand-albumin complexes. At molar ratios between ligand and albumin of 1:1 or less the greater part of the ligand molecules is bound to the high-affinity binding site, but a significant proportion of the ligand molecules is bound to secondary sites on the protein. Therefore binding of Phenol Red to its primary site could be unaffected by binding of octanoate, whereas binding of the dye to a secondary site (that is also the high-affinity fatty acid-binding site) could be competitively displaced. Binding of octanoate to its primary site as well as to its secondary site could be

unchanged. (Owing to the great difference of the association constants and the low concentration of free Phenol Red, it is probable that the Phenol Red that is supposed to bind to a secondary site cannot displace a significant proportion of octanoate from its high-affinity binding site.) An explanation of the apparent differences based on arguments derived from electrostatic effects does not seem probable. At pH 7.0 octanoate carries one negative charge, and Phenol Red is predominantly bound at its univalent form (Kragh-Hansen & Møller, 1973b). The effects of possible conformational changes of the albumin molecule accompanying binding of the ligands are difficult to evaluate. Probably the ligands can introduce different conformational changes due to differences in the location of the binding sites and in the association constants.

The decrease of Phenol Red binding in the presence of a few moles of long-chain fatty acids per mol of albumin is much lower than that caused by the medium-chain fatty acids. Theoretical considerations (Figs. 6 and 7) indicated that the high-affinity binding sites for both saturated and unsaturated long-chain fatty acids are different from that for Phenol Red. The diminished dye binding is probably caused by such indirect means as electrostatic repulsions and/or conformational changes of the protein.

At high fatty acid/albumin molar ratios the Phenol Red binding is diminished further. This

decrease can be brought about by various combinations of the three mechanisms mentioned.

Several authors (Cunningham *et al.*, 1975; Soltys & Hsia, 1978; Koh & Means, 1979) have suggested that the high-affinity binding sites for medium-chain fatty acid ions (e.g. octanoate) and long-chain fatty acid ions (e.g. palmitate) are not identical. This proposal is in accordance with the observation that the long-chain fatty acids, which are bound with much higher association constants, displace Phenol Red to a smaller extent than do the medium-chain fatty acids. The proposal is furthermore strongly supported by the observation that binding of 1 mol of stearate per mol of albumin has only a small influence on the displacement effect of octanoate on the binding of Phenol Red (Fig. 4). Cunningham *et al.* (1975) have proposed that the high-affinity binding site for medium-chain fatty acids corresponds to a site in the secondary class for long-chain fatty acids. The findings in the present study (Fig. 4) are in agreement with this suggestion. However, displacement of octanoate by stearate through indirect means cannot be excluded.

In the following an attempt is made to correlate the results obtained in the present study and data found in the literature, dealing with binding of fatty acid ions and a few other ligands to various preparations of albumin, to the model of the secondary structure of the protein proposed by Brown and co-workers (Behrens *et al.*, 1975; Brown,

Fig. 8. Model of the secondary structure of human serum albumin

The bending line represents the peptide chain of the protein, whereas the short horizontal lines show disulphide bridges. N and C denote the N-terminal and the C-terminal ends of albumin respectively. The numbers beneath the model indicate the positions of the nine double loops of albumin. The proposed locations of the high-affinity binding sites for long-chain fatty acid ions, tryptophan and octanoate, and bilirubin and Phenol Red are indicated by the bars. Furthermore, the positions of the cysteine-34 residue and the lysine-240 residue are indicated.

1977). The present schematic presentation of albumin (Fig. 8) is comparable with that published by Geisow & Beaven (1977). On the basis of studies with tryptic-digest and peptic-digest fragments of bovine serum albumin, Peters (1977) has proposed that the high-affinity binding site for palmitate is placed in double loop number 7 (Fig. 8). Berde *et al.* (1979) have reported results indicating that the first two molecules of long-chain fatty acids are bound side-by-side in the C-terminal part of human serum albumin. Displacement studies (Figs. 2, 3 and 4; Cunningham *et al.*, 1975; Soltys & Hsia, 1978; Koh & Means, 1979) show that octanoate probably does not have a high-affinity binding site in common with long-chain fatty acids. In contrast, competitive studies have indicated that octanoate and tryptophan bind to the same high-affinity binding site on bovine serum albumin (King & Spencer, 1970). Results of binding of tryptophan to chemically modified albumins (McMenamy, 1977) and to fragments of albumin (King & Spencer, 1970; Sjöholm & Ljungstedt, 1973; King, 1973; Geisow & Beaven, 1977) are consistent with a location of the high-affinity binding site at the 'top' of loops 3–5 as depicted in Fig. 8 (Kragh-Hansen, 1981). Displacement studies suggest that Phenol Red and bilirubin share a common high-affinity binding site on human serum albumin (Kragh-Hansen *et al.*, 1974). Jacobsen (1978) has obtained evidence that lysine-240 in human serum albumin may be located at the high-affinity binding site for bilirubin. This finding, together with results obtained on bilirubin binding to albumin fragments (Peters, 1977; Geisow & Beaven, 1977; Sjödin *et al.*, 1977; Reed *et al.*, 1975; Gitzelmann-Cumarasamy *et al.*, 1976), indicate that the bilirubin-binding site mainly is associated with the 'lower' end of loops 3–4 (Fig. 8) (Kragh-Hansen, 1981).

The observations made in the present study are in accordance with the results cited (summarized in Fig. 8). The high-affinity binding sites for medium-chain and long-chain fatty acids are not identical, and are different from that for Phenol Red. Furthermore, the location of the high-affinity binding sites for Phenol Red and octanoate in the same domain of the albumin molecule may account for the strongly inhibitory effect of this fatty acid by conformational changes and/or electrostatic interactions. By contrast, the remote location of the high-affinity binding site for long-chain fatty acids makes displacement of Phenol Red by such interactions less likely.

The displacement studies (Fig. 3) indicate that the high-affinity binding sites for long-chain saturated and long-chain unsaturated fatty acids could be identical. In contrast, some of the secondary sites seem to be different.

The results obtained by Reed *et al.* (1975)

indicate that one or more of the secondary sites for palmitate are placed in the N-terminal part of albumin. With stearate, and probably also palmitate, competition does not exist between the high-affinity binding site for Phenol Red and a secondary fatty acid-binding site. In contrast, a secondary site for long-chain unsaturated fatty acids could be identical with the primary site for the dye.

Noel & Hunter (1972) have observed that long-chain unsaturated fatty acid ions (oleate and linoleate), in contrast with long-chain saturated fatty acid ions, can cause oxidation of the thiol group of bovine serum albumin. These authors proposed that a binding site for long-chain unsaturated fatty acid ions is placed in the vicinity of the cysteine residue in albumin. The lone thiol group of serum albumin is placed in position 34 (Behrens *et al.*, 1975; Meloun *et al.*, 1975; Brown, 1977) (Fig. 8). The spatial relations between the thiol group and the high-affinity binding site for Phenol Red remain to be clarified.

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