The fatty-acid-induced conformational states of human serum albumin investigated by means of multiple co-binding of protons and oleic acid

Johannes H. M. DRÖGE, Lambert H. M. JANSSEN* and Jaap WILTING

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

The binding of oleic acid to human serum albumin causes progressive changes in (a) the pK of some amino acid residues, as detected by pH-stat titration and (b) the induced molar ellipticities of albumin-bound drugs (diazepam and oxyphenbutazone), as measured by c.d. It is concluded that albumin undergoes several conformational transitions as the amount of oleic acid bound increases from 0 to about 9 molecules/molecule of protein. At least three different conformations of the protein seem to be involved. These conformations can be linked with the three classes of oleic acid-binding sites on albumin.

INTRODUCTION

The binding of drugs to human serum albumin (HSA) continues to be a widely studied phenomenon (Vallner, 1977; Kragh-Hansen, 1981). Research carried out during the last few years has revealed the enormous complexity of the problem. The complexity is due to a number of factors.

(1) Albumin has two specific binding sites, the diazepam- and the warfarin-binding site (Sudlow *et al.*, 1976; Brodersen *et al.*, 1977; Fehske *et al.*, 1979, 1981; Müller & Wollert, 1979; Sjöholm *et al.*, 1979), also called 'site II' and 'site I' respectively.

(2) Ligands can bind to albumin in a ratio higher than 1. This implies the existence of both low-affinity and high-affinity sites.

(3) In albumin, conformational changes occur. Of these conformational changes, the proton-linked N–B transition (which has been demonstrated to occur in the pH region 6–9) (Leonard *et al.*, 1963; Harmsen *et al.*, 1971) is important for studies on binding at about physiological pH, because the affinity of drugs for either the N or the B conformation may be different (Wilting *et al.*, 1980a).

(4) Simple ions, such as Cl^- and Ca^{2+} , influence drug binding (Wilting *et al.*, 1980a, 1981). This influence may be either direct (displacement) or indirect (allosteric). Fatty acids or other bound drugs can give rise to similar effects [see Dröge *et al.* (1985) and references cited therein].

This combination of factors means that a measured ligand-binding constant (and any quantity related to it, such as free concentration) for any substance binding to albumin is an apparent one, that is to say, it is a function of the above-mentioned parameters. In other words, such a binding constant will be a function of the free concentration (or, better, the activity) of all the species involved in the binding (Wyman, 1964). Consequently, when studying the interaction of only one ligand with albumin, one should keep the free concentration of the

other ligands constant. For H^+ ions this is usually done by using buffers. For other ligands this requirement may give considerable experimental problems.

In a previous study (Dröge *et al.*, 1985) we reported on the binding of fatty acids (F), H^+ and drugs (D). In dealing with three ligands one needs several binding constants in order to describe the process leading from $HSA + F + D + H^+$ to the $HSA \cdot F \cdot D \cdot H^+$ complex. This is illustrated in Scheme 1. Note that in this scheme the H^+ concentration is kept constant. However, similar schemes can be arrived at by interchanging D, F and H^+ . It follows that:

$$\Delta G_1^0 + \Delta G_2^0 = \Delta G_3^0 + \Delta G_4^0 \quad (1)$$

where ΔG_i^0 ($i = 1-4$) represents the apparent standard free-energy change of the given reaction at constant pH. Changes in pH may cause a change in ΔG_i^0 . By taking the pH derivative of eqn. (1), one obtains the following expression (Wyman, 1964):

$$\Delta Z_1 + \Delta Z_2 = \Delta Z_3 + \Delta Z_4 \quad (2)$$

in which ΔZ_i ($i = 1-4$) represents the different number of protons bound by the species involved in the given reaction; so, for example,:

$$\Delta Z_1 = Z(HSA \cdot D) - Z(HSA)$$

This equation provides another approach to the study of pH-dependent processes.

Several steps in Scheme 1 have been studied. Reaction 1 was studied by measuring both the free concentration of warfarin (Wilting *et al.*, 1980a) and diazepam (Wilting *et al.*, 1980b) and ΔZ_1 as a function of pH (Janssen *et al.*, 1981). Reaction 4 has also been studied recently by measuring the free concentration of drug as a function of the ratio of F and as a function of pH (Dröge *et al.*, 1985). In the present paper we give particular attention to reaction 3. First of all we measure ΔZ_3 of this reaction at two pH values as a function of the ratio of F. Secondly, we observe the change in the conformation of

Abbreviations used: HSA, human serum albumin; F, fatty acids; D, drugs.

* To whom correspondence and reprint requests should be sent.

Scheme 1. Simplified scheme representing the binding of drug D, fatty acid F and H^+ to HSA

For details, see the text. Note that the reactions refer to the binding of one molecule of each species. However, any stoichiometry can be assumed.

the HSA·F complex at a fixed pH as a function of the amount of F. This conformational change was studied by measuring the change in molar ellipticity induced by the addition of a ligand. Both a site-I drug (oxyphenbutazone) (Fehske *et al.*, 1981) and a site-II drug (diazepam) (Sudlow *et al.*, 1976; Brodersen *et al.*, 1977; Fehske *et al.*, 1979, 1981; Müller & Wollert, 1979) are used as a label.

EXPERIMENTAL

HSA (infusion solution) was a gift from the Biotest-Serum-Institut G.m.b.H., Frankfurt am Main, Germany. We gave the batch lot no. 820512. The albumin was deionized before use and its concentration determined as described elsewhere (Dröge *et al.*, 1982). The deionizing procedure yielded a protein solution in which 0.9 mol of fatty acid is bound/mol of albumin. The method of measurement has been described previously (Dröge *et al.*, 1982). We used this albumin sample with 0.9 mol of fatty acid/mol of albumin as our starting material because we thought it might be advisable to work with albumin that contains some bound fatty acid (Dröge *et al.*, 1982). This value of 0.9 is included in those quoted in the present paper and also in the Figures shown. The identity of these fatty acids is not known. However, in order to avoid complicated formulations, it is assumed here that these residual fatty acids are oleic acid molecules.

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one; a gift from Hoffman-La Roche, Mijdrecht, The Netherlands), oxyphenbutazone [4-butyl-1-(4-hydroxyphenyl)-2-phenylpyrazolidine-3,5-dione], a gift from Ciba-Geigy BV (Arnhem, The Netherlands), ^{14}C -labelled diazepam (2.11 GBq·mmol $^{-1}$; Amersham Nederland BV, Utrecht, The Netherlands), ^{14}C -labelled oxyphenbutazone (1.14 GBq·mmol $^{-1}$; New England Nuclear, Boston, MA, U.S.A.) and oleic acid (sodium salt; Sigma Chemical Co., St. Louis, MO, U.S.A.) were used without further purification. All other chemicals were of analytical grade (Merck, Darmstadt, Germany, or J. T. Baker, Deventer, The Netherlands).

The affinity of diazepam and oxyphenbutazone was studied by means of equilibrium-dialysis experiments, which were carried out as described previously (Wilting *et al.*, 1980a). Free concentration of the ligand was determined by liquid-scintillation counting (Packard-Tri-Carb liquid-scintillation spectrometer, model 2425).

The dialysis experiments were performed by using 6.0×10^{-5} M-albumin solutions and a drug-to-protein ratio (r_D) of 0.3 at 25 °C. The results of the dialysis experiments were used to calculate the molar ellipticity of the ligand-albumin complexes.

C.d. experiments were performed as described previously (Wilting *et al.*, 1980b). The c.d. signals of the diazepam-albumin complex and the oxyphenbutazone-albumin complex ($r_D = 0.30$) were measured in phosphate and borate buffers (I.0.1) at 330 and 304 nm respectively. To calculate the molar ellipticity of the ligand-albumin mixtures from the measured c.d. signals, the concentration of the ligand-albumin complex of the various samples was taken into account. The c.d. experiments were performed with 6.0×10^{-5} M-albumin solutions at 25 °C.

The pH-stat experiments were carried out with the apparatus described by Janssen & Van Wilgenburg (1978). Oleic acid was added stepwise to an albumin solution with a fixed pH. Each time the oleic acid was added, the pH of the albumin solution was subsequently titrated back to its original value. The difference between the protonic charge of the albumin in the presence and in the absence of oleic acid (ΔZ_3) can be calculated (Janssen *et al.*, 1970; Janssen & Van Wilgenburg, 1978) from the amount of KOH or HCl needed to bring the pH back to its original value. The pH-stat experiments were done at 25 °C.

The experiments were carried out at pH 6.4 or at pH 9.2, since at these pH values the albumin exists mainly in one conformational state (N at pH 6.4 and B at pH 9.2).

RESULTS AND DISCUSSION

pH-stat experiments

The difference between the protonic charge of the albumin in the presence and in the absence of oleic acid (ΔZ_3) was measured by using a pH-stat technique. Positive ΔZ_3 values indicate that the pK values in protein and/or fatty acid shift to higher values when oleic acid is added, whereas negative ΔZ_3 values indicate a shift to lower values (Janssen *et al.*, 1970; Janssen & Van Wilgenburg, 1978; Dröge *et al.*, 1983).

Fig. 1 shows the relation between ΔZ_3 and the molar ratio of oleic acid to albumin (r_F) at pH 6.4 and 9.2. By using the binding parameters for the binding of oleic acid to albumin calculated by Goodman (1958), it can be demonstrated that, between $r_F = 0$ and $r_F = 9$, the value of r_F is approximately equal, within 10 %, to the number of oleic acid molecules bound to one albumin molecule (ν_F). This means that Fig. 1 can also be seen as the relationship between ΔZ_3 and ν_F . This Figure shows that ΔZ_3 varies considerably with r_F . Moreover, there is a remarkable difference between the ΔZ_3 -versus- r_F relationships at pH 6.4 and 9.2.

As was stated above, ΔZ_3 is related to pK changes in the protein and/or the ligand. If the negatively charged carboxy group of oleic acid interacts with some positively charged groups of albumin, it is reasonable to assume that, when oleic acid binds to albumin, the pK of oleic acid will decrease and the pK of any amino acid residue in the albumin will increase. Since the pK of oleic acid is 4.8 (Simpson *et al.*, 1974), such a pK shift in oleic acid will not be apparent in the ΔZ_3 -versus- r_F relationship at pH 9.2 and will make only a negligible contribution to

Fig. 1. Difference between the protonic charge of albumin in the presence and absence of oleic acid (ΔZ_3) as a function of the molar ratio of oleic acid to albumin (r_F)

○, pH 6.4; ●, pH 9.2. Results from duplicate experiments are shown.

the ΔZ_3 -versus- r_F relationship at pH 6.4. Therefore ΔZ_3 as given in Fig. 1 must be caused by pK shifts in the amino acid residues in the albumin molecule. It should be noted that the binding of each molecule of oleic acid can be accompanied by a pK shift in more than one residue. Moreover, these pK shifts can be different in both sign and magnitude for each molecule of oleic acid. Since no linear relationship is observed between ΔZ_3 and r_F at pH 6.4 or at pH 9.2, it can be concluded that (a) there must be more than one class of binding sites for oleic acid and (b) binding of oleic acid to one of these classes induced pK shifts in the albumin different from the pK shift induced by binding of oleic acid to any other class.

It has been suggested in the literature (Spector, 1975) that the ϵ -amino groups of lysine residues are involved in the electrostatic part of the interaction between fatty acid anions and albumin. Since these amino acid residues have pK values of about 10, increases in the pK of lysine side chains would have too small an effect on the uptake of protons to explain the observed ΔZ_3 values. However, in addition to causing pK shifts due to salt-bridge formation between the ligand and the albumin, the ligand can also cause pK shifts in the albumin by changing its conformation. Depending on the pH, albumin will be in either the N or the B conformation (Leonard *et al.*, 1963; Harmsen *et al.*, 1971; Wilting *et al.*, 1980a). Since the pK of some histidine residues in the N conformation is higher than the pK of those in the B conformation (Harmsen *et al.*, 1971; Janssen *et al.*, 1981; Dröge *et al.*, 1983), an oleic acid-induced shift of the N-B transition should lead to pK shifts. For instance, the negative value of ΔZ_3 at pH 6.4 can be explained by assuming that, owing to the presence of oleic acid, a larger fraction of albumin occurs in the B conformation. However, in order to explain the positive value of ΔZ_3 observed at pH 9.2, one has to assume that the albumin has shifted to the N conformation. If this is the

Fig. 2. Relationship between molar ellipticity $[\theta]$ of some ligand-albumin complexes and the molar ratio of oleic acid to albumin (r_F)

The ligands used were diazepam [pH 6.4 (□) and pH 9.2 (■)] and oxyphenbutazone [pH 6.4 (○) and pH 9.2 (●)]. The drawn lines are the relationships calculated on the basis of eqn. (3).

mechanism involved here, it is still difficult to explain the large positive pK shifts at pH 6.4 when r_F values are larger than 7.

Another striking feature of Fig. 1 is that both at pH 6.4 and at pH 9.2 the ΔZ_3 -versus- r_F relationship can be split up into three parts at about $r_F = 2$ and $r_F = 7$. It is striking that this classification is very similar to the classification given by Goodman (1958), namely two high-affinity sites, five medium-affinity sites and about 20 low-affinity sites.

C.d. experiments

Fig. 2 shows the molar ellipticity ($[\theta]$) of the diazepam-albumin and the oxyphenbutazone-albumin complex at pH 6.4 and pH 9.2 as a function of r_F . Note that the c.d. experiments were carried out with albumin samples labelled with a marker molecule. We chose to use diazepam, which binds to site II, and oxyphenbutazone, which binds to site I. It can be seen from Fig. 2 that the r_F dependence of $[\theta]$ of the diazepam-albumin complexes at pH 9.2 can be split up into three or four parts at $r_F = 3$, $r_F = 7$ and $r_F = 9$ or 10. All four molar ellipticities of the fatty acid-albumin complexes as depicted in Fig. 2 show that $[\theta]$ is independent of r_F at r_F values larger than 10. This means that, at r_F values larger than 10, i.e. ν_F values larger than 9, the bound oleic acid probably no longer changes the conformation of the albumin. Therefore the various stages of the r_F -dependence of $[\theta]$ of the diazepam-albumin complex at pH 9.2 point to three or four conformations. This conclusion can also be drawn from the ΔZ_3 -versus- r_F curves. Similarly, the split into three or four parts is in agreement with the classification of the oleic acid-binding sites proposed by Goodman (1958).

On the basis of these findings we can now try and explain the profiles observed in Fig. 2. The most simple

explanation is that $[\theta]$ is an additive parameter, the value of which changes in accordance with the successive binding of fatty acids to the several classes of binding sites. This can be represented by the following equation:

$$[\theta] = a_0 + a_1\nu_1 + a_2\nu_2 + a_3\nu_3 \quad (3)$$

where ν_1 , ν_2 and ν_3 stand for ν_F of the high-affinity, medium-affinity and low-affinity sites respectively, and a_0 , a_1 , a_2 and a_3 are constants dependent on the molar ellipticities of the fatty acid-albumin complexes. It should be noted that, in eqn. (3), it is assumed that the binding of oleic acid to albumin is very co-operative within each class of binding sites or that within each class of binding sites the binding of each molecule of oleic acid contributes equally to $[\theta]$. Since Ashbrook *et al.* (1975) proved that the binding of oleic acid to albumin does not have a co-operative character, the latter assumption is probably more correct.

With help of the data for the interaction of oleic acid with albumin obtained by Goodman (1958), we calculated ν_1 , ν_2 and ν_3 for every r_F value. Because of the r_F independence of $[\theta]$ at $\nu_F > 9$, a value of 2 was chosen for ν_3 . The four experimental curves of Fig. 2 were then fitted to the equation. It can be seen from this Figure that, at both pH 6.4 and 9.2, the fits made according to eqn. (3) are remarkably good, especially for the diazepam-albumin complex. The fits for the oxyphenbutazone-albumin complex are somewhat less satisfactory. If the medium-affinity sites of oleic acid are split up, the fits for the oxyphenbutazone-albumin complex at pH 6.4 and at pH 9.2 improve considerably. This means that there might be more than three N and B conformations.

The combined data obtained by means of a pH-stat technique and c.d. experiments point to the existence of oleic acid-induced conformational changes in albumin. The conformational changes observed by these methods are probably the same as those recently described as 'N*' and 'B*' (Wanwimolruk & Birkett, 1982; Dröge *et al.*, 1985). The existence of these conformational changes was deduced from experiments which are described by step 2 in Scheme 1; the experiments discussed here, however, are based on step 3 in Scheme 1. The present results also suggest that the course of these N-N* and B-B* transitions is determined by the classification of the oleic acid-binding sites proposed by Goodman (1958).

REFERENCES

- Ashbrook, D. J., Spector, A. A., Santos, E. C. & Fletcher, J. E. (1975) *J. Biol. Chem.* **250**, 2333-2338
- Brodersen, R., Sjödin, T. & Sjöholm, I. (1977) *J. Biol. Chem.* **252**, 5067-5072
- Dröge, J. H. M., Janssen, L. H. M. & Wilting, J. (1982) *Biochem. Pharmacol.* **31**, 3775-3779
- Dröge, J. H. M., Janssen, L. H. M. & Wilting, J. (1983) *Pharm. Weekbl. ('scientific edition')* **5**, 228-233
- Dröge, J. H. M., Janssen, L. H. M. & Wilting, J. (1985) *Biochem. Pharmacol.* **34**, 3299-3304
- Fehske, K. J., Müller, W. E. & Wollert, U. (1979) *Biochim. Biophys. Acta* **577**, 346-359
- Fehske, K. J., Müller, W. E. & Wollert, U. (1981) *Biochem. Pharmacol.* **30**, 687-692
- Goodman, D. S. (1958) *J. Am. Chem. Soc.* **80**, 3892-3898
- Harmsen, B. J. M., De Bruin, S. H., Janssen, L. H. M., Rodrigues de Miranda, J. F. & Van Os, G. A. J. (1971) *Biochemistry* **10**, 3217-3221
- Janssen, L. H. M. & Van Wilgenburg, M. T. (1978) *Mol. Pharmacol.* **14**, 884-889
- Janssen, L. H. M., De Bruin, S. H. & Van Os, G. A. J. (1970) *Biochim. Biophys. Acta* **221**, 214-227
- Janssen, L. H. M., Van Wilgenburg, M. T. & Wilting, J. (1981) *Biochim. Biophys. Acta* **669**, 244-250
- Kragh-Hansen, U. (1981) *Pharmacol. Rev.* **33**, 17-53
- Leonard, W. J., Jr., Vijai, K. K. & Foster, J. F. (1963) *J. Biol. Chem.* **238**, 1984-1988
- Müller, W. E. & Wollert, U. (1979) *Pharmacology* **19**, 59-67
- Simpson, R. B., Ashbrook, J. D., Santos, E. C. & Spector, A. A. (1974) *J. Lipid Res.* **15**, 415-422
- Sjöholm, I., Ekman, B., Kober, A., Ljungstedt-Påhlman, I., Serving, B. & Sjödin, T. (1979) *Mol. Pharmacol.* **16**, 767-777
- Spector, A. A. (1975) *J. Lipid Res.* **16**, 165-179
- Sudlow, G., Birkett, D. H. & Wade, D. N. (1976) *Mol. Pharmacol.* **12**, 1052-1061
- Vallner, J. J. (1977) *J. Pharm. Sci.* **66**, 447-465
- Wanwimolruk, S. & Birkett, D. J. (1982) *Biochim. Biophys. Acta* **709**, 247-255
- Wilting, J., Van Der Giesen, W. F., Janssen, L. H. M., Weideman, M. M., Otagiri, M. & Perrin, J. H. (1980a) *J. Biol. Chem.* **255**, 3032-3037
- Wilting, J., 't Hart, B. J. & De Gier, J. J. (1980b) *Biochim. Biophys. Acta* **626**, 291-298
- Wilting, J., Van Der Giesen, W. F. & Janssen, L. H. M. (1981) *Biochem. Pharmacol.* **30**, 1025-1031
- Wyman, J., Jr. (1964) *Adv. Protein Chem.* **19**, 223-286