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COOPERATIVITY OF WARFARIN BINDING WITH HUMAN SERUM ALBUMIN INDUCED BY FREE FATTY ACID ANION*

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Abstract—The effects of oleate ion, a free fatty acid anion, on the binding characteristics of warfarin with human serum albumin have been examined using fluorescence spectroscopy. The affinity constant of the warfarin–albumin complex was found to be increased without affecting the number of warfarin binding sites in the presence of a low molar ratio (~3) of oleate ion and serum albumin, irrespective of the concentrations of the albumin used (5–200 μM) in the study. These findings indicate the presence of a cooperative (allosteric) interaction between warfarin and the oleate ion for albumin binding and, further, suggest a conformational transition in the albumin molecule as a result of such interactions. However, the results of the polarization of fluorescence of warfarin-bound albumin indicate that such cooperative effects might not result from a conformational change of the local warfarin binding sites. On the other hand, in concentrated albumin (400–600 μM) or at a molar ratio of oleate ion to albumin of greater than 5, such cooperative interactions disappear and a simple competitive inhibition of the warfarin binding results. Correlations of these results with some clinical situations have been made.

The effects of free fatty acids (FFA) on the cooperative binding behavior of warfarin to bovine serum albumin (BSA) were reported by Chakrabarti *et al.* [1]. The results of such studies have shown that in a dilute solution of serum albumin (55 μM or less) the binding affinity of the warfarin–albumin complex was increased in the presence of 100 μM (or less) either of lauric or oleic acid or a mixture of these fatty acids, but higher concentrations of these fatty acids decrease such binding. Such a dualistic behavior of FFA was attributed to the cooperative (allosteric) interactions involving the fatty acid and warfarin for the albumin binding. On the other hand, the affinity constant of warfarin for the concentrated solution of serum albumin (~580 μM) was decreased in the presence of both low and high concentrations of FFA. In order to determine if such cooperative interactions might occur in other types of albumin as well, the present experiments were carried out, studying the binding characteristics of warfarin with various concentrations of human serum albumin (HSA) in the presence of oleic acid. Oleic acid was chosen as a representative long chain fatty acid in the present investigation for two reasons. First, it is one of the two long chain fatty acids which is present in a significant amount in mammalian plasma. Secondly, the relatively high solubility of the oleic acid anion makes it possible to use various concentrations.

Although dialysis and ultrafiltration methods are extensively used for the study of drug–protein binding, with some advantage, there are experimental problems associated with the use of membrane filters for detecting small amounts of free drugs such as leakage of protein through the filters and absorption of free drugs by the membranes. Since large changes in the fluorescence intensity of serum albumins are associated with warfarin binding [1–5], it is possible to determine the degree of binding saturation fluorometrically. In the present work, the direct binding titrations involving the fluorometric method were employed, therefore, to study the equilibrium reaction between HSA and warfarin. Such a fluorometric technique has already been used by other workers [2–4] to measure warfarin binding.

MATERIALS AND METHODS

Human serum albumin of a fatty acid free grade (<0.005%) was obtained from the Sigma Chemical Co., St. Louis, MO. The concentration of albumin was determined using the extinction coefficient of $E^{1\%}_{1cm} = 5.3$ at 280 nm [6]. Warfarin sodium of U.S.P. grade was used without further purification. Warfarin concentrations were measured with an absorption spectrophotometer (Unicam SP8000), using an extinction coefficient of $1.39 \times 10^4\,M^{-1}\,cm^{-1}$ at 308 nm [2]. The sodium salt of oleic acid was purchased from Sigma Chemical Co., St. Louis, MO, and was used without further purification. All solutions were made up in sodium phosphate buffer (0.05 M, pH 7.4) except for that of sodium oleate which was made in distilled water initially.

All fluorescence measurements were made at 22° in a Perkin–Elmer MPF-3A spectrophotofluorometer. The binding of warfarin to serum albumin was measured by the fluorescence titration method with an exciting wavelength of 320 nm and an emission wavelength of 380 nm. Titrations with warfarin were

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performed by the manual addition of a small volume (μl) of a concentrated warfarin solution to a standard solution of albumin in a 1-cm quartz cuvet. The binding constants for the albumin with warfarin were determined according to the equation developed by Klotz *et al.* [7]

$$\frac{1}{r} = \frac{K_d}{n}\frac{1}{(1-x)D} + \frac{1}{n} \qquad (1)$$

where r is the moles of warfarin bound per mole of protein, n is the number of binding sites per protein molecule, K_d is the apparent dissociation constant, D is the total warfarin concentration, and x is the fraction of warfarin bound. A plot of $1/r$ vs $1/(1-x)D$ will produce a straight line. All the experimental data were fitted to regression lines. From the slope and intercept, the values of K_d and n can be determined. For each concentration of warfarin, x was determined from the relation of x as the ratio $(F - F_1)/(F_0 - F_i)$, where F is the observed fluorescence, corrected for protein, F_i is the fluorescence of free warfarin at a given concentration of warfarin, and F_0 is the fluorescence intensity due to complete binding of the warfarin to the protein, determined by measuring the fluorescence of a constant amount of warfarin in the presence of varying amounts of albumin and calculating the fluorescence on extrapolation to infinitely high protein concentration. These experiments were done both in the absence and presence of sodium oleate. The value of F_0 was measured separately for each HSA–oleic acid system also. Intensities of fluorescence were corrected for self-absorption and dilution wherever necessary.

This approach requires three assumptions: (i) that all binding sites are identical in drug-binding properties, (ii) that all sites are independent, and (iii) that the fluorescence quantum yield of bound warfarin is the same at all sites.

Two film-type polarizers were used to measure the polarization of fluorescence of warfarin bound to albumin. The degree of polarization, P, was calculated as:

$$P = \frac{I_{\parallel} - fI_{\perp}}{I_{\parallel} + fI_{\perp}} = \frac{I_{EE} - I_{EB}(I_{BE}/I_{BB})}{I_{EE} + I_{EB}(I_{BE}/I_{BB})} \qquad (2)$$

where $I_{\parallel}$ or I_{EE} and $I_{\perp}$ or I_{EB} refer to the intensity of the fluorescence light polarized parallel ($\parallel$) and perpendicular ($\perp$) to the exciting light, and f or I_{BE}/I_{BB} is a correction factor which accounts for the selective transmission of the monochromators and the selective reflection of the sample cuvettes.

RESULTS

Effects of oleate ion on the fluorescence spectra of the warfarin–HSA complex. When warfarin was bound to the albumin, the fluorescence peak intensity of warfarin was considerably enhanced and the fluorescence peak of warfarin was shifted from 388 to 380 nm, indicating the formation of a warfarin–albumin complex. Furthermore, the position of the fluorescence peak as well as the shape of the fluorescence spectra due to the warfarin–albumin complex was not affected by the presence of the oleate ion except for a change in the peak intensity. The changes in the fluorescence peak intensity of the warfarin–albumin complex on gradual addition of increments of oleate ion to various solutions of the complex, each containing equimolar amounts of warfarin and albumin, are shown in Fig. 1. It is seen from the Figure that the fluorescence intensity initially increased with the first few additions of oleate ion and reached a maximum at a molar ratio of oleate ion and albumin of about 3.5, independently of the actual amounts of warfarin and albumin used. On the other hand, on increasing further the amounts of oleate ion, corresponding to a molar ratio of oleate ion and albumin of greater than 3.5, the fluorescence peak intensity of the warfarin–albumin complex decreased and finally reached a steady value. This phenomenon is the same as that observed in our previous works [1] with BSA.

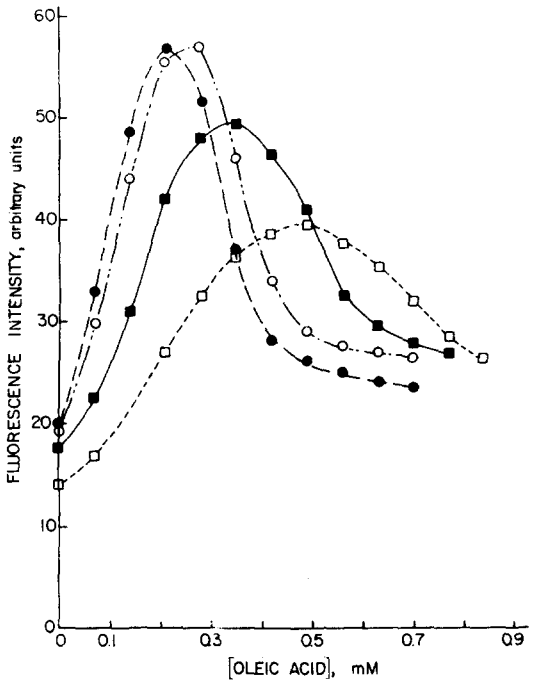

Fig. 1. Effects of oleate ion on the fluorescence peak intensity of the warfarin–human serum albumin (HSA) complex containing equimolar amounts of HSA and warfarin in phosphate buffer, pH 7.4, at 22°. Different solutions are: 60 μM HSA–60 μM warfarin (●), 80 μM HSA–80 μM warfarin (○), 100 μM HSA–100 μM warfarin (■), and 140 μM HSA–140 μM warfarin (□). Excitation and emission wavelengths are 320 and 380 nm respectively.

Binding constant and stoichiometry of the warfarin–albumin complex. The results in Fig. 1 suggest that the binding affinity of warfarin for HSA varies with the molar ratio of oleate ion and albumin. To look for such a possibility, the apparent binding constant was determined by the Klotz plot (eqn. 1) as obtained by the fluorescence titration technique. The results are summarized in Table 1 for the various concentrations of HSA used. A typical Klotz plot for the binding of warfarin to HSA at 22° is illustrated in Fig. 2. It is apparent from the table that the apparent dissociation constant of the albumin for warfarin depends on the concentration of albumin used, but there seems to be no direct correlation of the dissociation constant with the concentration of albumin

Table 1. Effects of sodium oleate on the binding of warfarin to human serum albumin at 22°C*

Serum albumin (μM)	Oleate ion (μM)	K_d† (μM ± S. E. M.)	K_d‡ (μM ± S. E. M.)	n
5	0	12.3 ± 0.6	14.6 ± 0.9	1.3
5	15	4.5 ± 0.2	6.7 ± 0.3	1.1
5	45	31.7 ± 1.2	29.1 ± 1.8	1.3
36	0	3.07 ± 0.3	2.5 ± 0.3	1.1
36	108	0.61 ± 0.02	0.9 ± 0.10	0.75
36	295	10.7 ± 0.9	15.2 ± 1.1	1.07
60	0	21.4 ± 2.8		1.2
60	180	5.5 ± 0.2		0.99
60	492	97.0 ± 6.2		1.17
120	0	33.8 ± 2.1	21.6 ± 3.0	0.99
120	360	14.2 ± 0.4	11.8 ± 1.1	0.92
120	965	58.0 ± 4.3	47.5 ± 3.5	1.05
200	0	45.6 ± 2.3		0.85
200	600	23.7 ± 1.5		0.68
200	1650	161.0 ± 5.4		0.83
400	0	39.0 ± 1.9	23.1 ± 2.20	0.90
400	1200	36.5 ± 0.8	20.5 ± 1.8	0.82
400	2800	182.0 ± 6.0	112.0 ± 5.0	0.98

* Results are the average of three sets of experiments. Total concentration of warfarin used is twice the concentration of the corresponding albumin in each case.

† K_d is the apparent dissociation constant of the warfarin–albumin complex for the warfarin-binding sites. n. K_d values are obtained from linear regression lines with correlation coefficients in the range of 0.92 to 0.99.

‡ Determined by ultrafiltration.

used. This probably indicates that the drug–protein binding depends not only on the protein concentrations but also on other factors such as conformational and/or subunit interactions involving the protein as well. On the other hand, the contributions of the secondary (weaker) warfarin binding sites (although not possible to evaluate by the present fluorometric titration which only measures the strong warfarin binding sites) to the total warfarin binding might be significant at high drug and protein concentrations, as observed by other workers [8, 9]. It is evident from the Table, however, that warfarin binds to one strong site per HSA molecule independently of the concentration of HSA used. The values of K_d and n reported in the present paper are found to be of the same order of magnitude as those reported by other workers [8–12]. Furthermore, such stoichiometry of the warfarin–HSA binding was found not to be affected by the presence of oleate ion (Table 1).

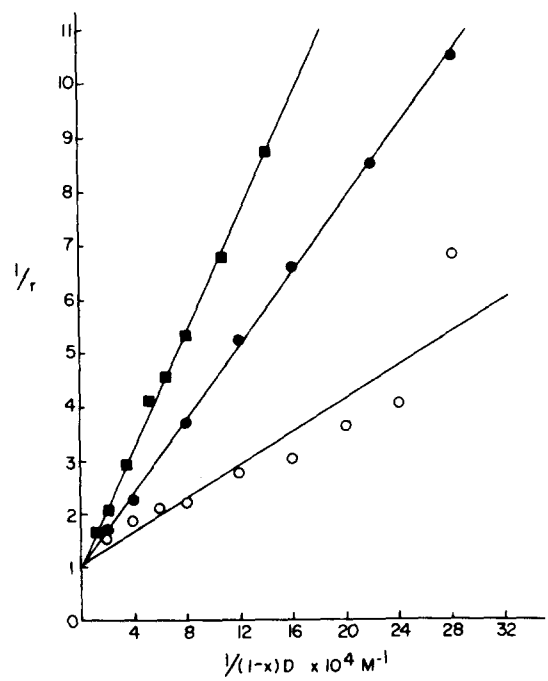

Fig. 2. Typical Klotz plot for warfarin binding to HSA (120 μM) in phosphate buffer, pH 7.4, at 22°, in the absence (●) and in the presence of low (○) (360 μM) and high (■) (965 μM) concentrations of oleate ion. Excitation and emission wavelengths are 320 and 380 nm respectively.

Table 1 further shows that, at a molar ratio of oleate ion to albumin corresponding to a value of 3, the binding affinity of warfarin for the albumin is always increased independently of the concentrations of albumin used in the present study. Some of the data when checked by the method of ultrafiltration gave rise to the same results as reported in the table, indicating the validity of such results. However, at much higher concentrations of HSA (400–600 μM), the binding constant of the warfarin–HSA complex was found to be unaffected by the presence of low concentrations of oleate ion corresponding to a molar ratio of oleate ion to albumin of about 3. On the other hand, at higher concentrations of oleate ion corresponding to a molar ratio of oleate ion to albumin of greater than 5, the binding constant of the complex was found to be always decreased independently of the amounts of HSA used. Some of these results are also presented in Table 1. This is in agreement with the works of Solomon *et al.* [10] using HSA, our previous works using BSA [1], as well as works by Rudman *et al.* [13].

Since warfarin U.S.P. is a racemic mixture, the binding of the two forms might be different. But in the absence of definite data in the literature, such a differential effect is yet to be recognized.

Studies of the conformational changes. Since the results in Fig. 1 might indicate a possibility of oleate ion-induced conformational changes of the warfarin-binding sites within HSA, as observed in our previous work [1], the polarization of fluorescence of the warfarin-bound albumin at its peak fluorescence intensity was measured, in the absence and presence of different amounts of oleate ion using the same experiments as presented in Fig. 1. Such measurements monitor conformational changes which may be induced by oleate ion in the environment near the protein-bound warfarin (where warfarin is used as a fluorophore). However, only a small but insignificant change in the polarization of fluorescence (from 0.27 in the control to 0.25 in the presence of oleate ion) of the albumin-bound warfarin was noticed in some cases, when the molar ratio of oleate ion and albumin was about 3.

DISCUSSION

The present results clearly demonstrate that a low molar ratio of oleate ion to albumin favors a cooperativity of the warfarin binding with human serum albumin, provided the albumin solutions are not too concentrated. This is in agreement with our previous works [1] with bovine serum albumin. The phenomenon of cooperativity of binding of human serum albumin with warfarin, although presently reported with oleate ion, is also realized in the presence of low concentrations of other fatty acid anions such as those of lauric, linoleic, and palmitic acids as well.

In spite of the realization that manifestation of cooperative behavior requires the presence of free fatty acid, the exact mechanism of the cooperativity of the warfarin binding is rather complex when one considers that such a phenomenon disappears in concentrated albumin solutions (400 μM or higher).

Human serum albumin is known [14] to possess at least two kinds of sites capable of binding hydrophobic structures. The primary or strong sites are responsible for the specific binding of free fatty acids and the secondary sites are specific for substrates or other organic ligands or drugs or free fatty acids. It is also known [14] that the binding affinities of free fatty acids for the albumin with regard to strong binding sites are much higher (100–1000 times) than those of most of the drugs. When a fatty acid binds to its strong sites in the defatted albumin, it changes the conformation of the albumin molecule [15–17] leading to formation of more stable hydrophobic regions due to certain rearrangements of the native structure. It has further been demonstrated [18] that the configuration and stability of the protein depend upon the presence of at least a molecule of fatty acid. A marked increase as well as a blue shift in the fluorescence maximum of warfarin on complexation with albumin (from 388 to 380 nm) strongly indicates the hydrophobic character of the warfarin-binding sites in the serum albumin. Since the fluorescence quantum yield of warfarin bound to the albumin is considerably higher than that of free warfarin, and is further enhanced by the addition of low concentrations of the oleate ion, it is quite possible that the oleate ion produces a change in the environment of the albumin-bound warfarin caused by a change of the protein conformation. However, since the polarization of fluorescence of the albumin-bound warfarin is not affected by the presence of oleate ion, it might indicate that the conformation of the local environment involving warfarin-binding sites is not altered. This is contrary to the observations reported in our previous works with bovine serum albumin [1]. However, the present results do not exclude the possibility of a gross conformational change of the albumin molecule induced by fatty acids [15, 16, 19].

Works by Shaklai *et al.* [20] have demonstrated that the binding of oxygen to haemocyanin (which has only one strong oxygen binding site) from *Levantian Lierosolima* was cooperative in the presence of calcium ions, even at the level of a subunit. Such a phenomenon has been explained by these authors as due to an interaction between oxygen and calcium ions and not directly related to the changes in the subunit association–dissociation equilibrium.

The currently available binding data suggest [21, 22] that the polypeptide chain of human serum albumin is folded into three or perhaps four globular regions. Although these globular regions are joined by peptide linkages, the molecule can in a sense be considered as consisting of subunits. It has also been shown that the interhelical attraction for a considerable portion of the albumin molecule is relatively weak [23, 24]. Such low interhelical attractions permit the R substituents of the amino acid residues to assume various orientations relative to each other. These arrangements could provide combining regions which can take a variety of configurations. The binding of a few molecules of oleic acid (at low concentrations of oleic acid) to these regions occurs within the albumin molecule and could cause further separation of the helices (when the albumin solution is either dilute or not too concentrated), a process which might facilitate the binding of warfarin by an indirect interaction and thus result in a positive cooperativity of warfarin binding. However, in the presence of high concentrations of oleic acid, a rather direct interference between the acid and warfarin could be possible, resulting in a negative cooperativity of such binding. The generation of cooperativity (positive/negative) through the interactions between two ligands has been treated by Wyman [25]. On the other hand, at higher concentrations of serum albumin, the system will consist of dimers and polymers in equilibrium. In that case, such positive cooperative interactions between oleic acid and warfarin, although possible, will probably be masked by the specific associations between dimeric and polymeric albumin molecules resulting in certain rearrangements of the tertiary structures of the monomers and, hence, resulting in a loss of positive cooperativity. Alternatively, the spatial geometry or the molecular symmetry of the dimeric and/or polymeric albumin molecules is such that the allosteric ligand (oleic acid) could be in closer proximity to the warfarin-binding site due to a probable significant dimer–polymer and/or site–site interaction resulting eventually in a rather direct interference between distinct but different binding sites. Consequently, a negative cooperativity or competitive in-

hibition of warfarin binding occurs in the presence of high concentrations of the albumin and moderate/high concentrations of oleic acid. The effect of fatty acids on the binding of benzodiazepines to bovine serum albumin has recently been studied by circular dichroism (CD). The induced CD spectra attributed to the benzodiazepines–bovine serum albumin complexes were modified upon addition of the fatty acids in a manner which suggested to the authors a conformation rather than a displacement phenomenon [26, 27] in agreement with our previous works [1] with warfarin.

The reasons why the polarization of fluorescence of warfarin bound to HSA remained unchanged in the present study whereas that with BSA was affected by the presence of oleate ion [1] could be traced to the works by Steinhardt *et al.* [28]. These authors showed that the two serum albumins differ quantitatively in their binding behavior and in the way in which complexing affects their conformations, as indicated by optical rotatory dispersion, viscosity and accessibility of prototropic groups. Their fluorescence data showed that there are large differences in the immediate environment of tryptophan residues of the two proteins. Although the amino acid compositions of bovine and human serum albumins are closely similar (except for extra tryptophan in BSA), the amino acid sequences in the neighborhood of tryptophan differ. HSA appears to unfold in either one stage or in an essentially continuous process, while BSA has been shown to exist in two distinguishable unfolded states [28].

Although direct demonstrations of clinically important cooperative drug–protein interactions involving endogenous substances are lacking at the present time, there are some clinical situations where such cooperative drug–protein interactions induced by endogenous substances seem to be present. Thus, Nilsen *et al.* [29] found an increased binding of quinidine to serum albumin in anuric rats. These authors believed that conformation changes might be induced in albumin by strongly bound endogenous substances accumulating during anuria. However, the increased binding of quinidine does not seem to be directly related to the different stages of uremia, since uremia develops over 48 hr, while binding returns to nearly base level between 30 and 48 hr. This was explained by these authors as first due to increased binding induced by endogenous substances below a certain concentration range and then, inhibition at higher concentrations. Second, Dromgoole [30] while studying the effect of hemodialysis on the binding capacity of albumin using anephric patients has shown that the binding capacities of methyl orange of both the pre- and post-dialysis sera were much lower than normal. Defatting of this post-dialysis serum to reduce its nonesterified fatty acids to within the normal range markedly increased its binding capacity to slightly above that of the normal pool. Third, while studying the uptake of bilirubin by human erythrocytes, Bratlid [31] observed that, when the bilirubin/human albumin ratios were below unity, oleate ion was found to increase cellular binding of bilirubin when the molar ratio of free fatty acid to albumin exceeded 6. With bilirubin–albumin ratios above unity, however, increased amounts of free fatty acid reduced the cellular binding of bilirubin.

The maximum concentrations of HSA used in the present study showing cooperativity of the warfarin–HSA binding by oleate ion correspond closely to the physiological concentrations of albumin present in human plasma in certain diseased states [32]. The present study, combined with the results of some clinical situations mentioned above, suggests the importance of the role of endogenous mediators such as fatty acids in drug–protein binding.

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