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LOCATION AND CHARACTERIZATION OF THE WARFARIN BINDING SITE OF HUMAN SERUM ALBUMIN

A COMPARATIVE STUDY OF TWO LARGE FRAGMENTS

OCTAAF J. M. BOS,* JOBINA P. M. REMIJN, MARCEL J. E. FISCHER, JAAP WILTING and LAMBERT H. M. JANSSEN

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

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Abstract—The warfarin binding behaviour of a large tryptic fragment (residues 198–585 which comprise domains two and three) and of a large peptic fragment (residues 1–387 which comprise domains one and two) of human serum albumin has been studied by circular dichroism and equilibrium dialysis in order to locate and characterize the primary warfarin binding site.

The induced ellipticity of the warfarin–peptic fragment complex turned out to be pH dependent. This pH dependence occurs in the region of the neutral-to-base transition of the albumin molecule. The induced ellipticity of the warfarin-tryptic fragment complex is pH independent. Difference CD-spectra showed that the peptic fragment and albumin have similar warfarin binding properties whereas the tryptic fragment has deviant warfarin binding properties. The equilibrium dialysis experiments showed that the affinity of warfarin to the peptic fragment and to albumin is practically the same whereas the affinity of warfarin to the tryptic fragment is a factor 2–8 lower than the affinity of warfarin to albumin. Our results indicate that the main part of the primary warfarin binding site is located in domain two of the albumin structure and that domain one plays an important role in the N–B transition of albumin.

Many endogenous and exogenous compounds reversibly bind to albumin,† which functions as a transport and depot protein in the blood circulation [1–5]. At therapeutic drug concentrations usually only a small number of the available binding sites on albumin are occupied. Most drugs bind to one of the specific high affinity binding sites on this protein. The best characterized drug binding sites are the warfarin site and the diazepam site, designated as site I and site II respectively by Sudlow *et al.* [6, 7]. Site I is shared by coumarin anticoagulants, sulphonamides, phenylbutazon and related compounds. It actually consists of two partly overlapping binding sites for warfarin and azapropazone respectively [8]. There is much evidence pointing to the participation of the lone tryptophan residue, Trp 214, in the non-overlapping part of the warfarin site [8]. The main part of site II (the binding site of e.g. benzodiazepines and tryptophan) is in all probability located in domain three of the albumin structure [3, 9]. However, it should be stressed that there are still many uncertainties regarding the exact location of the binding sites.

The binding of warfarin and diazepam to albumin was found to be very sensitive to the ionic composition and the pH of the solution. This sensitivity around physiological pH has been attributed partly to a conformational change in albumin, namely the neutral-to-base or N–B transition [10–18]. At pH 6 albumin is almost completely in the N conformation whereas at pH 9 the B conformation is predominant.

Albumin consists of one single peptide chain of 585 amino acid residues. Disulphide bridges force the albumin molecule into 9 loops. These loops form three more or less independent domains linked together by small peptide chains [3, 4, 19, 20]. These small peptide chains are relatively easily accessible for enzymes such as pepsin and trypsin. Therefore we were able to isolate a 46 kDa peptic fragment (domains one and two) and a 45 kDa tryptic fragment (domains two and three) of albumin [9, 21]. We did this in order to obtain more information about the molecular mechanism of the N–B transition and the location of the drug binding sites.

In this paper, we report on our studies with warfarin as a site I marker. The binding of warfarin to the fragments was investigated as a function of the pH by means of equilibrium dialysis and circular dichroism.

MATERIALS AND METHODS

Reagents. Warfarin (Sigma Chemical Company, St Louis, MO) and [^{14}C]warfarin (55 mCi/mmol, Amersham, Nederland BV, Utrecht, The Netherlands) were used without further purification. Q-Sepharose Fast Flow, Polybuffer exchanger 94 (PBE-94) and Polybuffer-74 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Trypsin (from bovine pancreas) was purchased from Sigma Chemical Company (St Louis, MO). Pepsin (from pig stomach mucosa) and trypsin-inhibitor (from hen egg-white) were purchased from Boehringer Mannheim GmbH (F.R.G.). All other chemicals

* To whom all correspondence should be addressed.

† Abbreviations used: By albumin we mean human serum albumin unless otherwise stated; T45 = the 45 kDa tryptic fragment; P46, the 46 kDa peptic fragment.

were of analytical grade (J. T. Baker, Deventer, The Netherlands; Janssen Chimica, Beerse, Belgium or Merck, Darmstadt, F.R.G.).

Human serum albumin. Albumin was isolated from human plasma according to the method described by Kremer [22]. The albumin was deionized before use by passing the protein solution over a mixed-bed ion exchange column (Amberlite IRA 400 and IR 120, volume ration 2:1) [23]. Albumin from one batch was used for all our experiments. The relative molecular weight of albumin was assumed to be 66,500 (in accordance with the literature [3, 11]). The extinction coefficient ($E^{1\%}_{1cm}$) at 278 nm was found to be 5.8. Protein concentrations were determined as described elsewhere [9, 21].

Isolation of the 46 kDa peptic fragment. The 46 kDa peptic fragment (the P46 fragment) was obtained after albumin had been treated with pepsin as described previously [21]. After isolation, the P46 fragment was immediately deionized and used for further experiments. For the calculations, we used a relative molecular weight of 46,000 and an extinction coefficient of 6.0 at 278 nm. The fragment represents residues 1–387 and therefore comprises domains one and two of the albumin structure.

Isolation of the 45 kDa tryptic fragment. Trypsin digestion was performed as described recently [9]. A three-step isolation procedure for the T45 fragment was described in the same manuscript. For the experiments reported in this paper, we made use of an improved two-step isolation procedure.

The solution obtained after the trypsin digestion was concentrated to 10–15 ml by ultrafiltration in an Amicon stirred cell equipped with a PM-10 filter. The concentrated protein solution was diluted 100 times in 20 mM Tris–HCl pH 7.9, concentrated to 10 ml by ultrafiltration and applied to a Q-Sepharose Fast Flow column (2.6 × 30 cm) equilibrated with a 20 mM Tris–HCl buffer pH 7.9. The applied digest solution was eluted with a 0.12–0.28 M NaCl gradient in the same buffer; the gradient had a total volume of 1000 ml. The T45 fragment was eluted from the column when the gradient reached about 0.2 M NaCl. The fractions containing the T45 fragment were pooled, diluted 20 times in 25 mM bis-Tris–HCl buffer pH 5.9 and concentrated to 5–10 ml by ultrafiltration. These last two steps were repeated once and the concentrated material obtained was applied to a PBE-94 column (1.6 × 30 cm) equilibrated with 25 mM bis-Tris–HCl pH 6.0. The proteins were eluted with 550 ml of 10 times diluted polybuffer-74 pH 4.6. The T45 fragment was found to be eluted from the column when the pH gradient reached a pH of about 5.2. The fractions containing the T45 fragment were pooled and concentrated to about 4 ml by ultrafiltration. After deionization, the obtained fragment solution was immediately used for further experiments. For the calculations, a relative molecular weight of 45,000 and an extinction coefficient of 5.0 at 278 nm were used. The T45 fragment consists of domains two and three of the albumin structure (amino acid residues 198–585).

Circular dichroism (CD). Circular dichroic studies of the interaction of warfarin with albumin, of warfarin with the P46 fragment and of warfarin with the T45 fragment were performed using a Dichrograph III (Jobin Yvon, Longjumeau, France) as described recently [9]. CD-spectra were recorded between 300 and 370 nm and CD measurements as a function of the pH were performed at 310 nm. The protein concentrations were $6 \cdot 10^{-5}$ M and warfarin-to-protein ratios (r) of 0.4 were chosen because this was found to give suitable signal-to-noise ratios. The linearity of the observed ellipticity (θ_{obs}) as a function of r was checked from $r = 0$ to 0.7. It appeared that θ_{obs} increases linearly with the warfarin concentration. Because it is known that there is only one high affinity warfarin binding site present on the albumin molecule (with an affinity several orders of magnitude higher than the secondary binding sites) [11], this linearity indicates that it is mainly the high affinity binding site which is involved in the warfarin binding.

The observed ellipticities are the differences between the CD-spectra of the drug–protein complexes and the protein alone at a given wavelength. Molar ellipticities ($[\theta]$) were calculated using the equation [13]: $[\theta] = 100 \cdot \theta_{obs}/l \cdot c$ (l is the path length in cm and c is the concentration of the warfarin–protein complexes in dmol/ml). The concentrations of the warfarin–protein complexes were calculated from the results of the equilibrium dialysis experiments.

By plotting the molar ellipticity of the warfarin–protein complex vs pH, one can visualise the N–B transition. For convenience, transitions in the fragments around neutral pH are also referred to as N–B transitions, although we do not know whether the same conformational changes occur. The N–B transition is characterized by the midpoint pH of the pH dependency of the molar ellipticity (pH_{mid}) and by the Hill coefficient (HC). At the pH_{mid} equimolar concentrations of the N and B forms of the proteins are present. The HC is the slope of the log $(f/1-f)$ vs pH curve at the pH_{mid}, where f is the fraction of the protein in the B conformation. The fractions f were calculated from the curves of the molar ellipticity vs pH as described previously [10].

Equilibrium dialysis. The equilibrium dialysis experiments were performed as a function of the pH in order to locate the primary warfarin site and to investigate the warfarin binding characteristics of albumin, the P46 fragment and the T45 fragment. The experiments were carried out as described previously [11], using a Dianorm Equilibrium Dialyzer (Diachema A.G., Rüschlikon, Switzerland) equipped with cells consisting of two compartments each having a volume of 1 ml. The compartments were separated by dialysis membranes of hydrated cellulose (Diachema, type 10.14, mw. cut-off 5000). The dialysis time was 15–20 hr, the temperature was 25° and the rotation velocity was 8 rpm.

The concentrations of the solutions of albumin, the T45 fragment and the P46 fragment were $6 \cdot 10^{-5}$ M and the drug-to-protein ratios were 0.2. We chose a ratio of 0.2, because at that ratio the contribution of the secondary binding sites to the total binding process is almost negligible [11]. The protein solutions were buffered with phosphate or borate buffers, $I = 0.1$. The percentage of bound and free warfarin was determined by liquid scintillation counting (Packard Tricarb liquid scintillation

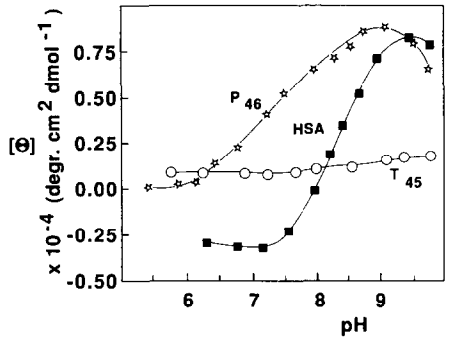

Fig. 1. CD measurements as function of the pH. The figure shows the molar ellipticity [θ] of the warfarin–T45 fragment complex (○), of the warfarin–P46 fragment complex (☆) and of the warfarin–albumin complex (■) as a function of the pH. The protein concentrations were $6 \cdot 10^{-5}$ M and warfarin was present in a drug-to-protein ratio of 0.4. Measurements were performed at 310 nm in a non-buffered system where the pH was adjusted with 0.1 N NaOH.

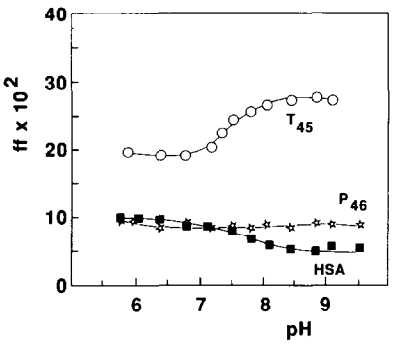

Fig. 3. Equilibrium dialysis experiments. The figure shows the percentage of unbound warfarin as a function of the pH in the case of the T45 fragment (○), the P46 fragment (☆) and albumin (■). Protein concentrations were $6 \cdot 10^{-5}$ M and warfarin was added in a drug-to-protein ratio of 0.2. The measurements were performed in phosphate or borate buffers, $I = 0.1$.

spectrometer, model A-4430). Since the drug-to-protein ratios are low, the affinity constants (K) can be calculated using [11, 18]:

$$K = \frac{[\text{warfarin–protein complex}]}{[\text{free warfarin}] \cdot [\text{free protein}]}.$$

RESULTS

Figure 1 shows the molar ellipticity [θ] of the three warfarin–protein complexes at 310 nm as a function of the pH. Because there are no changes known to occur in warfarin ($pK_a = 5.0$) in the pH region 6.0–9.0, the effect of the pH on [θ] must be due to a change in the protein structure [11]. With increasing pH, an increase in [θ] was observed for the warfarin–albumin and the warfarin–P46 fragment complexes. The warfarin–T45 fragment complex did not show a significant change in molar ellipticity over the pH range pH 5.5–9.5. To investigate the warfarin–protein interactions in more detail, we determined difference CD-spectra (θ_{obs} as a function of the wavelength, Fig. 2). Figure 2 shows that binding of warfarin to albumin and to the P46 fragment generates θ_{obs}-spectra which are similar in shape. By contrast, binding of warfarin to the T45 fragment does not show a significant induced ellipticity between 300 and 370 nm, not even if we correct for the lower affinity of warfarin for the T45 fragment.

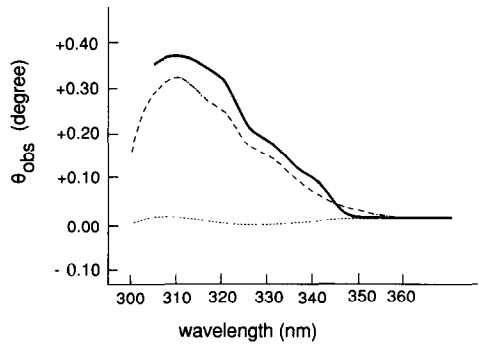

Fig. 2. CD-spectra of albumin, the T45 fragment and the P46 fragment. Shown are the differences between the CD-spectra (θ_{obs}) of the warfarin–albumin complex and albumin alone (——), the warfarin–T45 fragment complex and the T45 fragment alone (– – –) and the warfarin–P46 fragment complex and the P46 fragment alone (–·–·–). The spectra were recorded using $6 \cdot 10^{-5}$ M deionized protein solutions, adjusted to about pH 9.0 with 0.1 N NaOH. The molar ratio of warfarin to protein was 0.4 in all cases.

The results of the equilibrium dialysis experiments are shown in Fig. 3, where the free fraction (ff) of warfarin is plotted as a function of the pH. The affinity constants (K), calculated from the dialysis experiments, are summarized in Table 1. It follows from Fig. 3 and Table 1 that the binding of warfarin to albumin is pH dependent, which is in accordance with the literature [11]. A twofold increase in K was observed, from $K = 2.0 \cdot 10^5\,M^{-1}$ at pH 6 to $K = 4.2 \cdot 10^5\,M^{-1}$ at pH 8.5. The P46 fragment and albumin have almost the same affinity for warfarin, although it should be noted that the affinity of warfarin for the P46 fragment is pH independent. The affinity of warfarin for the T45 fragment is pH dependent and a factor 2–8 lower than for albumin.

DISCUSSION

From Figs 1 and 2 it is obvious that comparable conformational changes occur in albumin and the P46 fragment (domains one and two), but not in the T45 fragment (domains two and three). The CD results found for the warfarin–protein complexes are comparable to those found for the diazepam–protein complexes [9]. That is, binding of diazepam to the P46 fragment, like binding of diazepam to albumin, causes a clear pH-dependent induced ellipticity whereas the diazepam–T45 fragment complex has a small CD signal, which is difficult to measure.

It appeared that the T45 fragment contains the primary diazepam binding site while the P46 fragment contains one (or more) secondary diazepam binding site(s) [9]. This means that binding of diazepam to secondary sites also generates pH-

Table 1. The Hill coefficient, the midpoint pH (pH_{mid}) and the binding constant (K) of the warfarin–T45 fragment, the warfarin–P46 fragment and the warfarin–albumin complexes as determined by circular dichroism and equilibrium dialysis

	HSA	N	T45 fragment	N	P46 fragment	N
Domains present	1, 2 and 3		2 and 3		1 and 2	
Hill coefficient	1.3 ± 0.1	5	—[b]	3	0.9 ± 0.1	4
pH_{mid}	8.4 ± 0.1	5	—[b]	3	7.3 ± 0.1	4
$K \cdot 10^{-5}$ (M^{-1})	2.0 ± 0.1[a] to 4.2 ± 0.2	4	0.88 ± 0.05[a] to 0.53 ± 0.04	5	2.0 ± 0.1	4

All data are expressed as mean ± SD; N = number of determinations.
[a] pH dependent (pH 6.0–9.0).
[b] $[\theta]$ is pH independent (pH 6.0–9.0).

dependent induced ellipticities. For warfarin too, it has been deduced that binding to secondary sites on albumin generates pH-dependent induced ellipticities [11]. Therefore, the absence of a transition in the T45 fragment is likely not due to binding of warfarin to one or more secondary sites.

The most probable explanation for comparable conformational changes in albumin and the P46 fragment is that both proteins contain domain one. The T45 fragment lacks this domain and has deviant conformational properties. In other words, it looks as if domain one is very important for the N–B transition of albumin.

The N–B transition of albumin has been interpreted in terms of the two-state model of Monod *et al.* [24, 25]. When we apply this model to albumin, protons serve as the ligands and the transition is characterized by the pH_{mid} and the Hill coefficient (HC) [11, 13, 17]. Table 1 summarises these values for the warfarin–protein complexes. If we assume that the two-state model is also applicable for the P46 fragment, then the differences in the pH_{mid} and the HC values of the warfarin–albumin and the warfarin–P46 fragment complexes must be due to differences in the parameters describing this model (e.g. the number of proton binding sites). However, the information presented in this paper is insufficient to account for the observed phenomena. We are currently trying to obtain further information by doing acid/base titration and proton NMR experiments.

The fact that the P46 fragment and albumin nearly have the same affinity for warfarin (Fig. 3 and Table 1) indicates that the primary warfarin site (site I) must be located in the P46 fragment (domains one and two) and that domain three is not involved in the binding process. In contrast to albumin, no pH-dependent warfarin binding can be detected in the P46 fragment. This might seem contradictory if both proteins have the same binding site. However, it has been found that in the warfarin–albumin interaction there is only a small change in the free energy as a function of the pH, whereas relatively large changes were found in the enthalpy and entropy [26]. It is possible that in the absence of domain three (i.e. the P46 fragment), there are even smaller changes in the free energy as a function of the pH. This results in an almost pH independent affinity constant. In other words, a pH independent affinity constant does not rule out a pH dependent enthalpy and entropy. Therefore, the affinity constant (or the free energy) is apparently not a very suitable parameter with which to investigate the N–B transition of albumin.

Warfarin has a lower affinity for the T45 fragment than for albumin (Fig. 3 and Table 1). The warfarin binding is seen to be clearly pH dependent, although the direction of the pH dependence is reversed compared to albumin. This result is comparable to the situation described for the binding of diazepam to the T45 fragment [9]: in this case too, and in contrast to the situation for albumin, the affinity of the drug for the T45 fragment decreases with increasing pH (pH 6–9). The question might be whether the warfarin binding site on the T45 fragment corresponds to the primary warfarin site or to one or more secondary binding sites of albumin. Secondary affinity constants for the warfarin binding to albumin were found to be lower than the corresponding primary affinity constants by a factor of 15–100 [4, 11]. The difference in the affinity constants of warfarin for albumin and warfarin for the T45 fragment is only a factor 2–8 (pH 6–9). In view of the relatively high affinity constant, especially at pH 6, it can be concluded that the T45 fragment contains at least the main part of the primary warfarin binding site. This result combined with the high affinity of warfarin for the P46 fragment indicates that at least the main constituent of the warfarin binding site (drug binding site I) is located in domain two of the albumin structure. This finding supports the results found by Fehske *et al.* [27], who described the participation of Trp 214 (located in domain two) in the non-overlapping part of the warfarin binding site.

CONCLUSION

From the CD and equilibrium dialysis experiments, we can conclude that:

(1) it is very probable that domain one (present in the P46 fragment, absent in the T45 fragment) plays an important role in the N–B transition of albumin;

(2) at least the main part of the warfarin binding site (site I) is located in domain two of the albumin structure.

The warfarin binding to the proteins is influenced by domain one (and perhaps domain three). This proves that the domains do not act independently of each other (which is in agreement with the first

conclusion). This means that whereas the binding affinity of a drug to an isolated domain may be the same as for intact albumin, other binding characteristics may be quite different.

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