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LOCATION AND CHARACTERIZATION OF THE SURAMIN BINDING SITES OF HUMAN SERUM ALBUMIN

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Abstract—The objective of the present study was to investigate the location of the high-affinity suramin binding sites on the human serum albumin molecule. For this purpose, circular dichroism and equilibrium diaylsis experiments were performed on the interaction between suramin and a large peptic and a large tryptic fragment of albumin, the former comprising domains one and two of the albumin structure and the latter domains two and three. The equilibrium dialysis experiments revealed that albumin and the fragments have a comparable total affinity for suramin. Furthermore, all three proteins display a similar pH dependence of the unbound fraction of suramin. The circular dichroism experiments revealed that only the suramin–albumin and the suramin–peptic fragment complexes can undergo the pH dependent neutral-to-base or N–B conformational change, whereas the suramin–tryptic fragment complex lacks this ability. It is likely that the main parts of the high-affinity binding sites for suramin are located in domain two of the albumin molecule. The nature of these binding sites is discussed. The deprotonation of histidine and other positively charged residues taking part in salt bridges between suramin and albumin is, in all probability, the main cause of the decrease in affinity of suramin for albumin as the pH is raised from 6 to 9.

Many compounds bind reversibly and with high affinity to albumin† [1–10]. Albumin, the main protein component in blood, is a non-glycoprotein which is believed to be built up of three contiguous domains [1, 2]. On the albumin molecule there are at least two relatively specific binding sites for drugs present: site I (warfarin site) and site II (diazepam site) [3, 11, 12]. The binding characteristics of drugs can be dependent on the composition of the solvent. This is partly due to the occurrence of a conformational change in the albumin molecule around physiological pH. This conformational change is known as the neutral-to-base or N–B transition [13–18].

Suramin, a drug used in the treatment of human trypanosomiasis (sleeping sickness), is a polyanion with six sulphonic acid groups which are deprotonated under physiological conditions [19]. It has been shown that the drug binds with high affinity to a large number of proteins and enzymes, including albumin [19–23]. The binding of suramin to plasma proteins is in all probability an important factor for both the pharmacological effect and the pharmacokinetics of the drug [19, 24–26]. Furthermore, we think that binding to plasma proteins also plays a part in the toxic side-effects of the drug [26]. As albumin primarily accounts for the plasma binding of suramin in serum [19, 26], studies to obtain a better understanding of the nature of the binding of suramin to albumin are warranted. In a recent paper, we discussed the pH dependence of the binding of suramin to albumin [19]. It was found that the binding is sensitive to the N–B transition. This result was in line with earlier observations [22]. In this paper, we investigate the location of the high-affinity suramin binding sites on the albumin molecule. For this purpose, we studied the interaction between suramin and a large peptic fragment (comprising domains one and two) and a large tryptic fragment (comprising domains two and three) of albumin by means of circular dichroism and equilibrium diaylsis.

MATERIALS AND METHODS

Reagents. Suramin, obtained from Bayer (Leverkusen, F.R.G.) and [^{14}C]suramin (sp. act. 1.18 MBq/mg), kindly provided by the UNDP/World Bank/WHO-Special Programme for Research and Training in Tropical Diseases, were used without further purification. The purity of the [^{14}C]suramin was checked by means of TLC and was found to be more than 99%.

All other chemicals used were of analytical grade (J. T. Baker, Deventer, The Netherlands; Pharmacia, Uppsala, Sweden; Janssen Chemica, Beerse, Belgium; Sigma Chemical Co., St Louis, MO, U.S.A.; or Merck, Darmstadt, F.R.G.).

Preparation of the albumin and of the fragment solutions. Albumin was isolated from pooled human plasma according to the method described by Kremer [27]. The albumin used for all experiments was from one batch. The peptic fragment (the P46 fragment) and the tryptic fragment (the T45 fragment) were obtained as described previously [28–30]. The P46 fragment represents residues 1–387 and therefore comprises domains one and two of the albumin structure. The T45 fragment corresponds to residues 198–585 or domains two and three of the albumin structure. Protein concentrations were determined by the

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† By albumin we mean human serum albumin unless otherwise stated.

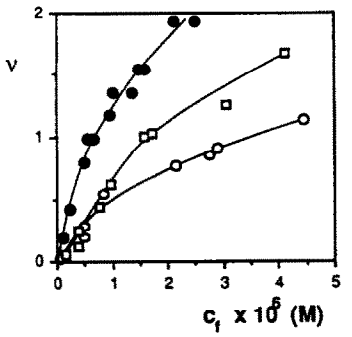

Fig. 1. Binding isotherms for the interactions of suramin with albumin (●), the P46 fragment (○) and the T45 fragment (□) at pH 6.0 (phosphate buffered, I = 0.1); 25°. The protein concentrations were 6×10^{-5} M. The figure gives the molar ratios of suramin bound to the proteins (ν) vs the unbound suramin concentration (c_f).

method of Hartree [31] and all protein solutions were deionized before use [30].

Circular dichroism and equilibrium dialysis. The circular dichroism (CD) spectra of the suramin–protein complexes were obtained between 300 and 400 nm. The protein concentration was 6×10^{-5} M and the suramin-to-protein ratio was 0.4. The observed ellipticities (θ_{obs}) are the differences between the CD-spectra of the suramin–protein complexes and the protein alone at 340 nm. The molar ellipticities ([θ]) were calculated as described previously [19].

The affinity of suramin for albumin and the fragments was determined by means of equilibrium dialysis, following the method with the [^{14}C]-labeled drug as described recently [19]. All data points were obtained at least in triplicate. The concentration of the protein solution was 6×10^{-5} M. The molar suramin-to-protein ratio (before dialysis) was 0.1 in the experiments where the pH dependence of the binding was measured; for the determination of the binding isotherms, molar ratios were varied between 0.0 and 3.0. The protein solution was buffered with phosphate or borate buffers, I = 0.1.

The experimental data on the binding were treated using the equation [19]:

$$\nu = \frac{n_1 K_1 c_f}{1 + K_1 c_f} + \frac{n_2 K_2 c_f}{1 + K_2 c_f} \qquad (1)$$

where ν is the molar ratio of suramin bound to the protein, c_f is the concentration of the unbound suramin and n_1 and n_2 are interpreted as the number of binding sites with the corresponding affinity constants K_1 and K_2. The binding parameters n_1, n_2, K_1 and K_2 were estimated by a non-linear least-squares computer program (Hewlett–Packard HP98820A-Statistical Library) on a Hewlett–Packard, model 9153, computer.

RESULTS

Figure 1 gives the isotherms for the binding of suramin to albumin and the fragments at pH 6. The binding parameters, as calculated with Eqn 1, are summarized in Table 1. For albumin the obtained binding parameters are in close agreement with those reported recently [19]. It can be seen that the total number of binding sites on the proteins is three. Both for albumin and the P46 fragment it was estimated that $n_1 = 2$ and $n_2 = 1$. For the T45 fragment it was estimated that $n_1 = 3$. Under the experimental conditions, no lower affinity binding sites could be demonstrated for the T45 fragment, which may be due to the fact that in the T45 fragment domain one of albumin is cut off. It should also be realized that for the T45 fragment the obtained set of data might be too small for a proper differentiation between high-affinity and low-affinity binding sites. Therefore, to make meaningful comparisons, we decided to evaluate the total affinity ($n_1K_1 + n_2K_2$) of the proteins (Table 1). The fact that the fragments have total affinities for suramin only about four to five-fold lower than albumin, might indicate that the fragments at least obtain the main parts of the high-affinity binding sites.

Figure 2 shows the pH dependence of the unbound fraction of suramin at the molar drug-to-protein ratio of 0.1. It can be seen that in all three suramin–protein solutions the unbound fraction has a comparable value. Furthermore, the unbound fractions display a comparable pH dependence.

In recent papers, it has been demonstrated that the pH dependence of the high-affinity suramin binding site is linked to the N–B transition of albumin [19, 22]. To determine whether this is also true for the binding of suramin to the fragments, we investigated the pH dependence of the molar ellipticity of the suramin–fragment complexes.

Binding of suramin to albumin gives rise to induced

Table 1. The binding parameters n_1, n_2, K_1 and K_2 of the binding of suramin to albumin and the fragments at pH 6.0

Protein	n	K (M^{-1})	SE	$n_1K_1 + n_2K_2$ (M^{-1})
Albumin	$n_1 = 2$	$K_1 = (1.4 \pm 0.1) \times 10^6$	0.13	2.9×10^6
	$n_2 = 1$	$K_2 = (1.3 \pm 0.1) \times 10^5$		
P46	$n_1 = 2$	$K_1 = (2.6 \pm 0.5) \times 10^5$	0.27	5.3×10^5
	$n_2 = 1$	$K_2 = (1.6 \pm 0.8) \times 10^4$		
T45	$n_1 = 3$	$K_1 = (2.2 \pm 0.6) \times 10^5$	0.07	6.6×10^5

The parameters were estimated by means of non-linear least-squares analysis, according to Eqn 1. The standard error represents the error of ν on c_f (see Eqn 1).

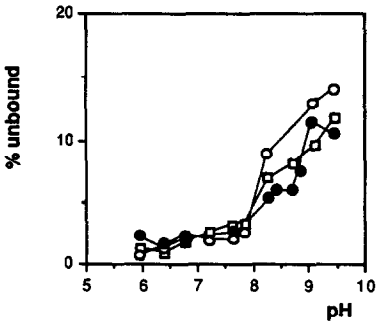

Fig. 2. The percentage of unbound suramin in solutions of suramin and albumin (●), of suramin and the P46 fragment (○) and of suramin and the T45 fragment (□) as a function of the pH. The protein concentrations were 6×10^{-5} M and suramin was added in a molar drug-to-protein ratio of 0.1. The measurements were performed in phosphate or borate buffers (I = 0.1).

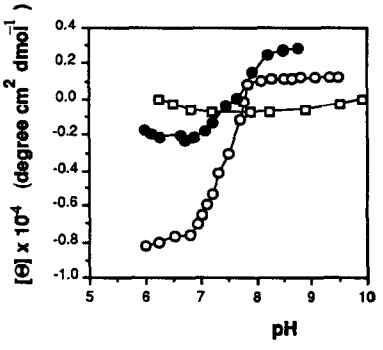

Fig. 3. The molar ellipticities $[\theta]$ of the suramin–albumin (●), the suramin–P46 fragment (○) and the suramin–T45 fragment (□) complexes as a function of the pH. The protein concentrations were 6×10^{-5} M and suramin was present in a drug-to-protein ratio of 0.4. The measurements were performed at 340 nm in phosphate or borate buffers (I = 0.1).

extrinsic Cotton effects between 300 and 370 nm with a maximum at 340 nm, i.e. θ_{obs} is at its maximum at 340 nm. The binding of suramin to the P46 fragment induces extrinsic Cotton effects as well, with a maximum at 340 nm (data not shown). By contrast, for the T45 fragment it was not possible to detect an induced Cotton effect between 300 and 370 nm upon binding of suramin, i.e. θ_{obs} equals zero at 340 nm.

The influence of the pH on the molar ellipticity is given in Fig. 3, where $[\theta]$ is plotted vs pH. From this figure it is evident that, between pH 7 and 9, $[\theta]$ increases for the suramin-albumin complex and the suramin–P46 fragment complex, whereas for the suramin–T45 fragment complex $[\theta]$ is practically independent of pH. The CD experiments are supported by preliminary UV difference measurements, which show a pH dependence which runs parallel with the pH dependence of $[\theta]$. Since no changes in the physico-chemical properties of suramin are known to occur in the pH region 6 to 9 [19], it is likely that the pH dependence of $[\theta]$ has its origin in changes in the structure of the proteins.

The results obtained therefore support previous findings, which indicated that albumin and the P46 fragment can undergo N–B-like conformational changes, whereas the T45 fragment lacks this ability [30, 32].

DISCUSSION

The equilibrium dialysis experiments presented in this paper reveal that the fragments have a total affinity for suramin of the same order of magnitude as albumin. Furthermore, the pH profile of the unbound fraction appears to be comparable for the three proteins. These results indicate that the main constituents of the high-affinity binding sites for suramin are present in the fragments as well. It might, therefore, be concluded that the main parts of the high-affinity binding sites are located in the second domain of the albumin structure, as this is the only domain which is present in all three of the proteins.

Brown and Shockley [2] proposed that each domain of albumin can be regarded as a cylinder formed by six helices in an anti-parallel orientation. The cylinder has a hydrophobic interior and a polar exterior. Around the opening of the cylinder especially basic residues are situated. The three domains of albumin are linked together by small peptide chains. This predicted structure of Brown and Shockley [2] was recently more or less confirmed by Carter *et al.* [33], who described the albumin to be an oblate ellipsoid of three contiguous domains with an overall molecular length of 137 Å. When we combine the aspect of the basic residues around the cylindrical opening of the domains with the findings that the distance of the two clusters of negative charge of suramin is approximately 40 Å [34] and the diameter of the second domain is approximately 50 Å [1, 2], it is conceivable that the suramin molecule binds to the top of the domain-two cylinder structure. This idea is supported by preliminary competition experiments with oleate, revealing that, up to a molar fatty acid-to-albumin ratio of 4, oleate is incapable of displacing suramin from its high-affinity binding sites. According to the literature [1, 2], the two primary oleate binding sites are located in the third domain whereas the two secondary binding sites are located in the second domain of albumin. Moreover, it is believed that the oleate binding sites are situated inside the cylinder structures of the domains [2]. As no competition occurs between suramin and oleate, it is plausible to conclude that the two high-affinity binding sites for suramin are located on the surface of the cylinder structure of albumin. The positively charged amino acid residues, present on both sites of the cylinder of domain two [1, 2], may be suitable candidates for binding of the negatively charged suramin.

We found that for all three proteins the affinity for suramin decreases when the pH is raised from 6 to 9 (Fig. 2). Furthermore, it was shown that only the suramin–albumin and the suramin–P46 fragment complexes have the potential to undergo an N–B-like conformational change, whereas the suramin–T45 fragment complex lacks this potential (Fig. 3).

These results suggest that the pH dependent binding of suramin to the proteins is dominated by the pH dependent changes in electrostatic interactions between the negatively charged suramin and the negatively charged proteins. In the pH region 6 to 9, suramin has six negatively charged sulphonate groups; the net negative charge of the proteins decreases from −5 to −24 for albumin, from −3 to −16 for the T45 fragment and from −4 to −22 for the P46 fragment [32]. So, it seems that the increase in electrostatic repulsion between suramin and the proteins is responsible for the decrease of the binding of suramin to the proteins, at increasing pH. However, we have discussed in a recent paper [19] that the pH dependence of the binding of suramin to albumin is linked to the N–B transition and that the change in electrostatic interaction is only of minor importance in the pH dependent binding of suramin. As outlined above, the T45 fragment lacks the potential to undergo an N–B-like transition but, at the same time, displays a pH dependent suramin binding profile comparable to that of albumin and the P46 fragment. It is questionable, therefore, whether the N–B transition indeed fully accounts for the pH dependent binding of suramin to albumin.

It is assumed here that, at low pH, one or more protonated amino acid residues take part in salt bridges between suramin and the proteins. Upon deprotonation of the involved amino acid residue(s), these salt bridges will be disrupted, evidently resulting in a decreased affinity between pH 8 and 9. This statement is based on the fact that the albumin molecule contains intramolecular salt bridges between positively charged histidine residues in domain one and negatively charged anionic residues in domains two and three. These salt bridges are severed in the N–B transition [32]. It is likely therefore that also in the binding of suramin to albumin, besides other positively charged amino acids, at least some histidine residues present in domain two are involved. The pK values of the amino acid residues involved in salt bridges between suramin and albumin are in all probability close to the histidine residues involved in the N–B transition. This would explain why the pH dependence of the binding of suramin to albumin and the N–B transition appear to be related phenomena. Moreover, the assumption that salt bridges are involved in the binding of suramin to albumin also explains why albumin in the B-conformation has a lower affinity for suramin than albumin in the N-conformation. This situation contrasts the situation found for other drugs, where higher affinities for the B-conformation were demonstrated [15, 16, 18, 35–37]. As suramin still displays a considerable affinity towards albumin at higher pH values, it is conceivable that hydrophobic interactions between albumin and suramin will at least partly play a role in the binding phenomenon. Such a view is in line with spectroscopic evidence reported earlier [22], indicating that binding of suramin to albumin moves at least parts of the suramin molecule into hydrophobic areas of the albumin molecule.

From the experiments presented in this paper, the following conclusions may be drawn. Firstly, it is likely that the main parts of the two high-affinity binding sites for suramin are located on the top and the bottom of the cylinder structure of the second domain. Secondly, it is probable that besides other charged amino acid residues having pK values around pH 8, positively charged histidine residues take part in salt bridges between suramin and albumin. Deprotonation of such groups might be the main cause of the decreased binding at high pH. Further research on the physico-chemical parameters of the binding of suramin to albumin is needed to elucidate the exact nature of this binding.

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