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COMMENTARY

THE LOCATION OF DRUG BINDING SITES IN HUMAN SERUM ALBUMIN

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After introduction into the circulation most drugs are bound to the different blood constituents (cells, proteins). Within the plasma proteins, serum albumin is undoubtedly the most important carrier for drugs and other small molecules. Therefore, the attention of many scientists has been drawn to the phenomenon of the interaction of drugs with the serum albumin. The literature dealing with this subject has been first reviewed by Goldstein in 1947 [1] and since that time many other reviews have been published [2–8].

Even today, nearly all of these papers can be categorized under five fundamental baseline questions outlined by Scatchard in 1954 [9] "How many? How tightly? Where? Why? What of it?", referring to the number of binding sites, the binding constants, the location of the sites, the binding forces, and the significance of the plasma protein binding for distribution and pharmacological activity. However, after going critically over the available literature it becomes clearly evident that the question "Where at the albumin molecule are the binding sites located?" has got much less attention than the other four. This lack of information was less important during the more descriptive phase of albumin binding studies, where the number of sites, the binding constants, and possible displacement reactions were determined and where these data were used to interpret the significance of the albumin binding for distribution and therapeutic activity.

However, because the HSA (Human Serum Albumin) primary structure has been known for several years and because it became more and more obvious during the last years that only a rather small number of sites mediates the binding of most drugs to HSA, the chance to investigate not only quantitative but also qualitative aspects of drug albumin interactions became more and more possible. This includes location and composition of drug binding sites within the HSA molecule. Although, as mentioned, these aspects may be less important for the more clinical aspects of drug albumin binding, there are at least two good reasons to carry out and continue these studies:

(1) Although the significance of the albumin binding of drugs has been overinterpreted in many ways during the last years it is now generally accepted that in certain but not all cases the albumin binding of a drug is a significant factor for the pharmacokinetics. Furthermore, the displacement of one drug by another from the albumin binding sites can at least contribute to drug interaction phenomena. An exact knowledge about location, size, and composition of the HSA binding sites seems now to be important for a rational understanding of the albumin binding and for any approach to systematize such interactions. This in turn could be helpful for predicting binding phenomena like displacement reactions and the sometimes dramatically changed albumin binding during several disease states, involving mainly liver and kidneys [10–14].

(2) In particular, the findings about the rather small number of drug binding sites of the HSA molecule and their some times receptor like properties [7] emphasize the value of serum albumin as a convenient model to study the molecular basis of specific ligand protein interactions. Thus, the experience and results obtained with this model system may be helpful for the understanding of other important ligand protein interactions in pharmacology and biochemistry, e.g. enzyme–substrate or receptor–ligand interactions.

Since the general properties of the most important ligand binding sites of HSA have been previously summarized [4, 6, 7, 15–17] the present commentary will concentrate on the structure and amino acid composition of the drug binding sites of HSA and their location within the secondary and tertiary structure of the protein.

STRUCTURE OF HSA

The complete primary structure of HSA has been known since 1975 [18, 19]. It consists of a single peptide chain of 585 amino acid residues which is formed into 9 double loops or subdomains by paired disulfide bonds [4, 18]. As proposed by Behrens *et al.* [18] the covalent structure of HSA consists of three domains, each formed by three loops. Since several similarities exist between the three domains with respect to amino acid composition, sequence, and covalent structure it has been suggested that HSA arose by duplication of a primordial single domain gene [20].

The secondary structure of HSA contains about 55 per cent α-helix, and about 15 per cent β-pleated sheet [4, 21–23]. Only for the first domain have probability profiles been calculated for helix, sheet, bend, and irregular structures from a prediction scheme based on single amino acid frequency distribution in several proteins of known structure [24].

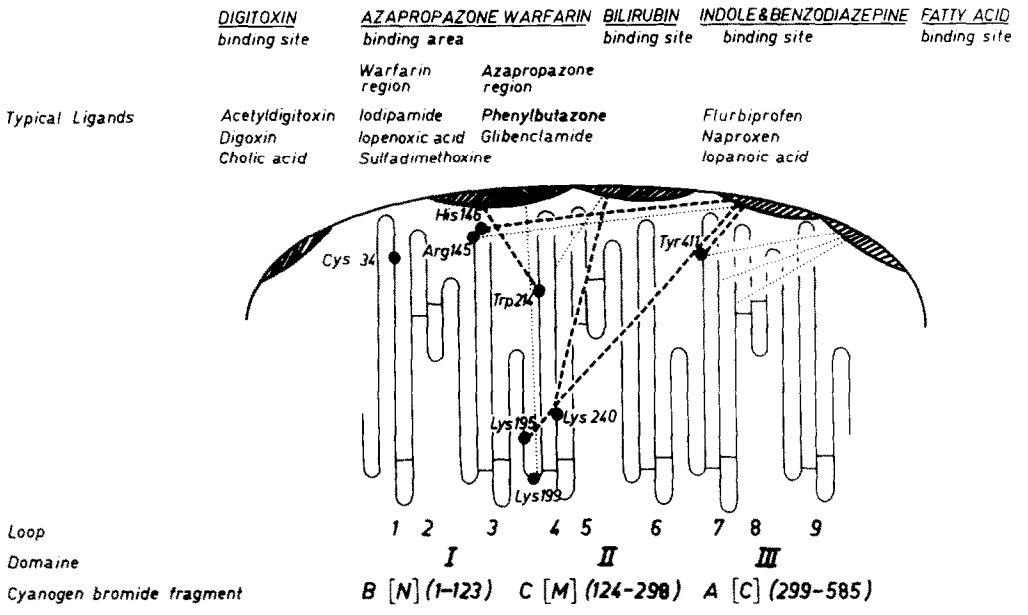

Fig. 1. The structure of human serum albumin and the possible location of drug binding sites. The model of the covalent structure of human serum albumin is given according to Behrens *et al.* [18], including the number of loops and domains and the three cyanogen bromide fragments, where A, B, C refer to the nomenclature used in reference [31] and C, N, M refer to the nomenclature of reference [19]. Some selectively modified amino acid residues (●) which are clearly involved in the five most important binding sites of the protein are indicated by the dashed line (– – –). Amino acid residues or loops which are possibly involved in the binding sites are indicated by the pointed (. . . .) line. For further details see the text.

In contrast to the secondary structure, the tertiary structure of HSA is not known except for some indirect data about the accessibility of amino acid residues for solvent perturbation measurements, chemical modification, or ligand interactions [25–30].

METHOLOGICAL ASPECTS

A large variety of biochemical techniques and experimental approaches has been used to investigate the location of ligand binding sites in HSA. Besides some spectroscopic methods, in most cases specific fragments of HSA or the modification of specific amino acid residues were used, and sometimes a combination of both methods.

The HSA molecule has been cleaved into definite fragments by the use of several different reagents like cyanogen bromide [19, 31, 32], brom succinimide [33], trypsin [34], and pepsin [21]. The binding of site specific ligands to definite fragments was then used to localize the ligand binding site in the fragments. However, the conformation (tertiary and even secondary structure) of the fragments might differ from that of the same sequence in the native HSA molecule. Thus, it can be difficult to decide if the failure to find binding to a specific fragment depends on the location of the binding site in another part of HSA or on a loss or destruction of the binding site due to conformational changes of the fragment investigated.

A variation of this method used the ligand as affinity label which is bound covalently to the HSA binding site before cleavage of the protein. The demonstration of the covalently bound ligand in a definite fragment of HSA is a strong indication of the location of the binding site within this fragment [35–38].

Since the findings that acetylsalicyclic acid specifically acetylates only one lysine residue of HSA [39, 40] many other amino acid residues of HSA have been more or less specifically modified, e.g., the lone tryptophan residue [23, 41, 42], the lone cysteine residue [40, 43, 44], the highly reactive tryosine residue [23, 43, 45], and lysine, histidine, or arginine residues sensitive to different reagents [35, 36, 40, 42, 46–49]. The binding of site specific ligands to the modified albumin derivatives clearly indicates whether or not the modified amino acid residues participate in the binding sites [15, 23, 41, 42, 44, 46–53].

The participation of specific amino acid residues in ligand binding sites has also been demonstrated by several spectroscopic techniques, including u.v. difference [25, 26, 29, 54] and fluorescence spectroscopy [54–56] or circular dichroism measurements [57, 58]. The distance between bound ligands and the lone tryptophan residue of HSA has been calculated from fluorescence energy transfer measurements [55, 56] and could be used to localize the binding site since the position of the lone tryptophan residue within the HSA sequence is known.

The most advanced approach to localize a binding site might be to rebuild this site synthetically. However, up to now only the copper binding site of HSA, which is located in the *N*-terminal end of the sequence, has been synthesized successfully [59].

BINDING SITES LOCATION

Indole and benzodiazepine binding site. The best characterized drug binding site of HSA is the indole and benzodiazepine binding site [7, 17], also called site II by Sudlow *et al.* [16]. This site binds several indole derivatives and benzodiazepines with a high degree of structural specificity [60–63]. The interactions of the enantiomers of tryptophan and of oxazepam hemisuccinate with this site are the two most pronounced examples of stereospecific binding phenomena on HSA [64, 65]. Specific marker ligands for this site are L-tryptophan [66], diazepam [17, 23, 51], dansylsarcosine [16, 67], iopanoic acid [68, 69], and flurbiprofen [16, 70]. Many other drugs of very different chemical structures also bind to the indole and benzodiazepine binding site with high affinity [15–17, 51, 66, 71].

The major part of this binding site is located in a cyanogen bromide fragment C (residues 124–298) as concluded from the binding of L-tryptophan and diazepam to this fragment, but parts of this site may be also located in a fragment A (residues 299–585) which binds both marker ligands, although to a much smaller extent [32, 36, 72]. By contrast, no binding of L-tryptophan and diazepam binding could be detected to a fragment B (residues 1–123) [32, 72]. Within fragment C, His 146 and Lys 194 are involved in this site as demonstrated by the covalent binding of a site selective affinity label [36]. One arginine residue whose modification strongly affects the diazepam binding [46] is probably Arg 145 [47], also located in fragment C. Within fragment A, the highly reactive tryosine residue of HSA is part of the benzodiazepine and indole binding site as can be concluded from the pronounced reduction of the binding of diazepam and L-tryptophan after its selective modification with tetranitromethane [23]. Evidence from several laboratories suggests that this tryosine is Tyr 411 [27, 43, 45, 50, 73]. Recently, Sjödin *et al.* [34] demonstrated that a part of the indole and benzodiazepine binding site must be located in a large trypsin resistant fragment of HSA (residues 182–585) because this fragment is still able to bind diazepam, though the affinity is much weaker. The highly reactive tryosine (Tyr 411) and Lys 194 are located in this fragment but not His 146 and Arg 145.

These data are definitely not consistent with previous assumptions that the indole and benzodiazepine binding site can be located in a single amino acid sequence of the HSA primary structure as has been found for the copper binding site [59]. On the contrary, these data indicate that sequences and amino acid residues of very different parts of the HSA primary structure are involved in this site. Accordingly, the indole and benzodiazepine binding site must be finally formed by the HSA tertiary structure.

Warfarin and azapropazone binding area. Besides the indole and benzodiazepine binding site the second most important drug binding site of HSA is the warfarin binding site [7, 17] also called site I by Sudlow *et al.* [16]. Structural parameters determining the selectivity of drugs for both sites are not yet known especially, since some drugs are bound specifically to both sites (dicoumarol, indomethacine, tolbutamide, ethacrynic acid) [15–17, 51, 71, 74] and in some cases only small changes of the chemical structure of drugs dramatically change the site selectivity [67–69]. Specific marker ligands for the warfarin site are warfarin itself [17, 51, 75–77], phenprocoumon [78], dansylamide [16, 67], iodipamide [68], phenylbutazon [71, 76, 77, 79, 80], azapropazone [71, 80], and iophenoxic acid [68, 69, 81].

In contrast to the indole and benzodiazepine binding site much less is known about the location of the warfarin binding site. At least one part of this binding site seems to be located in the cyanogen fragment C (residues 124–298), as has been concluded from experiments on the binding of phenylbutazone to this fragment [32]. In early studies on the binding of warfarin to HSA at different pH-values it has been suggested that one lysine residue might be involved in this site [48, 82]. And indeed, Lys 199, which is easily acetylated with acetylsalicyclic acid, seems to be involved in this site [39, 40]. However, its modification enhances the binding of some typical marker ligands [39, 48] so that this residue might be located only in the close neighbourhood of this site. The only amino acid residue clearly involved in the warfarin binding site is the lone tryptophan residue [18, 19, 30] of HSA. Its modification by two different reagents reduces the binding of warfarin and iodipamide to this site [51, 68] and its location within this site has been concluded from spectroscopic measurements [54, 55]. In large contrast to these findings are recent observations that the tryptophan modification does not affect the binding of phenylbutazone and of azapropazone [71], which are both known as potent ligands for the warfarin binding site [15–17, 75, 77, 79, 80]. It was concluded from these contradictory data that the warfarin binding site actually is a larger binding area, consisting of the overlapping binding sites of warfarin and azapropazone [71]. The lone tryptophan residue is located at one site of this area where drugs like iodipamide, warfarin, and sulfamethoxime are bound while the azapropazone site is located on the other site of this area, where drugs like phenylbutazon and glibenclamide are bound. Up to now, specific ligands for only one part of this large binding area have not yet been found since all drugs bound to one site of this area always displace marker ligands bound to the other part of this area, at least to a certain degree [71].

Digitoxin binding site. A third specific drug binding site of HSA is the digitoxin binding site which is clearly independent of the indole and benzodiazepine and of the warfarin binding sites [13, 17, 44, 80, 83, 84]. Only a few drugs also bind to this site, e.g., several other cardenolides and some bile acids [17, 44, 83]. Very little information is available about the possible location of this site. Neither the lone free SH-group [44, 84], nor the lone tryptophan residue (Trp 214), nor the highly reactive tyrosine seem to be involved in this site [84]. However, the modification of nine out of the eighteen tyrosine residues of HSA strongly reduces the binding of digitoxin to this site, indicating the possible involvement of tyrosine residues, whose location however is not yet known [84]. The digitoxin site is also not identical with the high-affinity long chain fatty acid

binding sites since the addition of fatty acids up to a molar ratio of three has no effect on the binding of digitoxin to HSA [44].

Bilirubin binding site. The very strong binding of bilirubin to HSA has been known for several years. Although not a drug, bilirubin binding will be discussed because of its definite clinical therapeutical relevance. Bilirubin binds to HSA via one high affinity and one or two low affinity sites [85]. However, it should be mentioned that the association constants of the secondary sites are still in the range of that of very strongly bound drugs [85]. The relative high affinity of the secondary site is a major handicap for locating the primary site by binding studies.

Bilirubin binding could be demonstrated to several quite different fragments of HSA. Bilirubin binding to fragment 1–386 is stronger than to fragment 49–307, but still weaker than to native HSA [21]. Strong bilirubin binding has also been observed to the trypsin resistant fragment 182–585, but circular dichroism measurements suggest substantial conformational changes of the binding site compared to native HSA [34]. Using a bilirubin affinity label [37], bound bilirubin has been identified in two further HSA fragments strongly indicating their significance for the bilirubin binding site (124–298 and 447–548) [38]. Thus, contradictory data exist about the location of the bilirubin binding site in fragments of HSA, especially about the significance of the C-terminal fragment. As mentioned before, some of the confusing data might be due to the relative high affinity of the secondary site. Only a little further information about the location of the bilirubin binding site is available from amino acid residue modification studies. While the lone tryptophan residue of HSA is clearly not involved and the significance of arginine residues is not yet clear, lysine residues are involved in this binding site [42, 46, 52]. One of these, Lys 240, could be identified after the covalent coupling of bilirubin to HSA with carbodiimide [35]. All these data together led to the conclusion that the main part of the bilirubin binding site might be located within the second domain possibily in loop 4, as suggested by Peters [4] and by the findings about the significance of Lys 240 [35]. Whether or not other parts of the HSA sequence also participate in this binding site is not yet finally known.

While the bilirubin binding site is clearly independent of the indole and benzodiazepine binding site of HSA [46, 71, 86] their relation to the warfarin–azapropazone binding area is not yet completely understood. While bilirubin at a molar ratio of one strongly reduces the binding of many drugs typical for the warfarin site (warfarin, azapropazone, phenylbutazon) [17, 71, 87], the binding of iophenoxic acid and of iodipamide (drugs bound to the tryptophan residue area of this binding site) remains unaffected by one mole bilirubin per mole HSA [71, 81]. In good agreement with these observations are findings of Jacobsen that the modification of the lone tryptophan residue of HSA does not affect the binding of bilirubin [42]. At the present state it is suggested that one bilirubin binding site overlaps the warfarin and the azapropazone part of this large binding area, but not the close neighbourhood of the lone tryptophan residue where iodipamide and iophenoxic acid are bound. The shape of several displacement curves [71, 81] and the effects of long-chain fatty acids on the bilirubin and the warfarin binding sites [88–91] suggest that this overlapping binding site is the second and not the first high-affinity site of bilirubin.

Fatty acid binding site. Although fatty acids are not drugs, the fatty acid binding site will be included in this commentary. This is justified by the accumulating evidence for the importance of the free fatty acid (FFA) level in the blood as determinant of individual and disease induced variations of the free blood levels of drugs. Fatty acids interact with different sites of the HSA molecule. Their binding can be associated with an enhanced or reduced binding of drugs to HSA, where both competitive displacement and allosteric effects have been reported [89–94]. Since the basic paper of Goodman [95] much additional evidence has been presented that the first two moles of fatty acids bind to a specific binding site of the HSA molecule where both are bound side-by-side in a antiparallel fashion [56]. This site is probably located in the third domain of the HSA structure around amino acid residue 422 as has been concluded from comparable observations with bovine serum albumin [4, 96, 97], from limited pepsine digestion experiments on HSA [21, 98], and from a calculation of the distance between this site and the lone tryptophan residue of HSA [56].

The relation of the fatty acid binding site to other specific drug binding sites of HSA is not yet clear. Using a spin label probe for the bilirubin binding site Soltys and Hsia suggested that the fatty acid binding site might be identical with the high affinity binding site of bilirubin [99]. However, these observations do not agree with findings of some other authors [88, 92] who favour more allosteric interactions between both sites.

In the case of the warfarin binding site, profound evidence has been presented that this site is not identical with the fatty acid binding site [89–91]. In the case of the indole and benzodiazepine binding site the relation to the fatty acid binding site is not so clear. However, although most data do not suggest that the two sites are identical, the effects of fatty acids on the binding of ligands specific for the indole and benzodiazepine binding site strongly indicate that they are located close together. This could explain the profound allosteric interactions between both sites [89, 100, 101]. This assumption is further supported by a definite reduction of the modification of the highly reactive tyrosine residue by one mole stearic acid per mole HSA, since this residue is clearly involved in the indole and benzodiazepine binding site [102]. In summary, most data reported so far for the high affinity binding site of long chain fatty acids (which dominate in human blood) are in general consistent with the model proposed by Goodman in 1958 indicating that this site is not identical with binding sites of other organic ligands, but that the binding of fatty acids to this site can profoundly affect other ligand binding sites of the HSA molecule via allosteric interactions.

Clearly different from the high affinity binding site of long chain fatty acids is the high affinity binding site for medium chain fatty acids [50, 95, 102], since the reactivity of the highly reactive tyrosine residue of HSA is strongly reduced in the presence of octan-

oate as well as chlordiazepoxide. Means and coworkers suggested that this site might be identical with the indole and benzodiazepine binding site of HSA [50, 102].

CONCLUSIONS

The data summarized in the present commentary clearly indicate the important developments made during the last ten years about the possible location of drug and other small ligand binding sites of the HSA molecule. However, the only ligand binding site of HSA which is completely known is the copper binding site at the *N*-terminal end of the protein. For all other ligand binding sites the complete structure and the final location are not yet known. The experimental evidence accumulated so far points to two major complications possibly responsible for these unsolved problems:

(1) There now exists increasing evidence that quite different parts of the HSA primary structure may be involved in organic ligand binding sites of the albumin. This assumption is best experimentally supported in the case of the indole and benzodiazepine binding site but some similar evidence also exists in the case of the warfarin and the bilirubin binding sites. Thus, it seems questionable whether binding sites can be located only within one fragment of the HSA amino acid chain. More likely, an important condition for final location and identification of binding sites might be a better knowledge of the HSA tertiary structure.

(2) Some of the drug binding sites seem to consist of several subsites, each with a certain degree of specificity for only a part of the ligands typical for the complete site. Thus, the expression "binding area" might better fit this type of binding site. It is clearly evident that with increasing size of such a binding area its location becomes more and more difficult. The best evidence for such a binding area exists in the case of the warfarin site.

Thus, the structure and the location of drug binding sites of HSA might be more complicated than it seemed a few years ago after the first promising reports about the presence and the properties of only a very limited number of sites mediating the binding of most drugs to HSA. However, we hope that despite these apparent complications the present commentary further stimulates scientists to continue with or to start projects dealing with the location of binding sites within the HSA molecule. The therapeutical and theoretical implications of this area of research have been outlined in the introduction. It is desirable and possible that another commentary on the same topic but several years later will give more definite answers about the location of drug binding sites of the HSA molecule.

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