Part I

Biochemical Aspects of Drug-Protein Binding

2

STRUCTURE OF BINDING SITES ON ALBUMIN

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INTRODUCTION

After introduction into the circulation, most drugs bind to the various blood constituents (cells and proteins), of which serum albumin is most important. Although the significance of the serum albumin binding for distribution and activity of drugs has been sometimes overinterpreted, it is now generally accepted that for some, but certainly not all drugs, binding to the albumin fraction plays an important role in their pharmacokinetics in man. This explains why we still want to know which molecular mechanisms are involved in the interaction of drugs with albumins, particularly with human serum albumin (HSA). In this respect we now have convincing evidence that only a very limited number of sites mediate the binding of most drugs and other small molecules (fatty acids, L-tryptophan, bilirubin, dyes) to HSA. Accordingly, an exact knowledge about structure and location of these binding sites is essential for the rational understanding of the albumin binding of drugs. This, in turn, will be very helpful for predicting therapeutically important binding phenomena—such as displacement reactions and the sometimes dramatically changed albumin binding in several disease states. For most cases of therapeutically relevant drug binding to HSA only, two different sites are important (see the preceding chapter). The present chapter will concentrate on structural aspects of these two drug binding sites (see

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TABLE 2.1
Different Designations of the Two Major Drug Binding Sites of HSA

Site I	Site II	Sudlow et al. (20)
Binding region 6	Binding region 2	Kragh-Hansen (2,21)
Azapropazone and warfarin binding area	Indole and benzodiazepine site	Fehske et al. (3,29)

(Table 2.1). Structural aspects of the other important ligand binding sites of HSA have been summarized elsewhere.[1,2,3]

BINDING SITES OR BINDING FUNCTIONS, OR DO PREFORMED BINDING SITES REALLY EXIST?

As already mentioned, many experimental observations strongly indicate that most drugs compete for mainly two binding sites on HSA at low molar drug/HSA ratios. These observations are usually explained by the presence of two binding sites or binding regions of the HSA molecule with well defined and sometimes receptor-like properties.[1,2,3] However, this model is not undisputed. Accordingly, before discussing the structure of these sites, some general remarks are appropriate about the major controversies in respect to possible mechanisms of drug binding to HSA.

Our site oriented model of drug binding to HSA is largely influenced by the use of the Scatchard transformation of ligand binding to macromolecules.[4] Brodersen, Honoré, and Larsen[5] and Honoré and Brodersen[6] have shown that many binding data could also be fitted by an alternative model of stoichiometric binding constants which does not imply the presence of given sites. This model would also take into account the large conformational flexibility of the HSA molecule, the pronounced conformational adaptability of some of the proposed binding sites, and mutual allosteric interactions between the proposed binding sites—three phenomena not perfectly in agreement with the assumption of given, preformed binding sites.[2,3] Thus, Brodersen, Honoré, and Larsen[5] and Honoré and Brodersen[6] prefer to talk about binding functions of the HSA molecule rather than about binding sites. However, they agree about the major importance of only two binding functions for the interaction of most drugs with HSA at low molar drug/HSA ratios. Thus, at least for the binding of drugs

at low molar drug/HSA ratios, the difference between both models is more semantic than fundamental, especially if we agree that even the assumption of a well defined and possibly preformed binding site does not necessarily indicate that this site can be localized within a single sequence of the HSA primary structure.

METHODOLOGICAL ASPECTS

General Remarks

The complete primary structure of HSA has been known since 1975,[7,8] and a possible model of its covalent structure consisting of three domains each formed by three loops is given in Figure 2-1. While some information is available about the distribution of helical and non-helical regions within the primary structure,[2] the tertiary

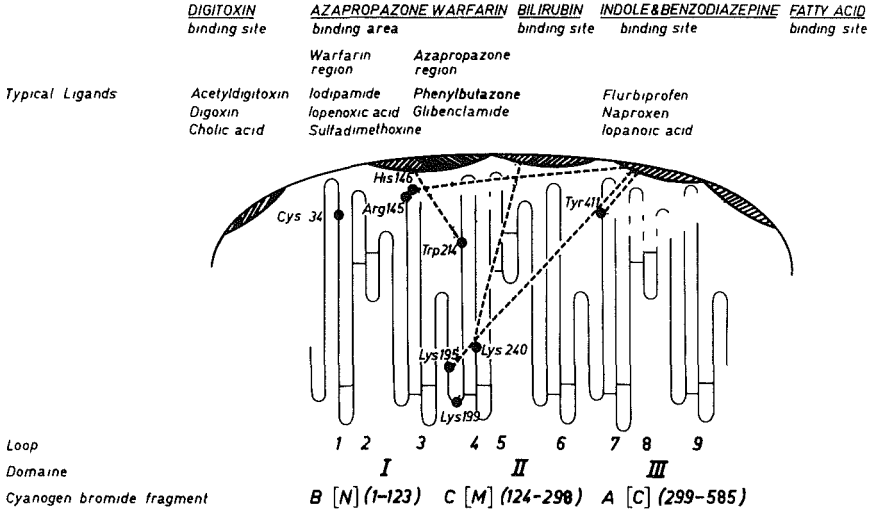

FIGURE 2-1
The structure of human serum albumin and the possible location of drug binding sites. Some selectively modified amino acid residues (●) which are clearly involved in the five most important binding sites of the protein are indicated by the dashed line (---). Amino acid residues or loops possibly involved in the binding sites are indicated by the pointed line (...).
Source:
Fehske, Müller, Wollert 1981. Reproduced with permission.

structure of HSA is still not known. A variety of different biochemical techniques has been used to localize ligand binding sites within the primary structure. Besides some spectroscopic methods, in most cases, specific fragments or the specific modification of amino acid residues has been employed.[2,3]

The specific binding of a ligand selective for only one site of HSA can be used to localize this site within only one fragment out of several different HSA fragments. However, the conformation of the fragments might differ from that of the same sequence within the native molecule. Thus, if no binding can be found to a specific fragment, it can be difficult to decide if this site is located in another fragment, or is located in the fragment but is destroyed by conformational changes. Up to now, none of the drug binding sites of HSA could be convincingly localized in only one specific fragment.

On the other hand, some amino acid residues of HSA have been selectively modified and some of these are specifically involved in only one of the drug binding sites.[2,3] In such cases, however, even a large decrease of the binding is not, in any case, indicative for a specific role of the modified residue within the binding site, since the modification could alternatively alter the properties of the binding site by allosteric mechanisms. However, the specific modification of amino acid residues is possible in some cases without measurable changes of the protein conformation and without measurable effects on drug binding sites except one. Thus, we still think that this approach has some advantages over the use of fragments for investigating the structure of binding sites of HSA.[9]

Tyrosine Modification

Of the 18 tyrosine residues of HSA, 9 can be modified with tetranitromethane (TNM).[10] Out of these, one reacts much faster and can be selectively modified. This residue has been identified as tyrosine 411 (see Fig. 2-1).[11,12,13] For most of our binding studies about the influence of the tyrosine modification on the drug binding properties of HSA, three derivatives were used (TNM-HSA I, II, and III) with degrees of modification of 2, 5, or 8 tyrosine residues respectively.[10] Similarly to HSA, 10 out of the 19 tyrosine residues of bovine serum albumin (BSA) can be modified by TNM using similar conditions.[14] Also similar to HSA, one tyrosine residue of BSA reacts much faster, and can be selectively modified.[14] This tyrosine very likely represents tyrosine 409.[15] For most of our binding studies about the influence of the tyrosine modification on the drug binding properties of BSA, three derivatives were used (TNM-BSA I, II, and III) with degrees of modification of 2, 8, or 10 respectively.[14]

Tryptophan Modification

The lone tryptophan residue of HSA (position 214) (see Fig. 2-1) can be selectively modified by 2-hydroxy-5-nitrobenzyl bromide (HNB bromide), as well as by o-nitrophenylsulfenylchloride (NPS chloride) under appropriate conditions.[10,16] Accordingly, the two tryptophan-modified HSA derivatives (HNB-HSA, NPS-HSA) used for the investigations reported, had a degree of modification of about one.[10,16]

Using methods similar to those described for HSA, the two tryptophan residues of BSA can be stepwise modified by both reagents[17] in agreement with their steric accessibility.[18] One tryptophan (residue 134) is accessible for modification under mild conditions (HNB-BSA I, NPS-BSA I) while the buried tryptophan 212 (which corresponds to tryptophan 214 in HSA) could additionally be modified after unfolding of the protein.[17] The tryptophan-modified BSA derivatives used for the binding studies presented, had degrees of modification of about one (HNB-BSA I, NPS-BSA I) or two (HNB-BSA II, NPS-BSA II).

Binding Experiments

The binding of the ^{14}C-labeled drugs to HSA and BSA was investigated by equilibrium dialysis at room temperature using .066 M phosphate buffer (pH 7.4), and albumin concentrations of 36.2 μM.[10,16]

THE INDOLE AND BENZODIAZEPINE BINDING SITE OF HSA

Binding Site or Binding Area

This site, also called site II (see Table 2.1) binds L-tryptophan, medium chain fatty acids, and several drugs with high affinity[1,2,20,21] (Fig. 2-1) and accounts for the two most pronounced examples of stereoselective ligand binding to serum albumins known so far.[21] Thus, this site justifies the term "silent receptor" for HSA even in this classical sense.[1] Nearly all present evidence indicates that this site represents a structurally well defined binding region, which is much less sensitive for conformational changes of the HSA molecule than the large binding area representing site I.[24,25] Moreover, all our attempts to alter the properties of this site by chemical modification had similar effects on the binding of all ligands

specific for this site.[23] On the basis of structure-binding relationships, Wanwimolruk, Birkett, and Brooks[26] have recently suggested that this site consists of a hydrophobic cleft 12-16 Å deep and 6-8 Å wide with a cationic group located near the surface of the protein. This model closely resembles the picture we have of a specific drug receptor. On the other hand, this does not mean that this specific site represents a completely rigid structure, since it still seems to have some conformational adaptability.[2,3] This is exemplified by our observations that the tryptophan modification decreases the binding of L-tryptophan to this site (see Table 2.2) by presumably allosteric mechanisms,[14] while the binding of diazepam to this site is much less (see Table 2.3), and that of some other drugs are not affected.[23]

TABLE 2.2
The Effect of Tyrosine and Tryptophan Modification on the Binding of L-Tryptophan to HSA and BSA

	HSA		BSA	
Albumin	n	$k \times 10^{-4}$	n	$k \times 10^{-4}$
Control	0.82	16.5	0.76	1.2
TNM I	0.66	0.4	-	-
TNM II	-	-	-	-
TNM III	-	-	-	-
NPS I	0.80	10.4	0.51	2.1
NPS II			0.64	1.7
HNB I	0.57	10.0	0.74	2.5
HNB II			0.71	2.1

Note:
The number of binding sites n and the association constants k (M^{-1}) were calculated from linear Scatchard plots by regression analysis.

Sources:
Fehske, Müller, Wollert 1979, 1978
Fehske, Schläfer, Müller 1981
Schläfer and Müller 1982

TABLE 2.3
The Effect of Tyrosine and Tryptophan Modification on the Binding of Diazepam to HSA and BSA

	HSA		BSA			
Albumin	n	$k \times 10^{-4}$	n_1	$k_1 \times 10^{-4}$	n_2	$k_2 \times 10^{-4}$
Control	0.9	133	0.9	2.5	4.3	0.07
TNM I	1.1	9	0.9	2.5	4.1	0.06
TNM II	1.3	8	0.6	2.9	3.2	0.07
TNM III	1.2	5	0.3	3.4	1.5	0.10
NPS I	1.0	116	0.6	3.7	3.5	0.09
NPS II			0.9	4.6	4.0	0.30
HNB I	0.9	73	0.6	3.6	3.9	0.10
HNB II			0.6	4.0	3.9	0.10

Notes:

Control for HNB-HSA I (Fehske, Müller, Wollert 1978) was $n = 1.0$, $k = 102 \times 10^4$ (M^{-1}). The number of binding sites n and the association constants k (M^{-1}) were calculated from linear Scatchard plots (HSA) by regression analysis or from curved Scatchard plots (BSA) by the graphical method of Pennock (1973).

Sources:

Fehske, Müller, Wollert 1978, 1979
Fehske, Schläfer, Müller 1981
Schläfer and Müller 1982

Location

Ligand binding studies to modified HSA derivatives clearly indicate that tyrosine 411 is part of site II of HSA.[10,11,13] These findings are in agreement with studies on HSA fragments indicating the presence of this site (although with considerably reduced affinity for typical ligands) in a cyanogen bromide fragment A (residues 299-585) and in a trypsin-resistant fragment (residues 182-585) (see Fig. 2-1 and reference 3). However, these findings do not agree with observations about the binding of L-tryptophan and diazepam to a cyanogen bromide fragment C (residues 124-298) and about the significance of arginine 145, histidine 146, and lysine 194 for ligand binding to this site as indicated by modification studies (Fig. 2-1).

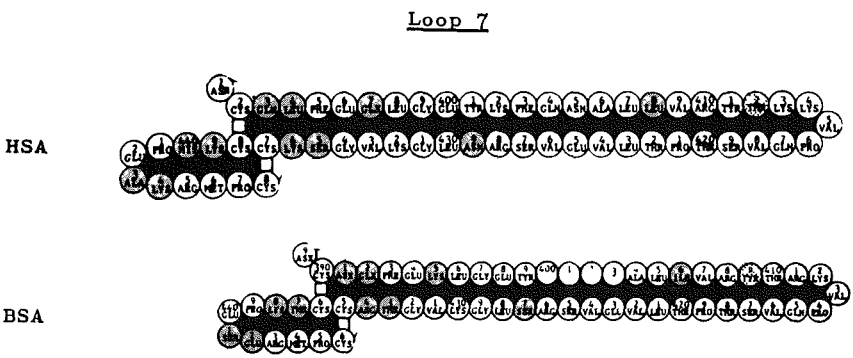

FIGURE 2-2
Sequences of loop 7 containing the highly reactive tyrosine residues of HSA (tyr 411) and BSA (tyr 409), both indicated by dotted pattern. Positions in both albumins which are different are indicated in gray. Specific differences between the two albumins are indicated by data for BSA in parentheses: GLN 393 (ASX 391), LEU 394 (GLX 392), GLX 397 (LYS 395), LEU 408 (ILE 406), LYS 413 (ARG 411), ASN 429 (SER 427), SER 435 (THR 433), LYS 436 (ARG 434), LYS 439 (THR 437), HIS 440 (LYS 438), ALA 443 (SER 441), LYS 444 (GLU 442). Sources: Kragh-Hanson 1981; Reed, Putnam, Peters 1980; Brown 1977.

Taken together, these data do not support the view that this site can be localized within a single sequence, but suggest that site II is finally formed in the tertiary structure by different parts of the primary chain. The sequences around residues 145, 146 and around tyrosine 411 are very likely involved (Fig. 2-1). This hypothesis gets some support from recent experiments on ligand binding to the indole binding site of BSA. Similarly to HSA, the binding of L-tryptophan to BSA is largely reduced after the modification of only two tyrosine residues (see Table 2.2), of which only the highly reactive tyrosine residue is involved.[14] This residue very likely represents residue 409.[15] Up to this point, many similarities to the indole site of HSA are obvious. Accordingly, only minor differences exist between the sequences surrounding the reactive tyrosines in both albumins (Fig. 2-2). It can be assumed that this sequence is also quite important for the indole site of BSA. This is consistent with the findings of King and Spencer[27] that stereoselective L-tryptophan binding is pretty well preserved in a large fragment of the C-terminal part (residues 306-582).[2] This contrasts to the above mentioned experiments with similar fragments of HSA, where ligand binding to this site was preserved only to a much smaller extent. Thus, for

BSA less evidence is present for a participation of different sequences of the primary chain within this site. Interestingly, the indole site of BSA which binds L-tryptophan and medium chain fatty acids similarly to the same site of HSA, does not bind several drugs specific for site II of HSA, e.g., diazepam (see Table 2.3) and flurbiprofen.[14] Thus, the substrate specificity of this site is more restricted in the case of BSA. We speculate now that the broader binding spectrum of this site in HSA is caused by the more complicated structure of this site in the human albumin. In other words, the sequences around the reactive tyrosines are mainly responsible for L-tryptophan and medium chain fatty acid binding in the case of both albumins, while the additional binding properties of this site in HSA are brought about by the participation of other parts of the primary structure in this site—e.g., the sequence around residues 145 and 146. The basic assumption that L-tryptophan binding and drug binding to this site of HSA might involve different parts of this site to a variable degree is supported by our findings about the allosteric effect of the modification of tryptophan 214 on ligand binding to this site, which is significant for L-tryptophan (Table 2.2), much less for diazepam (Table 2.3), and not present for flurbiprofen.[23]

In conclusion, we think that our data on ligand binding to the indole site of BSA support our concept that this site is finally formed in the case of HSA by the tertiary structure by at least two different sequences.

THE WARFARIN AZAPROPAZONE BINDING AREA

Binding Site or Binding Area

Similar to site II, a large variety of different drugs bind to this site[20,28,29] and stereoselective binding to this site has also been demonstrated.[22] Previous studies using warfarin as a typical ligand indicated that the lone tryptophan residue of HSA (Fig. 2-1) is part of this site, as suggested by the pronounced reduction of the warfarin binding after tryptophan modification (see Table 2.4). However, subsequent experiments showed that the binding of phenylbutazone (a classical inhibitor of the HSA binding of warfarin) and that of the analogue azapropazone were not affected by the tryptophan modification (see Table 2.5).[29] These findings are certainly not in agreement with the assumption of a common site for all three drugs as suggested by other experimental observers.[20,28] Both conflicting findings can be explained by assuming the presence of a warfarin azapropazone binding area (Fig. 2-1). All of our data and most data reported in the literature fit surprisingly well into this model.[31] The basic assumption

is that this area consists of the overlapping high-affinity binding sites of warfarin and azapropazone.[29] Thus, when both drugs are added to the HSA molecule at the same time, a mutual displacement occurs. On the other hand, both sites are not completely identical, since the tryptophan modification has no influence on the binding of azapropazone, phenylbutazone, and glibenclamide, although these drugs are potent displacers of warfarin and are displaced themselves by a number of drugs binding primarily to the warfarin site.[29] This suggests that the lone tryptophan is located within the nonoverlapping part of the warfarin region. Most ligands of this site bind preferentially, but not exclusively, to one or both parts of this binding area. We have not yet found drugs binding selectively to only one of both parts.

In agreement with Kragh-Hansen,[2,21] we do not think that this binding area is identical with the primary bilirubin binding site,[29] which is also not affected by the tryptophan modification.[32]

The proposed model of site I, which assumes the presence of a rather large binding area, makes it very unlikely that this site

TABLE 2.4
The Effect of Tryptophan and Tyrosine Modification on the Binding of Warfarin to HSA and BSA

	HSA				BSA			
Albumin	n_1	$k_1 \times 10^{-4}$	n_2	$k_2 \times 10^{-4}$	n_1	$k_1 \times 10^{-4}$	n_2	$k_2 \times 10^{-4}$
Control	0.89	124	1.9	5.2	0.88	32	2.3	2.0
NPS I	0.96	36	1.8	5.4	0.84	28	2.0	2.2
NPS II					0.84	27	2.0	2.1
HNB I	0.38	42	2.1	4.5	0.70	25	2.1	1.0
HNB II					1.0	6	1.7	0.7
TNM I	0.75	120	1.3	5.8	0.83	16	2.3	1.6
TNM II	0.49	139	1.1	3.9	0.80	5	1.5	0.7
TNM III	0.24	40	1.8	5.4	-	-	-	-

Note:
For experimental details see Table 2.3.

Sources:
Fehske, Müller, Wollert 1978
Fehske et al. 1982
Schläfer and Müller 1982

TABLE 2.5
The Effect of Tryptophan and Tyrosine Modification on the Binding of Phenylbutazone to HSA and BSA

	HSA				BSA			
Albumin	n_1	$k_1 \times 10^{-4}$	n_2	$k_2 \times 10^{-4}$	n_1	$k_1 \times 10^{-4}$	n_2	$k_2 \times 10^{-4}$
Control	1.02	82	2.0	2.2	0.87	164	1.9	2.9
NPS I	1.01	80	2.0	4.6	0.86	137	2.0	2.7
NPS II					0.90	142	1.9	3.7
HNB I	0.77	79	2.0	1.8	0.85	106	1.9	2.1
HNB II					0.83	51	1.5	2.5
TNM I	0.92	84	2.0	1.9	0.80	102	1.6	4.1
TNM II	0.98	70	2.1	2.2	0.69	36	0.9	3.9
TNM III	0.99	68	2.0	2.2	-	-	-	-

Note:
For experimental details see Table 2.3.

Sources:
Fehske, Müller, Wollert 1978
Fehske et al. 1979
Fehske et al. 1982
Schläfer and Müller 1982

represents only a small hydrophobic cleft as proposed for site II.[26] This is supported by findings indicating that ligand binding to site I is much more sensitive for allosteric effects than to site II—e.g., fatty acid binding[24] or the N → B transition.[33]

Location

Little is presently known about the final structure of this site. Binding studies with phenylbutazone indicate that at least one part is located in a cyanogen bromide fragment C (residues 124-298).[34] This agrees with our findings about the significance of the lone tryptophan (residue 214) and other observations about the possible relevance of lysine 199.[3] Taking all this evidence together, a sequence surrounding tryptophan 214 within loop 4 might be important.[2,3] Evidence for the participation of other parts of the HSA primary structure is missing, but seems likely in respect to the much larger size of this binding area relative to site II.

TABLE 2.6
Effect of Three Inhibitors (Molar Drug/HSA Ratios of 1.1 or 3.3) on the Binding of ^{14}C-Azapropazone, ^{14}C-Warfarin, and ^{14}C-Phenylbutazone (Molar Drug/HSA Ratio of 1.1) to HSA and BSA

Albumin	Ligand (^{14}C)	Inhibitors: Warfarin 1.1	Warfarin 3.3	Azapropazone 1.1	Azapropazone 3.3	Phenylbutazone 1.1	Phenylbutazone 3.3
HSA	Azapropazone (0.08)	159	363	114	340	-	-
	Warfarin (0.14)	73	175	37	61	-	-
	Phenylbutazone (0.11)	53	-	-	-	-	-
BSA	Azapropazone (0.32)	16	50	38	80	101	146
	Warfarin (0.17)	63	151	22	51	54	96
	Phenylbutazone (0.11)	21	60	33	72	117	226

Note:
Data are given as percent increase of the free fraction which is given in parentheses.

Sources:
Fehske et al. 1982
Schläfer and Müller 1982

Similarly to our experiments at site II, we tried to get further information about structure and location of site I by comparative studies on BSA. As with HSA, warfarin, phenylbutazone, and azapropazone compete for a common site on BSA at low molar concentrations (see Table 2.6). However, the modification of tryptophan 212 (only modified in NHB-BSA II and NPS-BSA II) has absolutely no effect on the azapropazone binding (data not shown), [17] has only a small effect on the phenylbutazone binding (HNB-BSA II but not NPS-BSA II in Table 2.5), and has a somewhat larger effect on the warfarin binding, but again only in the case of HNB-BSA II and not in the

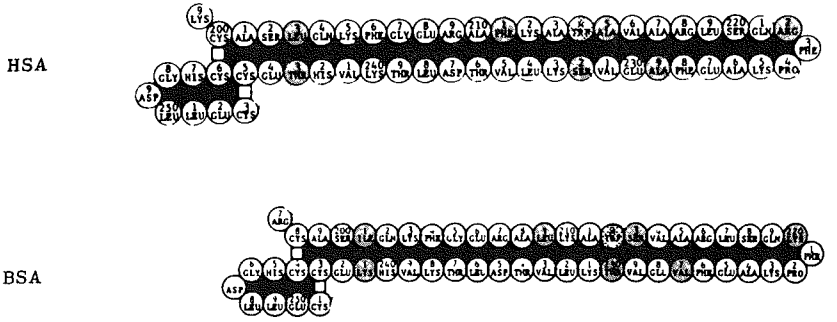

FIGURE 2-3
Sequences of loop 4 containing trp 214 (HSA) and trp 212 (BSA), both indicated by dotted background. Positions in both albumins which are different are indicated in gray. Specific differences between the two albumins are indicated by data for BSA in parentheses: LEU 203 (ILE 201), PHE 211 (LEU 209), ALA 215 (SER 213), ARG 222 (LYS 220), ALA 229 (VAL 227), SER 232 (THR 230), THR 243 (LYS 241). Sources: Kragh-Hanson 1981; Brown 1977.

case of NPS-BSA II (Table 2.4). Moreover, dicoumarol binding to BSA (molar ratio of one and three) is not affected by the modification of tryptophan 212 as indicated by circular dichroism measurements,[17] contrasting to the pronounced effects of the modification of tryptophan 214 in HSA under similar experimental conditions.[30] The sequences surrounding tryptophan 214 or 212 in HSA or BSA respectively exhibit distinct, but rather small differences (Fig. 2-3). We think that it seems rather unlikely that these small differences can account for all differences seen with respect to the binding properties just mentioned. Thus, we feel entitled to speculate that the comparative data on BSA give some, although premature, evidence that not only the sequence around tryptophan 214 is involved in site I. In conclusion, aside from this rather indirect evidence, little further information about the structure of this site can be taken from comparative experiments with BSA.

CONCLUSIONS

The data reported give fairly good evidence that both binding sites do really exist in terms of a given region within the tertiary

structure of the albumin. This point is strongly supported by our modification experiments, where a specific modification will only affect drug binding to one site with little, or possibly no, effect on drug binding to the other site. Thus, we think that it is justified to consider site I and site II of HSA as preformed regions of the HSA molecule.

However, there is also increasing evidence that both sites cannot be attributed to only one sequence of the amino acid chain, but consist of different parts of the primary structure and are finally formed by the tertiary structure of HSA. For both important drug binding sites of HSA, only some parts of the primary sequence involved are known. Thus, the final clarification of the structure of both important drug binding sites HSA will require further experimental work.

NOTES

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