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Five recombinant fragments of human serum albumin—Tools for the characterization of the warfarin binding site

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Abstract

Human serum albumin (HSA) interacts with a vast array of chemically diverse ligands at specific binding sites. To pinpoint the essential structural elements for the formation of the warfarin binding site on human serum albumin, a defined set of five recombinant proteins comprising combinations of domains and/or subdomains of the N-terminal part were prepared and characterized by biochemical standard procedures, tryptophanyl fluorescence, and circular dichroic measurements, indicating well-preserved secondary and tertiary structures. Affinity constants for binding to warfarin were estimated by fluorescence titration experiments and found to be highest for HSA-DOM I-II and HSA, followed by HSA-DOM IB-II, HSA-DOM II, and HSA-DOM I-IIA. In addition, ultraviolet difference spectroscopy and induced circular dichroism experiments were carried out to get an in depth understanding of the binding mechanism of warfarin to the fragments as stand-alone proteins. This systematic study indicates that the primary warfarin binding site is centered in subdomain IIA with indispensable structural contributions of subdomain IIB and domain I, while domain III is not involved in this binding site, underlining the great potential that lies in the use of combinations of recombinant fragments for the study and accurate localization of ligand binding sites on HSA.

Keywords: albumin; circular dichroism; fluorescence spectroscopy; ligand binding; warfarin

Human serum albumin (HSA), the most abundant serum protein, is composed of three homologous, predominantly helical domains (I, II, III), each of which contains two subdomains (IA, IB, IIA, IIB, IIIA, IIIB) (Fig. 1A–E). It interacts reversibly with a wide variety of endogenous and exogenous compounds, and thus serves as an important depot and transport protein. Among the endogenous substances bound with high-affinity are long- and medium-chain fatty acids, bilirubin, and hemin (Peters, 1996). The main and therefore best-characterized drug binding sites are site I or warfarin site, supposed to be centered in subdomain IIA, and site II (benzodiazepine site), formed by a binding pocket in subdomain IIIA (Sudlow et al., 1975b, 1976; He & Carter, 1992; Carter & Ho, 1994; Peters, 1996; Vorum, 1999). However, ultimate stringency of the exact binding locations of many ligands is still missing, as is detailed information on the contributions of discrete amino acid residues of the albumin molecule to the structural integrity and functionality of the different ligand binding sites.

Typical site I ligands are bulky heterocyclic anions with the charge situated in a central position of the molecule, whereas drugs that are bound in site II are generally aromatic carboxylic acids with an extended conformation and the negative charge located at one end of the molecule (Peters, 1996). Site I is shared by coumarin anticoagulants, sulfonamides, and salicylate, to mention just a few. From the diversity of acceptable ligands and the ability to bind more than one at the same time this site was interpreted as a large flexible region (Kragh-Hansen, 1988) or as a complex of different but overlapping sites (Fehske et al., 1982). Recently, it was pointed out by Yamasaki et al. (1996) that site I harbors at least three overlapping binding regions, and a new classification (site Ia, Ib, and Ic) was proposed. Employing the site directed mutants R218H, R218P, R218M of HSA, Petersen et al. (2000) demonstrated that the affinity of albumins that cause familial dysalbuminemic hyperthyroxinemia (R218H, R218P), to warfarin is fivefold decreased, leading to an elevated serum concentration of the free drug and to a fivefold reduction in the serum half-life of the drug.

Fragments of albumin, produced by enzymatic cleavage, have been used in the past in attempts to define the correct location of the high-affinity warfarin binding site on the native molecule. Bos

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Abbreviations: HSA, human serum albumin; BSA, bovine serum albumin; CD, circular dichroism; ICD, induced circular dichroism; DOM, domain; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

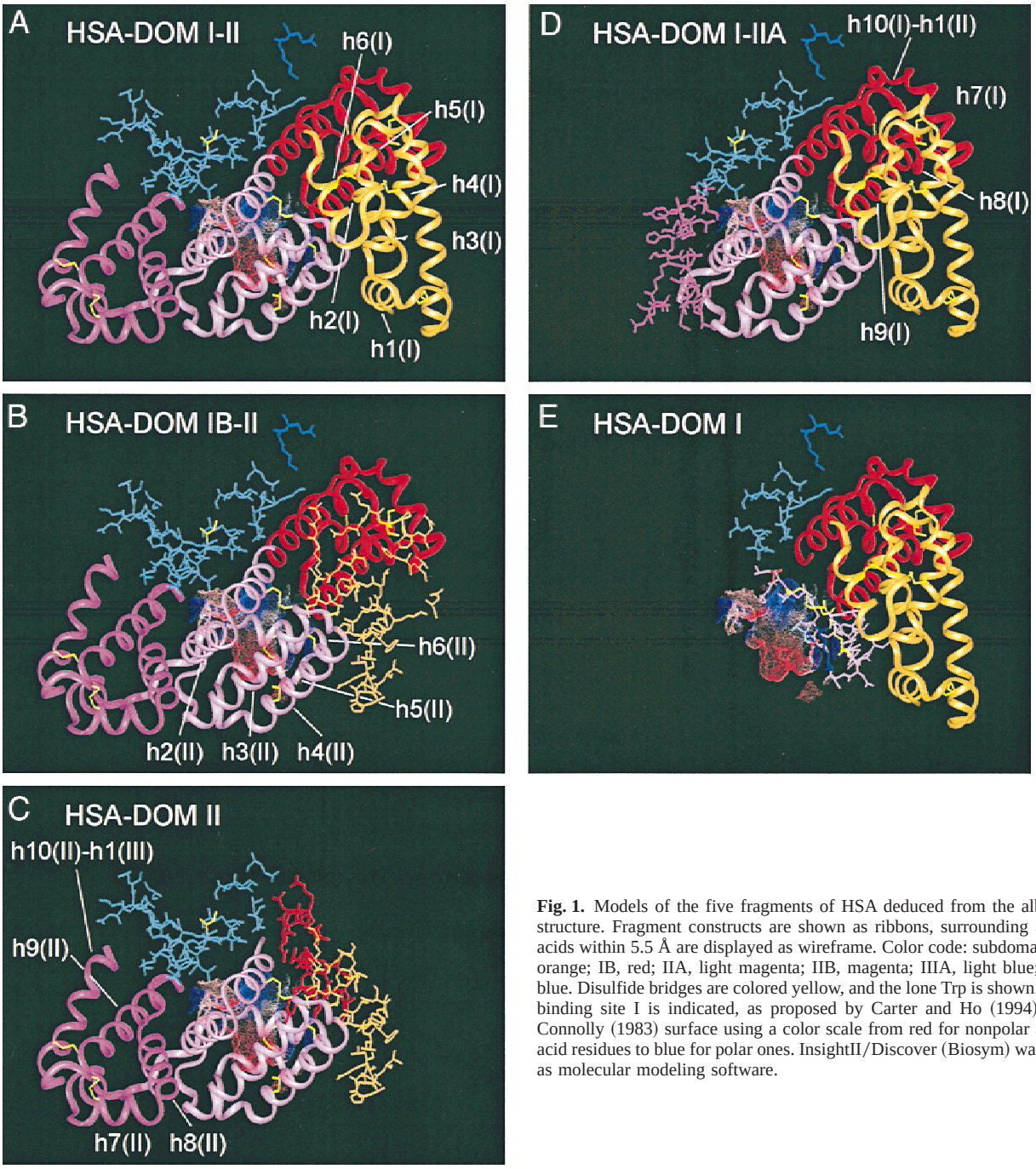

Fig. 1. Models of the five fragments of HSA deduced from the albumin structure. Fragment constructs are shown as ribbons, surrounding amino acids within 5.5 Å are displayed as wireframe. Color code: subdomain IA, orange; IB, red; IIA, light magenta; IIB, magenta; IIIA, light blue; IIIB, blue. Disulfide bridges are colored yellow, and the lone Trp is shown. Drug binding site I is indicated, as proposed by Carter and Ho (1994) by a Connolly (1983) surface using a color scale from red for nonpolar amino acid residues to blue for polar ones. InsightII/Discover (Biosym) was used as molecular modeling software.

et al. (1988, 1989) employed a peptic (domain I and II) and a tryptic fragment (domain II and III) of HSA and demonstrated that the peptic fragment harbors the entire primary warfarin binding site. Wilding et al. (1977) investigated the interaction of warfarin with two fragments of bovine serum albumin (BSA) consisting of the N-terminal and the C-terminal halves, respectively, of the native molecule. The binding affinity of each of the fragments was one order of magnitude lower compared to BSA, and a combination of the fragments raised the affinity but could not fully restore the drug binding to the extent of the native molecule. The authors concluded that the high-affinity binding site is located close to the center of the molecule. However, due to the absence of more suitable natural cleavage sites on the primary sequence of both HSA and BSA, a more detailed investigation toward a minimal fragment with warfarin binding affinity comparable to that of the native molecule was not possible.

In a recent study, we reported on the cloning, expression, and purification of the three domains of HSA as stand-alone proteins and were able to demonstrate their structural integrity and functionality as proteins with specific ligand binding properties (Dockal et al., 1999). The primary binding site for hemin was proven to be located in domain I, and that for diazepam in domain III, whereas

the high-affinity site for warfarin (site I) could not be assigned to domain II unambiguously. This was interpreted by missing contributions of at least the C-terminal part of domain I to the warfarin binding region.

These findings prompted us to study the warfarin–albumin interaction in more detail and, to pinpoint the structural features of this binding site, to attempt to prepare a recombinant albumin fragment containing those parts of the native molecule that are essential for the warfarin binding site. Three additional recombinant fragments of HSA were cloned and expressed, and characterized by standard biochemical procedures as well as by intrinsic tryptophanyl fluorescence and circular dichroic techniques. The affinity constants for the binding to warfarin of these novel fragments and of HSA-DOM I and HSA-DOM II in comparison to HSA were estimated by fluorescence spectroscopy, and the molecular interaction of the ligand with the proteins was further investigated by ultraviolet (UV) difference spectroscopy and induced CD.

Results

Biochemical characterization

The newly cloned fragments represent combinations of domains and/or subdomains of the first two domains of HSA and thus extend the set of recombinant fragments that we have described previously (Dockal et al., 1999, 2000). The fragment preparations yielded a purity greater than 98%, as indicated by the analysis of the Coomassie-stained SDS-PAGE gel (Fig. 2). Similar to the single domains of HSA, these novel proteins were analyzed by a number of biochemical standard procedures, and a summary of their physicochemical properties is presented in Table 1. Correct processing of the α-factor leader used for secretion from the host cell and the absence of posttranslational modifications are strongly suggested by N-terminal sequencing (leading Glu-Phe residues are due to the EcoRI restriction site used for cloning) and the MALDI-TOF-MS analysis, giving the expected molecular masses of the proteins. Furthermore, the isoelectric points are in close agreement with the predicted values and native-PAGE indicated that the fragments of HSA are present in our preparations as monomeric proteins.

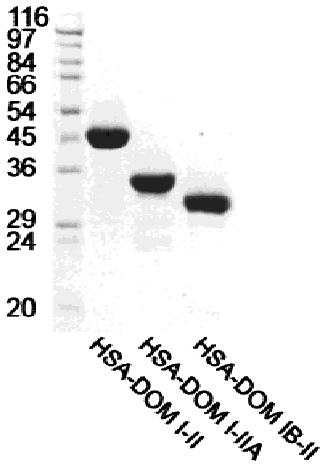

Fig. 2. SDS-PAGE. Polyacrylamide (12.5%) gel electrophoresis was performed in the presence of 0.1% SDS (according to Laemmli, 1970). Left lane: wide molecular weight standards (Sigma); lane 1, HSA-DOM I-II; lane 2, HSA-DOM I-IIA; lane 3, HSA-DOM IB-II (5 μg each per lane).

Structural characterization

To obtain information about the structural integrity, CD measurements in the far- and near-UV region were performed and spectra for HSA, HSA-DOM I-II, HSA-DOM I-IIA, HSA-DOM IB-II, HSA-DOM I, and HSA-DOM II are shown in Figures 3A and 3B, respectively. Minima of the spectra are given in Table 1. The results of the secondary structure resolved analysis are summarized in Table 2.

All HSA fragments display minima near 208 or 209 and 222 nm, indicative of predominantly α-helical structure (Fig. 3A; Table 2). The observed spectrum of HSA and the results of the secondary structure resolved analysis for the native molecule are in accordance with previous findings (Wetzel et al., 1980; Lee & Hirose, 1992; Uversky et al., 1997). HSA-DOM I-II and HSA have nearly superimposible CD spectra in the far-UV region, denoting a high degree of secondary structural similarity, which is further underlined by the calculated fraction of α-helical content (Table 2). The relative helicity of HSA-DOM I-IIA and HSA-DOM IB-II is intermediate between the native molecule and HSA-DOM I-II and the recombinant single domains of HSA (HSA-DOM I, HSA-DOM II) (Dockal et al., 1999, 2000). The β-sheet content of all HSA fragments is increased compared to that of HSA (Table 2), indicative for some structural rearrangement of the single newly exposed surface area of HSA-DOM I-II, HSA-DOM I-IIA, and HSA-DOM I, and patches at both termini of HSA-DOM IB-II and HSA-DOM II (Fig. 1A–E). Interestingly, the spectra of proteins with intact domain I (HSA-DOM I-II, HSA-DOM I-IIA, and HSA-DOM I) are similar in shape to the spectrum of HSA, differing from that of the spectra of HSA-DOM IB-II and HSA-DOM II (Fig. 3A).

To gain insight into the molecular environment of the disulfide bridges and aromatic amino acid side chains of the recombinant fragments, near UV-CD measurements were performed (Fig. 3B). All spectra are dominated by the large number of disulfide bridges of each of the proteins, leading to negative ellipticity in the performed wavelength range. The minima near 262 and 268 nm, which are observed for all proteins further denote their common origin. Spectra of proteins with two newly exposed surface areas (HSA-DOM IB-II and HSA-DOM II) (Fig. 1B,C) have less ellipticity compared to the spectra of HSA-DOM I-IIA and HSA-DOM I (Fig. 1D,E), which have an intact domain I and a similar relative content of aromatic amino acid residues.

The results of the far- and near-UV CD measurements indicate that the polypeptide chains of the novel recombinant fragments of HSA harbor sufficient information to adopt folds comparable to their structure in the context with the native molecule.

HSA has a single tryptophanyl residue, Trp214, located almost centrally in helix two of subdomain IIA (Carter & Ho, 1994). By exciting the intrinsic protein fluorescence of HSA, HSA-DOM I-II, HSA-DOM I-IIA, HSA-DOM IB-II, and HSA-DOM II at 295 nm, typtophanyl emission spectra were obtained, providing detailed information about the environment of this residue and the exposure of the fluorophore to the aqueous solvent (Halfman & Nishida, 1971a, 1971b; Steinhardt et al., 1971). An emission spectrum of the tryptophanyl residue of each of these proteins is presented in Figure 4. The position of the emission maximum of HSA, 340 nm, indicates that Trp214 is located in an amorphous protein

Table 1. *Physicochemical properties of HSA and its novel recombinant fragments*[a]

	HSA (defatted)	HSA-DOM I-II	HSA-DOM I-IIA	HSA-DOM IB-II
Molecular mass (MALDI-TOF MS)	(66,479)	44,461 (44,243)	34,568 (34,420)	32,146 (32,075)
Residue range	1–585	1–385[b]	1–299[b]	108–385[b]
Isoelectric point, pI	5.8[c]	5.2 (4.9)	5.3 (5.1)	5.4 (5.2)
Far-UV CD minima (nm)	222, 209	222, 209	222, 209	222, 208
Near-UV CD minima (nm)	268, 262	268, 262	268, 262	268, 261
$A^{0.1\%}_{278nm,1cm}$	0.58[d]	0, 70	0, 56	0, 79

[a]Theoretical or predicted values are given in parentheses. The physicochemical properties for HSA-DOM I, HSA-DOM II, and HSA-DOM III can be found in Dockal et al. (1999).
[b]Plus additional Glu-Phe at the N-terminus, due to the restriction site used for cloning.
[c]Peters (1996).
[d]Bos et al. (1988).

matrix with limited water accessibility, as was discussed previously (Eftink & Ghiron, 1977; Honore & Pedersen, 1989; Lee & Hirose, 1992). The emission of the lone tryptophanyl residue in HSA-DOM I-IIA has the same energy maximum as compared to HSA; however, the fluorescence intensity is 1.9 times reduced, indicative for a more solvent exposed environment (Halfman & Nishida, 1971a, 1971b; Eftink & Ghiron, 1977). All recombinant fragments of albumin with intact domain II display emission maxima shifted to a shorter wavelength compared to the native molecule (HSA-DOM I-II, 337 nm; HSA-DOM IB-II, 335 nm; HSA-DOM II, 333 nm). The fluorescence intensity at the emission maximum of HSA-DOM I-II is 14% triggered, whereas that of HSA-DOM IB-II and HSA-DOM II is quenched 1.6 and 2.5 times, respectively, denoting an altered environment of the tryptophan residue in these proteins.

Warfarin binding

The affinity of warfarin to all proteins studied was investigated by fluorescence spectroscopy. This method is based on the considerable increase in intrinsic warfarin emission that occurs when the ligand is transferred from a polar, aqueous environment to a hydrophobic binding site on the albumin molecule. In all cases, the binding of this anticoagulant drug resulted in a rise of warfarin fluorescence intensity accompanied by a shift of the emission maximum to shorter wavelength, as was previously reported for HSA (Maes et al., 1982; Kasai-Morita et al., 1987; Otagiri et al., 1987; Epps et al., 1995). However, this method was unapplicable to the investigation of the binding of warfarin to HSA-DOM I, because the fluorescence intensity increase observed was only fivefold compared to about 10-fold with the other recombinant fragments and the native molecule, indicating a binding mechanism with substantially different fluorescence properties. This observation was further corroborated by the results of the ICD experiments.

The binding isotherms of warfarin bound to HSA, HSA-DOM I-II, HSA-DOM I-IIA, HSA-DOM IB-II, and HSA-DOM II are shown in Figure 5, and the calculated stoichiometric binding constants K_i are given in Table 3. One high-affinity site and one with lower affinity were found for HSA in agreement with previous reports (Sudlow et al., 1975a; Maes et al., 1982; Kragh-Hansen, 1985; Larsen et al., 1985; Bos et al., 1989; Kosa et al., 1997). The order of affinity constants for the native molecule and its recom-

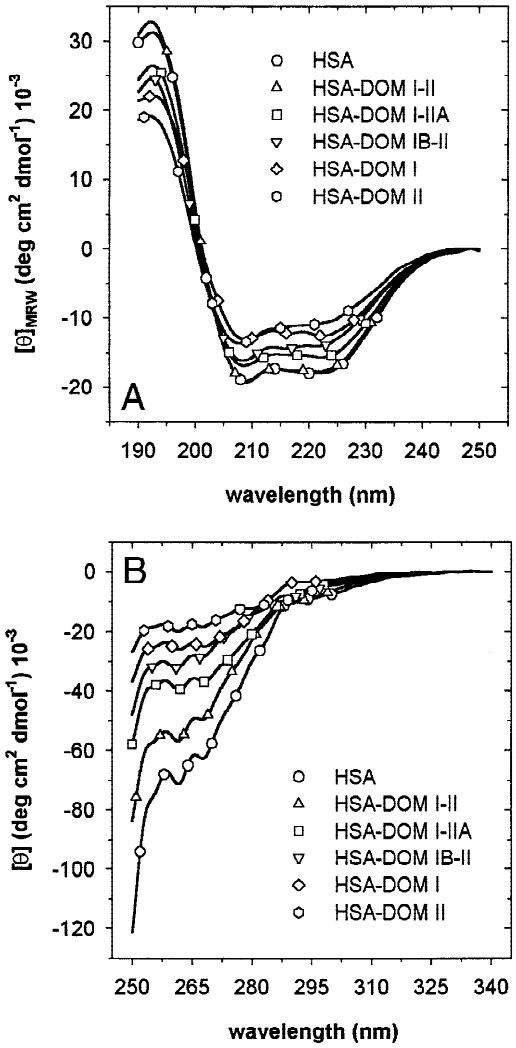

Fig. 3. A: Far-UV CD spectra of HSA (○), HSA-DOM I-II (△), HSA-DOM I-IIA (□), HSA-DOM IB-II (▽), HSA-DOM I (◇), and HSA-DOM II (⬡) in 10 mM sodium phosphate buffer, pH 7.4, 25 °C. Protein concentration for HSA and HSA-DOM I-II were 2 μM and for HSA-DOM I-IIA, HSA-DOM IB-II, HSA-DOM I, and HSA-DOM II 4 μM. **B:** Near-UV CD spectra. The protein concentrations were 20 μM in 0.1 M sodium phosphate buffer, pH 7.4, 25 °C. Symbols are as in **A**.

Table 2. *Fraction of secondary structural elements*[a]

Protein	f_α[b]	f_β[c]	f_R[d]
HSA (defatted)	0.66	0.06	0.28
HSA-DOM I-II	0.67	0.19	0.13
HSA-DOM I-IIA	0.62	0.22	0.16
HSA-DOM IB-II	0.55	0.28	0.16

[a] Fraction of secondary structural elements of HSA-DOM I, HSA-DOM II, and HSA-DOM III can be found in Dockal et al. (1999).
[b] f_α, fraction of α-helix content.
[c] f_β, fraction of β-sheet content.
[d] f_R, fraction of remainder structure elements.

binant fragments was found to be: HSA-DOM I-II = HSA > HSA-DOM IB-II > HSA-DOM II > HSA-DOM I-IIA. The high-affinity site of HSA is fully preserved in HSA-DOM I-II, as indicated by similar binding parameters, whereas binding to a secondary binding site is about four times weaker compared to HSA, demonstrating the dissection of the different binding sites of the whole albumin molecule.

The affinity of HSA-DOM IB-II to warfarin was found to be about fourfold lower compared to HSA, leading us to the conclusion that this fragment harbors sufficient information for the formation of the high-affinity warfarin binding site of HSA. The slight structural flexibility, which is indicated by the CD measurements in the far- and near-UV region (Fig. 3A,B), is likely to diminish the affinity for warfarin. In contrast, the affinity of HSA-DOM II and HSA-DOM I-IIA for warfarin is 23 and 36 times reduced, respectively, denoting that these two recombinant fragment are missing parts of the primary warfarin binding site of the native albumin molecule and/or that slight structural changes, which are especially obvious for HSA-DOM II (Fig. 3A,B), affect the ligand binding.

For a more detailed investigation of the molecular mechanism of the warfarin/protein interaction, we performed UV difference

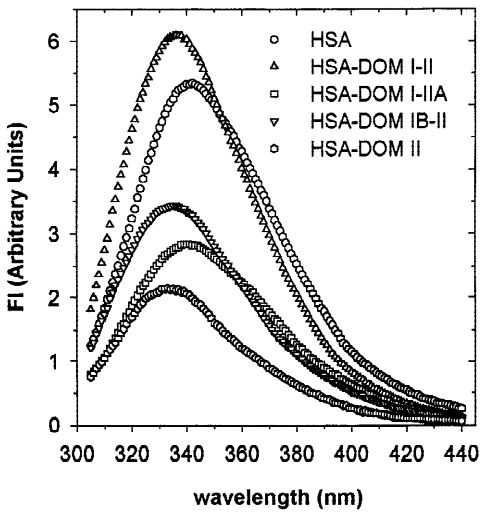

Fig. 4. Tryptophanyl emission spectra. The protein concentrations were 4.5 μM in 10 mM sodium phosphate buffer, pH 7.4, and 25 °C. Excitation wavelength, 295 nm. Symbols are as in Figure 3A.

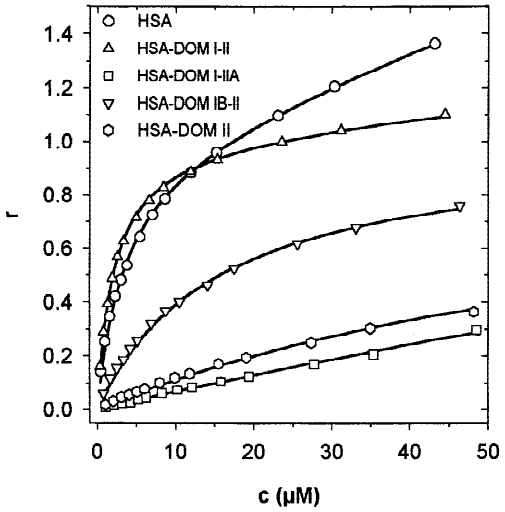

Fig. 5. Calculated warfarin binding isotherms. Observed points (mean), *r* vs. *c*, and the binding curves derived from the stepwise binding equilibrium equation (Adair equation). Experiments were performed in 67 mM sodium phosphate buffer, pH 7.4, and 25 °C. Symbols are as in Figure 3A.

(Fig. 6) and induced CD measurements (Fig. 7). Upon binding of warfarin to all proteins (molar ratio protein/warfarin: 3/1), a shift in the warfarin absorbance to a longer wavelength was observed. Similar difference spectra arose for all proteins with a maximum at 333 nm, a positive shoulder near 320 nm, and minima near 290 and 300 nm, which is in accordance with previous findings for HSA (Maes et al., 1982). The titration course, plotted in the inset of Fig. 6 (Δ Absorbance at 333 nm vs. total protein concentration), cleary reflects the binding behavior of the proteins as determined by the fluorescence studies, showing the strongest, specific interaction of warfarin with HSA-DOM I-II and HSA on the one hand, as indicated by the attainment of saturation at higher protein/warfarin ratio, and, on the other hand, a weak, hydrophobic interaction with HSA-DOM I.

Binding of warfarin to all proteins studied alters the CD spectra. By subtracting the ellipticities of the proteins without added ligand, induced CD spectra are obtained, which was reported to arise solely due to the binding of warfarin to the macromolecules (Brown & Müller, 1978). As shown in Figure 7, binding of war-

Table 3. *Binding of warfarin to HSA and its recombinant fragments*[a]

	Stoichiometric binding constants	
Protein	K_1 (M^{-1})	K_2 (M^{-1})
HSA	$2.8 \pm 0.1 \times 10^5$	$1.4 \pm 0.2 \times 10^4$
HSA-DOM I-II	$4.6 \pm 0.5 \times 10^5$	$3.6 \pm 0.6 \times 10^3$
HSA-DOM IB-II	$6.6 \pm 0.6 \times 10^4$	
HSA-DOM II	$1.2 \pm 0.1 \times 10^4$	
HSA-DOM I-IIA	$7.7 \pm 0.7 \times 10^3$	

[a] The given values are means ± SD of five experiments.

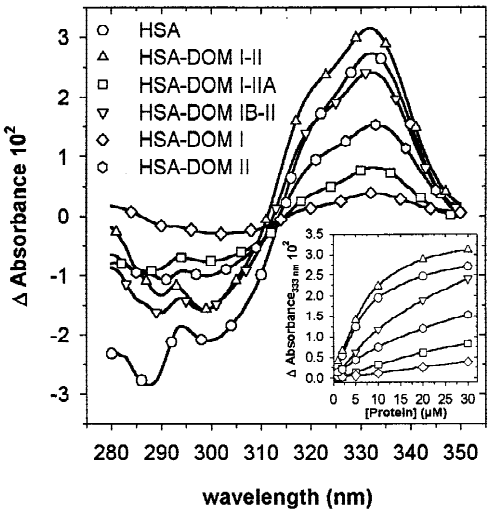

Fig. 6. Ultraviolet difference spectra of protein-bound minus free warfarin. The warfarin concentration was 10 μM, the protein concentrations were 30 μM in 67 mM sodium phosphate buffer pH 7.4, at 25 °C. **Inset:** Difference absorbance at 333 nm vs. total protein concentration. Symbols are as in Figure 3A.

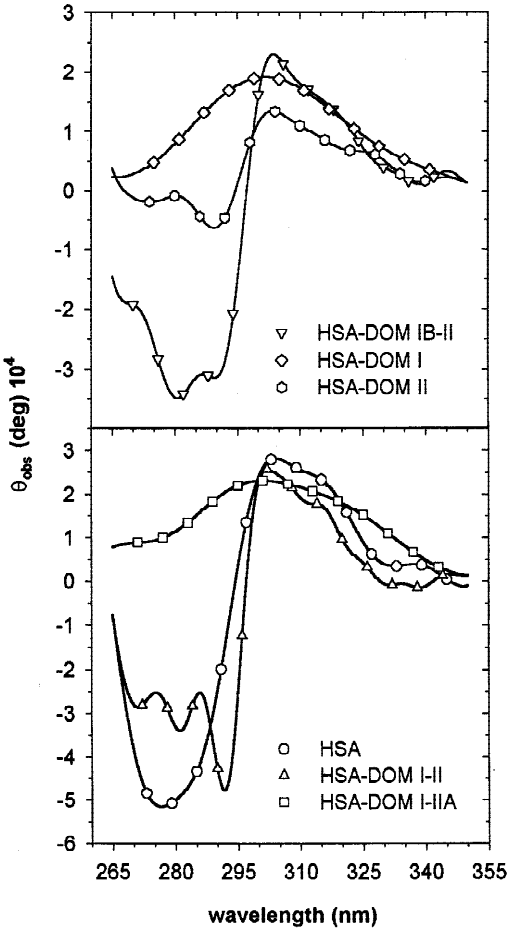

Fig. 7. ICD (expressed in degrees) of warfarin bound to the different proteins at a molar ratio (warfarin/protein) of 0.5. Protein concentrations were 20 μM in 0.1 M sodium phosphate buffer, pH 7.4, at 25 °C. Symbols are as in Figure 3A.

farin to HSA generates weak extrinsic Cotton effects with a positive band at 305 nm, a negative one at 277 nm, and a positive shoulder near 340 nm, as was recently demonstrated by our group (Dockal et al., 1999) in accordance with previous findings (Brown & Müller, 1978; Fehske et al., 1979, Otagiri et al., 1987; Bos et al., 1988). Warfarin, bound to all recombinant fragments with intact domain II (HSA-DOM I-II, HSA IB-II, HSA-DOM II), shows similar ICD spectra, especially with respect to positive Cotton effects; however, a slight shift of the positive maximum to 303 nm is obvious for all three proteins. Negative Cotton effects are clearly split into additional minima, which is most pronounced for HSA-DOM I-II. These findings might indicate that in this wavelength region the CD signals do not solely arise from bound warfarin, but probably are affected by interaction of the ligand with aromatic amino acid residues. Due to the structural differences indicated above, we cannot exclude that these influences are different for each of the proteins studied, representing a combination of ellipticities arising from warfarin binding on the one hand, and from perturbations of aromatic amino acid residues and disulfide bridges on the other hand. The interaction of warfarin with HSA-DOM I-IIA and HSA-DOM I gives a broad positive band with a maximum near 302 nm for both proteins, indicative for a diverse binding mechanism.

Discussion

The binding of a drug to HSA influences its metabolism, distribution, and elimination from the circulation. Cobinding of two drugs or displacement of one drug by another may alter the therapeutic drug level and can lead to serious health conditions. Thus, detailed knowledge of the binding site(s) of a drug on albumin and of their relative strengths is important. Besides classical methods for the determination of binding affinities that do not yield any information on which binding site is occupied by a ligand, a way to tackle this problem is by performing competitive binding experiments with ligands of known binding site. Another approach is to measure the binding parameters of natural or cloned mutants of albumin or to study the binding characteristics of fragments of HSA.

In a recent study, we reported on the structural characterization and the ligand binding properties of the three recombinant domains of HSA (Dockal et al., 1999). Warfarin was chosen as an interaction partner with HSA-DOM II, because many authors described that warfarin binds in drug binding site I (for reviews, see Fehske et al., 1981; Kragh-Hansen, 1981, 1990; Peters, 1996; Vorum, 1999), proposed to be located in subdomain IIA of the native molecule (He & Carter, 1992; Carter & Ho, 1994). However, although HSA-DOM II was found to be intact from a structural point of view, binding of warfarin to this recombinant protein did not fully reflect the qualitative characteristics of binding of warfarin to HSA, which we interpreted by the lack of important contributions of C-terminal parts of domain I to the functionality of site I of HSA (Dockal et al., 1999). To verify the location of the warfarin binding site and to identify those parts of HSA that are essential for the structural and functional integrity of this site, three additional fragments of HSA, combining the two N-terminal domains and/or subdomains, were cloned, and the interaction with warfarin was analyzed in a qualitative and quantitative fashion.

We decided to use a racemic mixture of warfarin in the present study because this is the form given clinically, although it was

demonstrated previously that the affinity of HSA for the (S)-enantiomer of warfarin is higher than for the (R)-form (Loun & Hage, 1996).

Fluorescence spectroscopy was chosen to analyze the binding of warfarin to the recombinant fragments and HSA, because the arising fluorescence spectra provide not only quantitative binding data but also qualitative information about the structural environment of the ligand molecule, which is advantageous compared to other methods such as equilibrium dialysis or ultrafiltration that yield only quantitative results. The fluorescence quantum yield of warfarin bound to all proteins increases significantly, and the emission maximum is shifted to shorter wavelength, which was ascribed to the diminution of the internal rotation of the acetonylbenzyl group of the warfarin molecule as it is fixed by the macromolecule in a rigid position in the hydrophobic binding site (Kasai-Morita et al., 1987). Additional indications for the transfer of the drug to a hydrophobic surrounding on the recombinant proteins and the native albumin are provided by difference spectroscopic measurements (Fig. 6), because dissolving warfarin in ethanol, which has a lower dielectric constant and is less polar than water, was reported to yield similar difference spectra compared to our results (Maes et al., 1982). Even though interaction of warfarin with HSA-DOM I leads to a difference spectrum, denoting hydrophobic contacts, the rise in fluorescence intensity when all of the ligand molecules are bound to this protein is two times less compared to that of HSA and the other fragments harboring subdomain IIA of HSA, indicating a different binding mechanism, which is also supported by ICD experiments discussed below. Therefore, the fluorescence spectroscopic method was inapplicable for the estimation of the binding parameters of HSA-DOM I.

The fact that HSA-DOM I-II and the native molecule have similar affinity for their first binding site (Fig. 5; Table 3) denotes that this double domain has sufficient information for forming the primary warfarin binding site, and that domain III is not essential for the binding process. The folding pattern of HSA-DOM I-II clearly resembles that of the native molecule, indicative to be domain III independent, as deduced by far- and near-UV CD spectroscopy (Fig. 3A,B), which yield information on the secondary structure and the asymmetry of the structure around the aromatic amino acid side chains and disulfide bridges, respectively. Reduced ellipticity and slight differences in the fine structure in the near-UV CD spectrum of HSA-DOM I-II allude to the reduced number of disulfide bridges and different relative content of aromatic residues. The increased β-sheet content of this fragment compared to HSA is explained by two possible structural alterations: either one of the C-terminal interdomain helix, which is newly exposed in this recombinant protein (Fig. 1A) and stabilized in the native molecule by both a hydrogen bond (Glu383, h10(II)-h1(III)-Arg485, h6(III)) and some hydrophobic contacts to helix 4 in subdomain IIIA (Val381, Pro384, h10(II)-h1(III)-Met446, h4(III)), and/or by rearrangements of portions of the surface patch located almost centrally between subdomain IB and IIB, which, in the context of the whole protein is covered by parts of subdomain IIIA and two side chains of subdomain IIIB (Fig. 1A) (Carter & Ho, 1994; Curry et al., 1998; Sugio et al., 1999). Slight structural changes in this region are further indicated by the increase in tryptophanyl fluorescence intensity and the shift of its emission maximum to lower wavelength in this protein (Fig. 4), denoting a more hydrophobic surrounding (Halfman & Nishida, 1971a, 1971b) and/or sandwiching of the indole side chain in a more rigid portion of the protein matrix compared to HSA (Eftink & Ghiron, 1976). However, these alterations, with respect to an altered environment of the tryptophanyl residue, do not dramatically change the binding orientation and mechanism of warfarin, which is indicated by the similarity of the ICD spectra of HSA-DOM I-II and HSA, especially in the wavelength region above 300 nm. Brown and Müller (1978) reported that BSA, which has quantitative warfarin binding parameters similar to HSA (Panjehshahin et al., 1992; Kosa et al., 1997), displays a fundamentally different ICD spectrum of warfarin, denoting a clearcut difference in the binding orientation and chemistry of the ligand. Below 300 nm, the ellipticities are clearly split into additional minima, which might indicate that in this wavelength region the CD signals do not solely arise from bound warfarin, but probably are affected by interaction of the ligand with aromatic amino acid residues, which is corroborated by findings of Larsen et al. (1985), who ascribed a small shoulder at 285 nm in the bound warfarin absorption spectrum to a perturbation of the tyrosine absorption in albumin, also explaining the deviations in the UV difference spectra of warfarin bound to HSA-DOM I-II and HSA, respectively (Fig. 6). Like HSA, HSA-DOM I-II harbors a secondary binding site for warfarin (Fig. 5; Table 3). The fact that the binding constant of this secondary site is four times lower compared to that of HSA indicates that either a secondary binding site located on this fragment has been truncated due to the absence of domain III or that a binding site, presumably located on domain III of the native molecule, is naturally absent in HSA-DOM I-II underlining the usefulness of albumin fragments for the dissection of drug binding sites with different affinity for the same ligand.

HSA-DOM IB-II has a four times lower affinity for warfarin compared to the high-affinity constant of albumin. Because the affinity of this ligand to the secondary binding site on HSA was found to be 20-fold lower than the corresponding primary affinity constant, which is in accordance with the findings of Sudlow et al. (1975a), we believe that the primary warfarin binding site is largely preserved on this fragment. However, slight structural alterations, indicated by differences in the shape of the far-UV CD spectra (Fig. 3A) as well as by the reduced α-helical, and the increased β-sheet content (Table 2) may interfere with the binding process, leading to reduced affinity. The emission spectrum of the tryptophanyl residue clearly shows that the microenvironment of this residue is modified (Fig. 4). The reduction of fluorescence intensity and the shift of the energy maximum to a lower wavelength can be ascribed to collisional quenching caused by surrounding solvent molecules, denoting a more hydrophilic environment (Halfman & Nishida, 1971a, 1971b). An impairment of the structure of helix 2 in subdomain IIA, which harbors the lone tryptophanyl residue, as indicated by the reduced helical content of HSA-DOM IB-II compared to HSA and HSA-DOM I-II (Table 2) as well as by the different shape of the far-UV CD spectrum (Fig. 3A), could further explain the reduced fluorescence intensity (Cowgill, 1968). Moreover, it seems that this recombinant fragment has a more fluctuating structure, as can be deduced from the transitions of the aromatic amino acid residues and the disulfide bridges (Fig. 3B), because HSA-DOM IB-II has a reduced ellipticity over the whole wavelength region compared to HSA-DOM I-IIA, which is a protein with a similar relative content of aromatic amino acid residues and disulfide bridges. Beyond this, it is striking from the results of the CD spectroscopic measurements in the far- and near-UV region that proteins with two newly exposed surface patches (HSA-DOM IB-II, HSA-DOM II) have a more flexible structure compared to proteins with only one such new surface area (Fig. 1B,C). It is

obvious from the crystal structures (Carter & Ho, 1994; Curry et al., 1998; Sugio et al., 1999) that residues clustered in helix 4 of subdomain IIA, which are highly conserved (Carter & Ho, 1994), are stabilized by hydrophobic contacts and H-bonds to residues in helices 1, 2, and 4 of subdomain IA in the native molecule. The fact that HSA-DOM IB-II is missing these contacts may lead to a more flexible structure with negative effects on the hydrophobic binding pocket, which in turn would affect the affinity of the ligand and the fluorescence behavior of the tryptophanyl residue. However, the orientation of the bound ligand seems to be largely intact, as indicated by the similarity of the ICD and UV difference spectra with those given by HSA and HSA-DOM I-II (Figs. 7, 6).

HSA-DOM II does not have the potential to bind to warfarin in a manner similar to the native protein, as indicated by the quantitative binding parameters, which show a 23-fold reduced affinity for this ligand. In consideration of the fact that HSA-DOM I-II and, to a lesser extent, HSA-DOM IB-II are functional as warfarin binding proteins, the loss in affinity of this recombinant domain can be either ascribed to the absence of important residues located in subdomain IB of the native molecule and/or to structural flexibility, which is demonstrated by the results of the CD spectroscopic measurements (Fig. 3A,B). Carter and Ho (1994) reported on the participation of the side chain of Tyr150 of subdomain IB to binding site I, the phenolic side chain of which is stabilized by Arg257 by cation–π interactions (Dougherty, 1996; Mecozzi et al., 1996) in the native structure. The structures of warfarin and several of its derivatives complexed with HSA have recently been determined by X-ray structural analysis and should aid in the clarification of other residues involved in the binding process (D.C. Carter et al., unpubl. obs.). In addition to the contacts that are lost due to the absence of subdomain IA, as discussed above, three additional electrostatic contacts and/or H-bonds to residues located in subdomain IB, which stabilize the interdomain helix h10(I)–h1(II) (Asp108 to Arg197), the loop between h5(II) and h6(II) (Lys159 to Glu285), as well as h6(II) itself (Glu153 to His288) are lost as deduced from the cystal structures (Carter & Ho, 1994; Curry et al., 1998; Sugio et al., 1999), which may affect the warfarin binding further. Interestingly, in proteins with intact domain II and successively fewer parts of domain I (HSA-DOM I-II, HSA-DOM IB-II, and HSA-DOM II), the tryptophanyl fluorescence is reduced, and the emission maximum is shifted to a higher energy, indicating a further impairment and/or exposure of helix 2, as discussed above. The nonnative end of the N-terminal helix (h10(I)–h1(II)), which lacks its natural helix termination residues, may lead to destabilization of this element and to a reduction of protecting contacts in the vicinity of the tryptophanyl residue, thereby exposing this residue further. In the native structure, Trp214 is tightly packed against highly conserved hydrophobic residues of helices 2, 3, and 9 of domain II (Ala215, Phe228, Val231, Leu238, Val343, Val344, Leu347), some of which are participating in the binding process (Ala215, Leu238), as described by Carter and Ho (1994). Nevertheless, the binding mechanism and orientation of warfarin bound to HSA-DOM II seems to be principally intact, as indicated by the results of the ICD experiments (Fig. 7).

Similar to HSA-DOM II, it is obvious from the binding parameters (Fig. 5; Table 3) that HSA-DOM I-IIA has lost the high-affinity binding to warfarin, even though the secondary and the tertiary structure is intact (Fig. 3A,B) and the fluorescence behavior of bound warfarin is similar to the other proteins, harboring an intact domain II. In the native molecule, hydrophobic residues clustered in helix 2 (Ala213–Ala217) and helix 3 of subdomain IIA (Phe228, Val231, Leu238) are tightly packed against hydrophobic ones located in helix 8 (Val325, Met329, Leu331, Tyr332) and helix 9 (Val343, Val344, Leu346, Leu347) of subdomain IIB (Fig. 1A,D), all of which are highly conserved in the albumin gene family. The deprivation of these important hydrophobic packing contacts, due to the fragmentation, may result in a more fluctuating structure at the entrance of the binding pocket and, therefore, to a loss in binding strength, corroborating the great importance of subdomain IIB to the structural and functional integrity of drug binding site I. A more hydrophilic environment of the tryptophanyl residue compared to the native molecule is strongly suggested by the 1.9 times reduced fluorescence intensity by adoption of the same emission maximum (Halfman & Nishida, 1971a, 1971b; Eftink & Ghiron, 1976). In contrast to the ICD spectra of HSA and fragments harboring an intact domain II, HSA-DOM I-IIA displays extrinsic Cotton effects similar to HSA-DOM I (Fig. 7), which suggests that either native contacts to subdomain IIB are important for the binding orientation or the binding mechanism, or more likely, that HSA-DOM I harbors parts of the primary binding site with different binding properties, as discussed recently (Dockal et al., 1999). However, there are differences in the binding behavior of warfarin bound to HSA-DOM I-IIA and HSA-DOM I, such as the fact that warfarin bound to HSA-DOM I-IIA displays fluorescence properties comparable to HSA and the fragments harboring intact domain II, and that the UV difference titration reaches a saturation level, underlining the importance of the contact region between domain I and subdomain IIA to the binding process either in an active manner or solely by stabilizing the structure of the warfarin binding site.

Conclusion

Using a molecular biological approach, we have successfully dissected the albumin molecule and were able to identify those parts of HSA that are essential for the formation of a fully functional warfarin binding site, concluding that domain III does not participate in the binding process. With our curent series of papers (Dockal et al., 1999, 2000), we present the first attempt of such a systematic and targeted approach, and together with the study and observation of the atomic coordinates of albumin, a new opportunity for gaining additional insight into this traditionally exceptionally well-studied protein is available. Although the five recombinant fragments of albumin have folds comparable to the native molecule, we show here that parts of subdomain IB and IIB perform stabilizing functions and/or are themselves part of warfarin binding site, making them indispensable for warfarin binding to be qualitatively and quantitatively comparable to HSA. To a lesser extent, this is also true for subdomain IA, which strongly argues that only combinations of overlapping fragments can provide sufficient information on the exact localization of a drug binding site on albumin. The same kind of approach should also prove fruitful for the investigation of drug binding site II, which is centered on subdomain IIIA of HSA and to this aim experiments are currently in progress in our lab.

Materials and methods

Materials

HSA (essentially free of fatty acids and globulin) was from Sigma (St. Louis, Missouri, cat. No. A 3782) and was further purified by

size-exclusion chromatography over Superdex 200 prep grade and delipidated by passing over a Lipidex-1000 column. Racemic warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) was from Sigma (cat. No. A 4571) and was used as received. The concentrations of all warfarin solutions were checked using 13,900 M^{-1} cm^{-1} for the molar absorption at 308 nm (Maes et al., 1982). Blue Sepharose 6 Fast Flow, Superdex 200 prep grade and Butyl Sepharose 4 Fast Flow were from Amersham Pharmacia Biotech (Uppsala, Sweden). Lipidex-1000 was from Canberra-Packard (Schwadorf, Austria) and TSKgel Ether-5PW from TosoHaas (Stuttgart, Germany). All other reagents were of analysis grade.

Cloning, expression, purification, and characterization

All domains (HSA-DOM I and HSA-DOM II) and combinations of domains and/or subomains (HSA-DOM I-II, HSA-DOM I-IIA, and HSA-DOM IB-II) were cloned and expressed as has been described before in detail (Dockal et al., 1999). The recombinant fragments encompassed the following amino acid residues of HSA: HSA-DOM I-II, 1–385; HSA-DOM I-IIA, 1–299; HSA-DOM IB-II, 108–385; HSA-DOM I, 1–197; HSA-DOM II, 189–385. Due to the restriction site used for cloning, all recombinant fragments had Glu-Phe as the N-terminal amino acids.

The purification of HSA-DOM I and HSA-DOM II was performed as reported, using Cibacron Blue affinity chromatography as the first step in the procedure (Dockal et al., 1999).

For HSA-DOM I-II, HSA-DOM I-IIA, and HSA-DOM IB-II the first purification step was performed by hydrophobic interaction chromatography. Ammonium sulfate was added to the protein containing culture supernatants (batch size 1.8 L, total amount of protein applied: 100–300 mg) to a final concentration of 1.8 M for HSA-DOM I-II and HSA-DOM IB-II, and 1.0 M for HSA-DOM I-IIA, respectively. Subsequently, the pH was adjusted to 7.0 with sodium hydroxide. For HSA-DOM I-II and HSA-DOM IB-II, a TSKgel Ether-5PW column (XK26, 70 mL gel) was used, while HSA-DOM I-IIA was purified over a Butyl Sepharose 4 Fast Flow column (XK26, 70 mL gel). The columns were pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing the appropriate concentration of ammonium sulfate. After washing with 10 column volumes of equilibration buffer, the proteins were eluted by a linear gradient against 50 mM sodium phosphate buffer, pH 7.0 in 10 column volumes. Fractions containing the protein of interest were pooled and concentrated by ultrafiltration (Centriprep 10, Amicon, Millipore, Bedford, Massachusetts). Subsequently, the concentrated protein solutions were applied to a size-exclusion column (XK26/60, Superdex 200 prep grade) using 50 mM sodium phosphate buffer, pH 7.0 containing 0.4 M NaCl as running buffer. For delipidation, a chromatography over Lipidex 1000 was performed at 37 °C according to Glatz and Veerkamp (1983). After extensive dialysis of the protein samples against water, the preparations were concentrated as above and lyophilized. The purity and homogeneity of the protein preparations were checked by SDS-PAGE, native-PAGE, and isoelectric focusing according to standard procedures.

Relative masses of the proteins were determined by MALDI-TOF-MS with a Dynamo instrument (Thermo BioAnalysis, Santa Fe, New Mexico) using 3,5-dimethoxy-4-hydroxycinnamic acid as the UV-absorbing matrix. Calibration of the instrument was performed with carbonic anhydrase.

Protein concentrations were measured by their absorbance at 278 nm on a Hewlett Packard HP8453 spectrophotometer. For HSA, an absorption coefficient ($A^{0.1\%}_{278nm,1cm}$) of 0.58 was used (Bos et al., 1988). The absorption coefficients of the recombinant fragments of HSA were determined using the method of Gill and von Hippel (1989) and were found to be 0.70 for HSA-DOM I-II, 0.56 for HSA-DOM I-IIA, 0.79 for HSA-DOM IB-II, 0.50 for HSA-DOM I, and 0.79 for HSA-DOM II.

Spectroscopic measurements

Equilibrium binding studies, fluorescence spectroscopy

Fluorescence was measured in 67 mM sodium phosphate buffer at pH 7.4 with an Hitachi F-4500 spectrofluorimeter equipped with a thermostated stirred cell holder at 25 °C in a 0.5 × 1 cm cell (0.5 cm at the excitation and 1 cm at the emission side) at 380 nm with the excitation wavelength set to 320 nm. The bandwidths of both the excitation and emission were 5 nm. To determine the binding affinity of the warfarin–protein interaction, four separate titrations, each of them repeated five times, were performed and evaluated according to Maes et al. (1982). For estimating the intrinsic molar fluorescence of protein-bound warfarin, only data points at a protein/warfarin molar ratio over 10/1 were used to keep the error of the extrapolation under 1%, as described by Rajkowski (1990). All measured fluorescence intensities were corrected for inner filter effects according to Chignell (1972) and for buffer baseline fluorescence.

The stoichiometric association constants (K_i) were calculated by fitting the obtained number of mol of warfarin bound/mol of protein (r) to the concentration of free warfarin (c) with a nonlinear least-squares regression analysis according to the stoichiometric binding equation:

$$r = \frac{cK_1 + 2c^2K_1K_2 + 3c^3K_1K_2K_3 + \cdots + Nc^NK_1K_2K_3\cdots K_N}{1 + cK_1 + c^2K_1K_2 + c^3K_1K_2K_3 + \cdots + c^NK_1K_2K_3\cdots K_N}.$$

In this equation, K_i is the stoichiometric constant for step i, and N is the maximal value of r, when $c \rightarrow 8$.

To obtain the fluorescence spectra of the lone tryptophanyl residue of HSA, HSA-DOM I-II, HSA-DOM I-IIA, HSA-IB-II, and HSA-DOM II the proteins were excited at 295 nm, to provide that the light was absorbed almost entirely by this residue. All spectra were recorded at least twice in a 0.5 × 1 cm thermostated stirred cell (0.5 cm at excitation and 1 cm at emission side) at 25 °C, with the excitation and the emission slit width set to 5 nm. The protein concentrations were 4.5 μM in 10 mM sodium phosphate buffer, pH 7.4. Each spectrum was corrected for buffer baseline fluorescence.

UV difference spectra

All spectra were recorded with a Hewlett Packard HP8453 spectrophotometer equipped with a thermostated stirred cell holder at 25 °C. The baseline was set to zero with buffer (67 mM sodium phosphate, pH 7.4). To obtain difference spectra of warfarin bound to the proteins, 10 μM warfarin was titrated with a solution containing 160 μM protein and 10 μM warfarin to avoid dilution of warfarin. In a reference titration, a 160 μM protein solution was added stepwise to buffer to reach the same final protein concentrations as above. All spectra were recorded three times. Subtraction of the warfarin spectrum (10 μM) and of the protein reference spectra from the warfarin/protein spectra resulted in difference spectra, which arise from warfarin bound to protein.

CD spectroscopy

Measurements were performed using a Jasco J-600 spectropolarimeter equipped with a thermostated cell holder at 25 °C. In the far-UV region (250–190 nm), scans of 2 μM HSA and HSA-DOM I-II and 4 μM HSA-DOM I-IIA, HSA-DOM IB-II, HSA-DOM I, and HSA-DOM II in 10 mM sodium phosphate buffer, pH 7.4 were carried out in a 1 mm cell as described recently (Dockal et al., 1999, 2000). The data were expressed as mean residue ellipticity ($[\theta]_{MRW}$, deg cm^2 dmol^{-1}), using the mean residue weights of 114.0 g mol^{-1} for the intact molecule, 114.3 for HSA-DOM I-II and HSA-DOM I-IIA, 114.6 for HSA-DOM IB-II, 114.9 for HSA-DOM I, and 113.2 for HSA-DOM II.

The fractional content of the secondary structure elements of the proteins was calculated from the far-UV CD spectra using the procedure of Provencher and Glöckner (CONTIN) with a set of 16 reference proteins (Provencher & Glöckner, 1981; Provencher, 1982a, 1982b).

CD measurements in the near-UV region and ICD experiments were performed in a 10 mm cell at 25 °C at a protein concentration of 20 μM for all tested proteins in 0.1 M sodium phosphate buffer, pH 7.4, as reported previously (Dockal et al., 1999).

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