The Three Recombinant Domains of Human Serum Albumin

STRUCTURAL CHARACTERIZATION AND LIGAND BINDING PROPERTIES

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Michael Dockal§, Daniel C. Carter§, and Florian Rüker‡

From the §Institute of Applied Microbiology, University of Agricultural Sciences, Muthgasse 18, A-1190 Vienna, Austria and ¶New Century Pharmaceuticals Inc., Huntsville, Alabama 35824

In an attempt to systematically dissect the ligand binding properties of human serum albumin (HSA), the gene segments encoding each of its three domains were defined based on their conserved homologous structural motifs and separately cloned into a secretion vector for Pichia pastoris. We were able to establish a generally applicable purification protocol based on Cibacron Blue affinity chromatography, suggesting that each of the three domains carries a binding site specific for this ligand. Proteins were characterized by SDS-polyacrylamide gel electrophoresis, isoelectric focusing, gel filtration, N-terminal sequencing, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, as well as near- and far-UV CD. In addition to the affinity chromatography ligand Cibacron Blue, binding properties toward hemin, warfarin, and diazepam, each of which represents a standard ligand for HSA, respectively, were investigated by the measurement of induced circular dichroism. Clear experimental evidence is provided here for the location of the primary hemin binding site to be on domain I of HSA, and for the primary diazepam binding site to be on domain III. Furthermore, secondary binding sites were found for hemin to be located on domains II and III, and for diazepam on domain I. The warfarin binding site was located primarily on domain II, while on domain I, a secondary binding site and/or parts of the primary binding site were found.

Human serum albumin (HSA) is a globular protein of 585 amino acids, which accounts for about 60% of the total protein in blood serum. In the serum of human adults, the concentration of albumin is ~40 mg/ml. It has an exceptional binding capacity for a wide range of endogenous and exogenous ligands, and its abundance makes it an important determinant of the pharmacokinetic behavior of many drugs (1, 2).

HSA is composed of three structurally similar globular domains, each of which contains two subdomains, denominated subdomain IA, IB, IIA, IIB, IIAB, and IIIB (Fig. 1). It is generally accepted today that albumin evolved from an ancestral protein of about 190 amino acids by triplication of this primordial domain. This view is most impressively documented by the striking internal homology of the atomic structure of the protein (3, 4), by the unique arrangement of disulfide loops that repeat as a series of triplets (1), and by the symmetrical location of the introns and exons of the albumin gene on chromosome 4 (5).

Although still partly controversial, consensus exists today that there are two high affinity binding sites (6) for small heterocyclic or aromatic compounds (located on subdomains IIA and IIB), two to three dominant long-chain fatty acid binding sites (located on subdomains IB and IIIB) (1), and two distinct metal-binding sites, one involving Cys-34 and the other the N terminus, making a total of six dominant areas of ligand association to albumin (2). Recently, a crystal structure was reported that showed five molecules of the medium-chain-length fatty acid myristate bound in different pockets of the HSA molecule (7).

Based on the observation of the quasi-independence of the three HSA domains, attempts have been made early on to use fragments of HSA produced by proteolytic or chemical cleavage to define the binding sites for several ligands (Refs. 8–10; see Ref. 11 for a review). However, these studies have been dictated by the location of the cleavage sites on the primary sequence and did not allow for a deliberate selection of the exact size of the domains and their boundaries. Bos and colleagues (12–14) used a peptic fragment of HSA encompassing domain I and II (residues 1–387) and a tryptic fragment encompassing domain II and III (residues 198–585) to study the binding of warfarin and diazepam. Hrkal et al. (15) have investigated the binding of hemin to the three large cyanoogen bromide fragments of HSA. Compagnini et al. (16) have used cyanoogen bromide fragments of HSA in an attempt to define the Cibacron Blue binding sites by a chromatographic procedure and reported that two molecules of Cibacron Blue may be located in the hydrophobic pocket corresponding to subdomain IIA, with a third molecule in the hydrophobic site corresponding to subdomain IIIA. In a recent study, Kjeldsen et al. (17) have expressed domains I and III in Saccharomyces cerevisiae, but were unable to produce domain II in this expression system. Ligand binding studies with myristate and an insulin analogue acylated with myristic acid showed that the latter bound to both domains while myristate alone interacted only with domain III.

Details of the molecular interactions of ligand binding to albumin are known for only a small number of cases (1). Insight gained from such studies shows promise in improving the design of many pharmaceuticals and diagnostic agents.

In the present study, all three domains of HSA were produced by molecular cloning in a completely predetermined manner, based solely on the natural boundaries of the domains. This definite advance over the previous studies using either albumin fragments produced by proteolytic or chemical cleavage or incomplete sets of recombinant domains is expected to allow an in-depth study of the folding of the domains and of
Amersham Pharmacia Biotech. Lipidex-1000 was purchased from Can-berra-Packard. All other reagents were of analytical grade.

**Cloning**—The gene segments coding for the HSA domains were amplified by polymerase chain reaction with Pfu polymerase (Stratagene) using a cDNA clone of HSA as template. EcoRI restriction sites for the subsequent cloning into the secretion vector pPICZaA (Invitrogen) were attached at the same time. Recombinant plasmids were transfected into *Pichia pastoris* GS115 by electroporation following standard procedures (Invitrogen). All clones were confirmed by polymerase chain reaction and by DNA sequencing. Domains encompassed the following amino acids: HSA-DOM I, 1–197; HSA-DOM II, 189–385; HSA-DOM III, 281–585. All clones contained an extra two N-terminal amino acids (Glu-Phe) due to the restriction site used for cloning.

**Expression**—Standard protocols and media formulations were used (Invitrogen). Briefly, clones were grown up on YPD medium and transferred to BMMY medium for induction with methanol in baffled shake flasks. All incubations were performed at 30 °C on an orbital shaker at 150 rpm.

**Purification**—All manipulations were performed at 4 °C unless stated otherwise. Culture supernatants were harvested by centrifu-gation, followed by filtration and dialysis against PBS. 1800 ml of protein containing dialysate were passed through a Blue Sepharose column (150 ml gel volume in a XK50 column) preequilibrated with PBS. After washing with 10 volumes of PBS, elution was performed by 1 M KSCN in PBS. After dialysis against PBS and concentration by ultrafiltration (Centriprep 10, Amicon), samples were adjusted to a final NaCl concentration of 0.25 M and were applied to a Superdex 75 prep grade XK26/60 column (Amersham Pharmacia Biotech) using PBS containing 0.25 M NaCl as running buffer. For delipidation, a chromatography over Lipidex 1000 was performed at 37 °C according to Glatz and Veerkamp (18). Then, the protein samples were extensively dia lyzed against water. Finally, the preparations were concentrated by ultrafiltration as above and lyophilized. All purification steps were monitored with protein assay and SDS-PAGE. The Coomassie-stained SDS-PAGE gels were scanned, and the purity of the preparations was evaluated using the program Non-Linear-Dynamic LTD (Phoretica).

**Protein Solutions**—Protein concentrations were measured by their absorbance at 278 nm on a Hewlett Packard HP8453 spectrophotometer. For HSA, an absorption coefficient \( \varepsilon_{278\text{nm}} \) of 0.58 was used (12). For the HSA domains, the molar extinction coefficients were determined using the method of Gill and Hippel (19). A Bio-Rad protein assay with HSA standard solutions (Sigma) was used to confirm the photometric results.

**Analytical Gel Filtration**—Analysis was performed on a LKB instrument equipped with a variable wavelength monitor set at 214 nm for detection. Data were analyzed using a Nelson Analytical 900 Series interface and Turbochrom3 software. Samples (100 µg/ml) were run in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.15 M sodium chloride at a flow rate of 1 ml/min on a Superdex 75 HR10/30 column (Amersham Pharmacia Biotech). For calibration, a low molecular weight calibration kit (Amersham Pharmacia Biotech) was used.

**Matrix-Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI-TOF)**—Relative masses of the proteins were detected by MALDI-TOF with a Dynamo instrument (Thermo BioAnaly-sis) using 3,5-dimethoxy-4-hydroxycinnamic acid as the UV-absorbing matrix. Calibration of the instrument was performed with carbonic anhydride.

**For UV-CD Measurements**—Measurements were made using a Jasco J-600 spectropolarimeter using 1-mm cells at 25 °C in a thermostatic cell holder. Scans were made from 250 to 190 nm. The slit width was programmed for a half-bandwidth of 1 nm, and the dynode voltage never exceeded 0.6 kV. All spectra were recorded at least twice. The data were expressed as mean residue ellipticity \( \theta \) (degrees \( \times \text{cm}^2/\text{dmol r} ) \), using the mean residue weights of 113.7 for the intact molecule, 114.9 for HSA-DOM I, 113.2 for HSA-DOM II, and 113.0 for HSA-DOM III. The secondary structure elements of the proteins were determined from the CD spectra (1-nm interval) using the procedure of Provencher and Göckler (20, 21).

**Near UV-CD and Induced CD Measurements**—Measurements were made using a Jasco J-600 spectropolarimeter using 10-mm cells at 25 °C in a thermostatic cell holder. The slit width was programmed for a half-bandwidth of 1 nm and the dynode voltage never exceeded 0.4 kV. Over 250 nm, the results are expressed in molar ellipticity \( \theta \) (degrees \( \times \text{cm}^2/\text{dmol r} ) \). In the ICD experiments, all solutions were scanned from a wavelength at which no induced ellipticity was observed (hemin, 450–360 nm; warfarin, 360–260 nm; diazepam, 360–250 nm). Stock solutions of the ligands were prepared as follows and added stepwise to the protein solutions: 2 mM hemin in 10 mM NaOH, 4 mM...
warfarin in 0.1 M sodium phosphate buffer, pH 7.4; 4 mM diazepam in absolute ethanol (ethanol concentration in the ICD measurements never exceeded 1% and the effects of the organic solvent on the CD measurements were undetectable). Protein solutions were 10 μM for the hemin measurements and 20 μM for the experiments with warfarin and diazepam, in 0.1 M sodium phosphate buffer, pH 7.4. All spectra were recorded at least twice.

The induced ellipticity was defined as the ellipticity of the drug/protein mixture minus the ellipticity of the protein alone at the same wavelength and was expressed in degrees. Intrinsic ellipticity was shown to be undetectable for all ligands at the relevant wavelengths.

RESULTS

Cloning, Expression, Purification, and Biochemical Characterization

Expression of the domains was achieved as secreted proteins (due to the α-factor leader sequence present in the vector pPICZαA) in the supernatant of the recombinant Pichia clones with yields between 65 and 300 mg/liter. Based on the assumption that HSA harbors several Cibacron Blue binding sites, with each of the domains containing at least one of them, however weak, we successfully employed Blue Sepharose affinity chromatography as the central step in our purification procedure. Binding of HSA-DOM II and HSA-DOM III to the Blue Sepharose column was strongest, since hardly any protein could be detected in the flow-through of the column. The efficiency was somewhat lower for HSA-DOM I, but the overall yield of purified protein was in the same order of magnitude in all three cases. Evaluation of scans of the Coomassie-stained SDS-PAGE gel indicated a purity of the domain preparations greater than 98% (Fig. 2).

The chromatographic elution profile (data not shown) of the analytical gel filtration indicated that the HSA domains are present in our preparations as monomeric proteins. MALDI-TOF analysis gave the expected molecular masses of the proteins, and N-terminal sequencing resulted in the expected sequence. A summary of the physicochemical properties of the HSA domains is given in Table I.

Far and Near UV-CD Measurements

The CD spectra in the far ultraviolet region relate to the polypeptide backbone structures. A comparison of the spectra of the HSA domains with that of intact HSA on a mean residue weight basis is shown in Fig. 3. Minima of the spectra are given in Table I, and the results of the quantitative analysis using the CONTIN program (21) are given in Table II. The results obtained with defatted HSA are in good agreement with published data (22-24). The spectra of HSA-DOM I and HSA-DOM III are similar in shape with HSA, but have reduced intensity, indicating reduced α-helix content, which is supported by the quantitative analysis. This is more pronounced in HSA-DOM I than in HSA-DOM III. Evaluation of the results obtained with HSA-DOM II indicates that its structure resembles that of an α + β protein rather than that of an all α protein. This could be due to increased instability of the newly exposed long helical regions at the N and C termini of this domain, as can be seen in Fig. 1.

To obtain insight into the molecular environment of the aromatic side chains of HSA as well as the domains, near UV-CD measurements were performed. As can be seen in Fig. 4, HSA and its domains all have negative ellipticity in this wavelength region. For both HSA and each of the domains, we found negative extrema at 268 nm and 262 nm, confirming data that have previously been reported for HSA (25). The sum of the molar ellipticities of the three domains shows the same minima and shape as HSA, especially at wavelengths above 265 nm. Below 265 nm, deviations are observed, which could be due to the additional phenylalanine residue that has been added to the N terminus of each of the domains as a result of the cloning procedure.

Taken together, the results of the CD measurements indicate that the domains have secondary and the tertiary structures with significant contribution of α-helical conformation. However, it is clear that β-sheet and random structure elements have increased in percentage as compared with native HSA. This observation is most pronounced for HSA-DOM II. The observed differences to the HSA signals, in particular as seen in the far UV-CD measurements, are probably the result of the disruption of non-covalent bonds stabilizing the albumin tertiary structure in the native molecule rather than of incorrect folding or irreversible denaturation of the domains, as further supported by the ligand binding data shown below.

Induced CD Measurements

Hemin—The method of ICD is based on the observation that an optical activity arises from dissymmetry in the ligand induced by its binding to the protein, since the free ligand has either no asymmetric center (hemin, diazepam) or is a racemic mixture (R,S-warfarin) and therefore gives no signal in solution.

Table 5A shows the ICD spectra of hemin complexed with the

TABLE I

Summary of the physicochemical properties of the HSA and its recombinant domains

<table>
<thead>
<tr>
<th></th>
<th>HSA (defatted)</th>
<th>HSA-DOM I</th>
<th>HSA-DOM II</th>
<th>HSA-DOM III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass (MALDI-TOF)</td>
<td>(66,479)</td>
<td>22,966 (22,860)</td>
<td>22,413 (22,519)</td>
<td>23,320 (23,383)</td>
</tr>
<tr>
<td>Isoelectric point, pI</td>
<td>5.8 (Ref. 2)</td>
<td>5.2 (5.3)</td>
<td>5.4 (5.1)</td>
<td>6.6 (6.5)</td>
</tr>
<tr>
<td>Residue range</td>
<td>1–585</td>
<td>1–197</td>
<td>189–385</td>
<td>381–585</td>
</tr>
<tr>
<td>Far UV-CD minima (nm)</td>
<td>222, 209</td>
<td>222, 209</td>
<td>209</td>
<td>223, 209</td>
</tr>
<tr>
<td>Near UV-CD minima (nm)</td>
<td>268, 262</td>
<td>268, 262</td>
<td>268, 262</td>
<td>268, 262</td>
</tr>
<tr>
<td>A&lt;sub&gt;278nm,1cm&lt;/sub&gt;</td>
<td>0.58 (Ref. 10)</td>
<td>0.50</td>
<td>0.79</td>
<td>0.30</td>
</tr>
</tbody>
</table>
three domains and with HSA. A ligand/protein molar ratio of 0.5 was selected to ensure that all of the ligand is bound to the primary binding site, avoiding interactions with secondary, weaker binding sites. Hemin complexed with HSA-DOM I and HSA, respectively, shows very similar spectra sharing the same minimum at 397 nm, indicating the location of the primary hemin binding site to be on HSA-DOM I. With this ligand, HSA-DOM II and HSA-DOM III display ICD spectra with different shapes, suggesting the presence of one or more secondary, weaker binding sites. Hemin complexed with HSA-DOM I and HSA-DOM III display ICD spectra with a primary binding site, avoiding interactions with secondary, weaker binding sites. Hemin complexed with HSA-DOM I and HSA-DOM II shows very similar spectra sharing the same minimum at 397 nm, indicating the location of the primary hemin binding site to be on HSA-DOM I. With this ligand, HSA-DOM II and HSA-DOM III display ICD spectra with different shapes, suggesting the presence of one or more secondary, weaker binding sites. However, it cannot be fully excluded that new binding sites, which are not present on native HSA, were created by exposing the domains as isolated proteins.

The results of the titration of HSA and HSA-DOM I with hemin are presented in Fig. 5B. No saturation of the ICD signal was achieved under our experimental conditions; however, a shift in the induced wavelength minimum was observed, which is in accordance with the experiments conducted with HSA by Beaven et al. (26). Starting at a molar ratio of hemin to HSA of 2, the negative extrinsic Cotton effect shifts from 397 nm to 400 nm with HSA, while under the same conditions HSA-DOM I causes a shift from 397 nm to 402 nm, which is already finished at a molar ratio of 2. The shift in the induced ellipticity is interpreted by the binding of hemin to one or more secondary binding sites with different binding properties and optical characteristics. This notion is further supported by similar observations in titration of HSA-DOM II and HSA-DOM III, which also show wavelength shifts upon titration with hemin (data not shown).

Warfarin—Fig. 6A shows the ICD spectra of warfarin complexed with the three domains and with HSA at a molar ratio of warfarin/protein of 0.5. The binding of warfarin to HSA generates extrinsic Cotton effects with a positive band at 308 nm, a negative one at 278 nm, and a positive shoulder near 340 nm, as previously shown by Fehske et al. (27) and Otagiri et al. (28). Warfarin binding to HSA-DOM II shows a similar spectrum with respect to positive and negative Cotton effects. However, the intensity of the bands is reduced, the wavelength of the positive and the negative bands is slightly shifted to 310 nm and 281 nm, respectively, and no shoulder is observed in the region of 340 nm. HSA-DOM I shows significant ellipticity with a broad band in the positive region (maximum at 306 nm), whereas no significant signal is observed for HSA-DOM III.

The results of the titration of HSA-DOM I and HSA-DOM II with warfarin are shown in Fig. 6 (B and C, respectively). A concentration-dependent signal is observed with both domains tested. HSA-DOM II yields a signal that is qualitatively comparable to that of HSA. However, it is clear that the level of the ICD signal obtained with this domain is lower than that obtained with HSA. Based on the concentration-dependent signal observed with HSA-DOM I, it can be concluded either that secondary binding site(s) are located here or, more likely, that a part of the primary binding site is present on this domain, as was previously suggested by Bos et al. (12, 13). An unambiguous interpretation of the interaction between warfarin and HSA-DOM III is not possible based on the data presented here, since the absence of an ICD signal with this domain could mean either the absence of binding or a binding event that does not produce an ICD signal.

Diazepam—The ICD spectrum of diazepam bound to HSA shows a strong positive band at 260 nm and a negative one at 317 nm, which is in accordance with the findings of Wilting et al. (29). Similarly, but with reduced intensity, binding of diazepam to HSA-DOM III shows a positive band near 260 nm and a negative one near 315 nm. In contrast, the induced ellipticity of diazepam bound to HSA-DOM I is negative at 260 nm and positive at 315 nm, with considerably lower intensity. Diazepam with HSA-DOM II shows no significant ICD spectrum in the observed wavelength range (Fig. 7A).

In the titration experiment (Fig. 7B), saturation of the signal obtained with HSA was observed at molar ratios higher than 2. However, no such saturation was obtained using HSA-DOM III, indicating that binding of diazepam to this domain, although qualitatively very similar, is probably taking place with reduced affinity as compared with HSA.
pH 7.4. Increasing the probability for impaired structural integrity and cleavage specificity of the reagents used for their production, structure or function, since they have been dictated by the literature, all of which have been aimed at gaining insight into the structure/function relationships of the different regions of the albumin molecule (e.g. Refs. 8, 10, 12, 15, 25, and 30–33). The production of fragments employed standard methods, including cyanogen bromide cleavage and proteolytic digestion by pepsin or trypsin.

Drug binding studies have for a long time relied on the use of albumin fragments, which proved to be valuable research tools, allowing the dissection and separation of the complex array of different binding sites present on HSA from each other, thereby facilitating the study of the binding of ligands that have more than one binding site on albumin (10, 12, 13, 25, 33, 34).

However, the majority of the albumin fragments reported so far do not have boundaries that can be justified on grounds of structure or function, since they have been dictated by the cleavage specificity of the reagents used for their production, increasing the probability for impaired structural integrity and potentially leading to a loss of functionality.

A recent report by Kjeldsen et al. (17) describes the cloning and expression of HSA domains I and III in S. cerevisiae. Attempts to produce domain II have not been successful. This is probably due to specific properties of the expression system used by these authors, which made use of a synthetic LA19 preproleader sequence for secretion of the recombinant protein from the host cell, since we were able to show here that domain II can in fact be produced in high yield with our expression system. Interestingly, Kjeldsen et al. found that domain I did not compete with immobilized HSA for binding to myristate and they concluded that domain I, when expressed as a separate entity, does not possess a myristate binding site. It should be noted that the domain preparations used in these experiments have not been subjected to any defatting or other delipidization procedure during their purification. In contrast, recent experiments in our laboratory2 showed that palmamate bound to domains I and III and, to a lesser extent, to domain II, based on competition studies with the fluorescent long-chain fatty acid cis-parinaric acid. These findings are in accordance with the crystal structure of HSA complexed with myristate described by Curry et al. (7).

Based on the primary, secondary, as well as tertiary structure of albumin, natural borders between the domains can be defined. We propose here to set the boundaries as follows: HSA-DOM I, residues 1–197; HSA-DOM II, residues 189–385; HSA-DOM III, residues 381–585. These partially overlapping regions of the domains have been suggested by the atomic structures of various ligand complexes with albumin (1, 35),3 and each contains the major conserved portions of the relevant interdomain helix (h10DOM I-h1DOM II for HSA-DOM I, h10DOM II-h1DOM III for HSA-DOM III, and both for HSA-DOM II). They include cysteine residues only as needed for the formation of the intradomain disulfide bridges and have previously formed the basis for selecting essential portions of recombinant subdomains (IIA and IIIA) (36).

To overcome the serious limitations imposed by the cleavage reagents available, we have undertaken to produce custom designed HSA domains by gene cloning and expression techniques, allowing the preparation of all three domains of HSA as stand-alone unit molecules. As an expression system, we chose to use the methylotrophic yeast P. pastoris, which has previously been shown to be an excellent host for the production of various recombinant proteins, including HSA (37). Under the shake flask conditions used, the HSA domains were expressed and secreted at levels similar to HSA,4 ranging from 65 to 300 mg/liter. From the crude culture supernatant, the proteins were purified by a uniform procedure, employing Cibacron Blue affinity chromatography as a central step. After the final steps of the purification procedure, which consisted of preparative gel filtration and defatting, the purity of the preparations was estimated to be higher than 98% and therefore suitable for all further studies discussed below.

All three domains of HSA were purified using this affinity matrix, indicating the presence of Cibacron Blue binding sites on each of the domains. Compagnini et al. (16, 38) examined the binding behavior of cyanogen bromide fragments of HSA to immobilized Cibacron Blue F3G-A and found that a fragment containing residues 1–123 did not interact with the dye, while a fragment spanning residues 124–298 did. This fragment was interpreted by these authors to harbor two interaction sites, both located in the major hydrophobic binding pocket corresponding to subdomain IIA. Since we found that HSA-DOM I

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2 A. Schweinberger, unpublished data.
3 D. C. Carter, unpublished data.
4 M. Dockal, unpublished data.
(residues 1–197) bound to Cibacron Blue under similar conditions, we predict that one of the two binding sites postulated by Compagnini in subdomain IIA is actually located in the C-terminal part of domain I.

A number of biochemical and biophysical techniques were employed for characterization of the domain preparations. The results of these measurements, which are presented in Table I, show that the proteins are present as monomers with a molecular weight and isoelectric point as expected, according to MALDI-TOF analysis, analytical gel filtration, as well as isoelectric focusing, indicating not only the absence of major unexpected posttranslational modifications, including glycosylation, but also correct processing of the \( \alpha \)-factor leader peptide. This processing event was further corroborated by determination of the N-terminal sequence by Edman degradation.

Far UV-CD—To obtain an insight into the structure of the domains as compared with HSA, CD spectra were studied for each of the separate domains. In the far ultraviolet region, such spectra relate to the polypeptide backbone structures. A com-

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**Fig. 6.** A, ICD (expressed in degrees) of warfarin bound to HSA-DOM I, HSA-DOM II, HSA-DOM III, and HSA at a molar ratio of 0.5. Line symbols are as in Fig. 3. Protein concentrations were \( 20 \mu M \) in 0.1 M sodium phosphate buffer, pH 7.4. B, ICD (expressed in degrees) of warfarin bound to HSA-DOM I and HSA at molar ratios ranging from 0.5 to 3 as indicated by the arrows. Protein concentrations were \( 20 \mu M \) in 0.1 M sodium phosphate buffer, pH 7.4. C, ICD (expressed in degrees) of warfarin bound to HSA-DOM II and HSA at molar ratios ranging from 0.5 to 3 as indicated by the arrows. Protein concentrations were \( 20 \mu M \) in 0.1 M sodium phosphate buffer, pH 7.4.

**Fig. 7.** A, ICD (expressed in degrees) of diazepam bound to HSA-DOM I, HSA-DOM II, HSA-DOM III, and HSA at a molar ratio of 0.5. Line symbols are as in Fig. 3. Protein concentrations were \( 20 \mu M \) in 0.1 M sodium phosphate buffer, pH 7.4. B, ICD (expressed in degrees) of diazepam bound to HSA-DOM III and HSA at molar ratios ranging from 0.5 to 3 as indicated by the arrows. Protein concentrations were \( 20 \mu M \) in 0.1 M sodium phosphate buffer, pH 7.4.
parison of the spectra of HSA domains with the parent mole-
cule on a mean residue weight basis is shown in Fig. 3, and a
quantitative evaluation is given in Table II. HSA gives a result
as expected (22–24). HSA-DOM I and HSA-DOM III show a
similar signal typical for a predominantly α-helical protein,
although with slightly reduced intensity as compared with
HSA. The spectrum observed for HSA-DOM II is characterized
by a different shape, indicating significant α-helical as well as β-sheet structure as further supported by the quantitative
analysis. Since the domain boundaries selected by us are
located centrally in the long interdomain helices (h10DOM
I-h1DOM II, h10DOM II-h1DOM III), this cleavage could give rise in
these regions to some loss of α-helical conformation for β-sheet
and coil structure. As HSA-DOM II contains two such unnat-
ural termini, the relative contribution of this effect could be
more pronounced than for HSA-DOM I and HSA-DOM III, each
of which contains only one such site. The C-terminal helix
(h10DOM I) of HSA-DOM I is partly buried in the native HSA
molecule. If exposed, as is the case in a single domain, this
could give rise to a destabilization of this region, manifesting
itself by reduced ellipticity in the far UV-CD. In the case of
HSA-DOM III, the interdomain helix connecting to HSA-DOM
II is located on a more solvent-exposed patch, thereby maybe
causing less perturbation to the molecule when exposed.

Furthermore, the isolated domains contain surface areas
that are not solvent-exposed in the native protein. This newly
created surface, which is predominantly hydrophobic in char-
acter, equals ~7.0% of the total molecular surface for HSA-
DOM I and 5.4% for HSA-DOM III, whereas HSA-DOM II
contains an extra 11.4% previously unexposed surface. This
can be taken as another explanation for the specific structural
behavior of the three domains.

Near UV-CD—Conformational changes around the aromatic
amino acid residues and S-S bridges of the domains can conve-
niently be studied by CD measurements in the near ultraviolet
region above 250 nm. The main contributions to the ellipticity
from tryptophan and tyrosine groups are seen above 265 nm
generally, with the largest maxima at 279, 284, and 291 nm for
tryptophan (39) and at about 277 nm for tyrosine residues (40).
The contribution from phenylalanine is most evident at about
255, 261, and 268 nm (41). When the CD spectra of the three
recombinant domains were added and compared with that of
native HSA, only small differences were noted above 265 nm,
while the changes below 265 nm were somewhat larger. Thus,
the structure around the lone tryptophan and the tyrosines
largely remains intact in the fragments. However, the changes
below 265 nm can be due to conformational changes around the
S-S bridges and/or phenylalanines. In addition, due to the
cloning procedure, there is an additional phenylalanine residue
at the N terminus of each domain, which in sum may contrib-
ute to the ellipticity in this region providing further explana-
tion for our findings.

In conclusion, we assume that the primary peptide structure
contains sufficient information to fold the recombinant do-

Hemin Binding—We chose to compare the shape, minima,
and maxima of the ICD spectra at a low molar ligand/HSA
ratio, since under these conditions, predominantly the main
interaction site on the protein is populated by the ligand, with
only minor contributions from secondary sites. Based on this
assumption, we clearly show here experimental evidence that
HSA-DOM I harbors the primary binding site for hemin, as
shown by spectra which are qualitatively very similar to those
obtained with native HSA, albeit with reduced intensity (Fig.
5A). This decrease in signal strength could indicate that the
absence of the neighboring parts of the albumin molecule gives
rise to a reduced binding affinity. Hrkal et al. (15) used cyan-
ogen-bromide fragments of HSA to study the interaction with
hemin. In contrast to our domains, however, none of these
fragments, which spanned residues 1–123, 124–298, and 299–
585, respectively, yielded ICD spectra or affinity constants
comparable to those of native HSA, indicating that neither of
these fragments carries a fully functional binding site as pre-
ceived on our HSA-DOM I. Unpublished crystallographic re-
results support this view.

In the titration experiments with HSA (Fig. 5B), we observed
a shift of the minimum to higher wavelength, in accordance
with Beaven et al. (26). This is a clear indication of the presence
of more than one binding site, since at higher concentrations
of the ligand, the first binding site is saturated and secondary
binding sites that exhibit altered optical properties become
populated. Indeed, we found that hemin shows induced ellip-
ticity not only upon binding to HSA-DOM I, but also to HSA-
DOM II and HSA-DOM III, as indicated in Fig. 5A. These
signals differ clearly from that of the postulated primary bind-
ing site. Furthermore, we were able to demonstrate that hemin,
when added in increasing amounts to either of the three do-

HSA-DOM II as used in our experiments contains an extra 9 N-
terminal residues compared with the tryptic fragment used by
Bos and colleagues. Since, in contrast to these authors, we see
a clear ICD signal with HSA-DOM II and warfarin, our find-

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nings provide additional support for the importance of the N-terminal portion of HSA-DOM II and of the border region between subdomains IB and IIA for the warfarin binding site.

Diazepam Binding—Here we provide further experimental evidence that the primary diazepam binding site is located in HSA-DOM III, since the ICD signal obtained with this domain closely resembles that of HSA upon binding to diazepam (Fig. 7A). This is in accordance with Bos et al. (10), who used the peptic (residues 1–387) and the tryptic (residues 198–585) HSA fragments (see “Warfarin Binding” above) corresponding to domains I + II and domains II + III, respectively, to study the binding of diazepam. Crystallographic proof of the primary diazepam binding site was provided by Carter and Ho (1, 35).

In addition, we detected an ICD signal when diazepam was bound to HSA-DOM I. However, this signal is opposite in sign compared with HSA and HSA-DOM III, suggesting that the binding of diazepam to HSA becomes saturated at a ligand/protein ratio of 2, which is not the case with HSA-DOM III, where a signal increase can still be detected at higher ligand concentration (Fig. 7B). These observations may be explained by a reduced binding affinity of HSA-DOM III as compared with HSA, in analogy to the equilibrium dialysis results obtained by Bos et al. (10) employing the proteolytic fragments.

In conclusion, we were able to show that the three recombinant domains of HSA exhibit secondary and tertiary structure comparable to HSA, suggesting that their conformation is similar to their native structure in the context of HSA. From the evolutionary point of view, the domains as produced here can be seen as potential models of the primordial albumin, which is analogous to their native structure in the context of HSA. From the evolutionary point of view, the domains as produced here can be seen as potential models of the primordial albumin, which is analogous to their native structure in the context of HSA.

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REFERENCES
The Three Recombinant Domains of Human Serum Albumin: STRUCTURAL CHARACTERIZATION AND LIGAND BINDING PROPERTIES
Michael Dockal, Daniel C. Carter and Florian Rüker

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