Studies on the Tryptophan and Drug-binding Properties of Human Serum Albumin Fragments by Affinity Chromatography and Circular Dichroism Measurements*

INGVAR SJÖHOLM AND INGRID LJUNGSTEDT

From the Department of Biochemistry, Faculty of Pharmacy, University of Uppsala, S-751 22 Uppsala, Sweden

SUMMARY

After cyanogen bromide digestion of human serum albumin, three fragments, A, B, and C, are obtained. Two different types of affinity columns of Sepharose with L-tryptophan were prepared, one with a free carboxyl group and one with a free amino group. Only Fragment C showed affinity to the Sepharose column with the carboxyl group of tryptophan free, while the column having the amino group free did not extract any fragment from the cyanogen bromide digest. Fragments A, B, and C were separated and studied by circular dichroism. Large parts of the native structure are preserved in the fragments. Human serum albumin contains 45 to 50% α helix, and totally about 35% is found in the fragments. Fragment A contains about 42% α helix and about 15 to 22% β structure, B contains 22 to 26% α helix and 9 to 14% β structure, and C contains 28 to 36% α helix and 11 to 32% β structure, the significance of which is discussed.

The binding of L-tryptophan and drugs to the different fragments was studied by circular dichroism and equilibrium dialysis; only Fragments A and C were found to be active. The apparent association constants, $K_{\text{app}}$, for the binding of L-[W]tryptophan at pH 9.0 and 10° were $0.16 \times 10^4 \text{ M}^{-1}$ and $0.17 \times 10^4 \text{ M}^{-1}$ for Fragments A and C, respectively. The lone tryptophan residue of human serum albumin is found in Fragment C, the importance of which is discussed.

One of the main physiological functions ascribed to serum albumin is to work as a transporting macromolecule and to form depots for low molecular weight substances in blood (1). A prerequisite for such functions of the serum albumin is that the substance to be transported or stored in the blood form strong complexes with the albumin molecule. Indeed, such a condition is fulfilled by many endogenous substances, e.g. tryptophan (2, 3), as well as by several compounds foreign for the organism such as drugs of different chemical structure (4, 5). For many drugs, binding to serum albumin will be of great importance for their pharmacokinetic properties. Thus, for instance, a very high binding constant will decrease the free concentration of the drug and consequently also the pharmacodynamic effect and prolong the duration (6). In contrast to small ions, which generally are bound to more numerous sites on the albumin surface (7), drugs are bound to a limited number of sites only, and often enough only one site is of pharmacodynamic importance, i.e. when the binding constant is high enough to effectively influence the drug distribution (6). Moreover, it has been shown in several cases (8) that some drugs can affect the binding of other drugs by competition for a common site on the albumin surface. In the same way, tryptophan has been shown to interact with the binding of acetylsalicylic acid (9), indomethacin, and other antirheumatic drugs (10). A common structural feature of these substances is combination of a hydrophobic aromatic ring system with a charged side chain. Therefore, it is reasonable to suppose that the albumin structure has at least one site with strong affinity for such substances. Such a site would consist of a hydrophobic center with charged groups in close proximity. One of the purposes of the present work was to isolate a fragment of albumin which has high affinity for tryptophan. Isolation of such a fragment would be a first step towards the identification of the binding site and a better understanding of protein binding of small molecules.

The binding of tryptophan to HSA has earlier been quantitatively studied with equilibrium dialysis methods by McMenamy et al. (2, 11) and with circular dichroism measurements by Sjöholm and Grahnén (3). Thereby the primary apparent association constant was found to be $8 \times 10^4$ at pH 9.0 and 10° (8) and $4 \times 10^4$ at pH 7.95 and 2° (2). McMenamy et al. (12) have also presented methods for the degradation of HSA with cyanogen bromide and for the isolation of a total of seven different fragments. This paper describes the isolation of a tryptophan-binding albumin fragment by affinity chro-

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matography on agarose-bound tryptophan and the drug-binding capacity of the fragments as well as some spectropolarimetric studies on the isolated fragments.

**EXPERIMENTAL PROCEDURE**

**Materials**

Human serum albumin was kindly provided by AB KABI, Stockholm. Carboxypeptidase A (twice crystallized with an activity of 30 units per mg) was purchased from Sigma Chemical Co., St. Louis, Mo. Sepharose 4 B was obtained from AB Pharmacia, Uppsala. The drugs used were received as gifts from the different manufacturers, and were not further purified.

**Preparation of Affinity Columns**

Two different affinity columns were prepared, one having the carboxyl group of tryptophan free, the other one with the amino group free. The general procedure is outlined in Fig. 1 and followed essentially the method of Axén et al. (13) as described by Cuatrecasas (14).

**Sepharose-Trp-COOH—**Packed Sepharose 4B (90 ml) was activated by cyanogen bromide (7.5 g) at pH 11 and 20°. The temperature and pH were held constant by addition of ice and 3 M NaOH. After 20 min, the suspension was quickly washed with cold 0.1 M sodium borate buffer, pH 9.5 (400 ml). Within 90 s the coupling agent, ethylenediamine (2.7 g in 30 ml of borate buffer), was added. The Sepharose was stirred at 4° for 20 hours and was then carefully washed with large amounts of water. Water (30 ml) and succinic anhydride (3 g) were then added to the Sepharose suspension and the pH was adjusted to 6 with 6 M NaOH. After 70 min, the pH was constant and the suspension was stirred overnight at 4°. The Sepharose was then washed copiously with water in a column. L-Tryptophan (125 mg) was dissolved in 20 ml of water and added to the Sepharose suspension, the pH of which was adjusted to 5.0 with 2 M HCl. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (400 mg in 4 ml of water) was then added during 10 min, while the pH was kept constant at 5.0 with 0.1 M HCl. The Sepharose was left for 20 hours at room temperature, and then washed with large amounts of water. The coupling procedure with L-tryptophan was repeated once.

The amount of L-tryptophan coupled to the Sepharose-Trp-COOH gel was determined with carboxypeptidase A. To 1.5 ml of packed Sepharose in 0.1 M Tris-HCl, pH 8.6 (total volume 3.0 ml), 5 μl of carboxypeptidase A (1500 units per ml) were added at 37°. After 3 hours, the suspension was centrifuged and the tryptophan content of the solution was determined spectrophotometrically. By this method the Sepharose-Trp-COOH gel was found to contain 0.94 μmole of tryptophan per ml of packed Sepharose.

**Sepharose-Trp-NH₂—**After the cyanogen bromide activation of Sepharose 4B and the washing with borate buffer, pH 9.5, as described above, β-alanine (4 g in 30 ml of borate buffer) was added to the Sepharose. After stirring the suspension overnight at 4°, the Sepharose was washed copiously with water and treated with ethylenediamine (150 mg in 30 ml of water). The pH was adjusted to 5.0 with 2 M HCl and held constant with 0.1 M HCl during the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (1.6 g in 10 ml of water) for 10 min. The reaction mixture was left at room temperature for 20 hours. The gel was then washed with water and with the coupling procedure with ethylenediamine repeated. The final coupling step with L-tryptophan was carried out as described above for Sepharose-Trp-COOH.

The tryptophan content of Sepharose-Trp-NH₂ was determined after alkaline hydrolysis with 4 M NaOH at room temperature for 18 hours. The released tryptophan was determined spectrophotometrically after neutralization and centrifugation and corresponded to 0.15 μmole per ml of packed Sepharose gel.

**Fragmentation of HSA and Isolation of the Fragments**

HSA was freed from fatty acids by treatment with activated charcoal according to Chen (15) at pH 3. The monomer of HSA was then treated with cyanogen bromide. This fragmentation and the further isolation of the resulting fragments A, B, and C₁ obtained before reduction, were performed according to McMenamy et al. (12).

Amino acid analyses were carried out in an automatic amino acid analyzer, Biochrom BC-200, BioCal, after hydrolysis for 20 hours in 6 M HCl at 110° in evacuated glass tubes.

Polyacrylamide-gel electrophoresis was performed with a gel concentration of 7 or 9% at pH 8.3, mainly according to Davis (16). Only the separation gel was used and sucrose was added to the samples, which were layered on top of the gels.

**Spectropolarimetry**

Circular dichroism measurements were performed with a JASCO J-20 spectropolarimeter, Japan Spectroscopic Co., Tokyo. The instrument was calibrated with D-10-camphorsulfonic acid and tested daily with the internal test-signal system. Rectangular cells with a path length of 0.25 to 10 mm were used in order to optimize the measuring conditions. The photomultiplier voltage was not allowed to exceed 600 volts. Thermostated cell holders were used at 10° or 25° and the samples were filtered through a Millipore filter (0.22 μm). The results are expressed in molar ellipticity (θ), degrees cm² × decimole⁻¹ over 250 nm. The concentrations of the fragments needed for these calculations were determined from amino acid analyses. The molecular weights of the fragments are seen in Table II. Of the substances whose binding capacities were studied, only tryptophan shows ellipticity above 250 nm.
The difference spectra have consequently been corrected for the tryptophan ellipticity. Below 250 nm the circular dichroism data are expressed as the mean residual ellipticity, $\theta_m$, with the mean residual weight of 112, 113, and 114 for the HSA Fragments A, B, and C, respectively (12). The mean residual weight of HSA is 113 as calculated from the amino acid composition (17).

**Determination of Binding Constants**

The binding of tryptophan to the HSA fragments was studied by equilibrium dialysis. The cells consisted of cylindrical compartments with a volume of 1 ml on each side of the membrane. One side contained the protein solution with the same concentration in all cells (0.5 X 10^-4 to 1.0 X 10^-4 M). The other side contained radioactive tryptophan solutions of different concentrations. The buffer used in all of the solutions was 0.1 M KCl, 0.005 M phosphate, pH 9.0. The cells were shaken at 10° for 9 hours.

The distribution of tryptophan between the two cell compartments was determined from the radioactivity content. A 250-μl aliquot was withdrawn from each compartment, dissolved in 4 ml of absolute ethanol and 10 ml of a scintillation solution (toluene, 1000 ml, dimethyl 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) 0.3 g, 2,5-diphenyloxazole (PPO) 5 g) and the radioactivity was measured in a Packard liquid scintillation spectrophotometer, model 3315. The apparent binding constant $k$ can be determined from the slope of the line when $A/D$ is plotted against $n$.

$$\frac{n}{D} = \frac{N}{k} - \frac{n}{k}$$

where $n$ = number of tryptophan molecules bound to 1 protein molecule, $D$ = concentration of unbound tryptophan, and $N$ = number of binding sites. The binding constant $k$ can be determined from the slope of the line when $n/D$ is plotted against $n$.

**RESULTS**

**Affinity Chromatography**—The digest obtained after cyanogen bromide degradation of HSA was passed through a column of Sephadex G-25 in 1% propionic acid to remove cyanogen bromide. The protein fraction was applied to the affinity columns, Sepharose-Trp-COOH and Sepharose-Trp-NH$_2$, at pH 9.0 in 0.001 M sodium phosphate, 0.1 M KCl. The same buffer was used to wash out unadsorbed material. Glycine buffer (0.1 M, pH 3.0) with KCl (1.0 M) was then used to elute the adsorbed protein. The columns were finally washed with 5 ml acetone. Fig. 2 shows that some of the applied protein material was adsorbed on Sepharose-Trp-COOH and subsequently desorbed with the glycine-KCl buffer. However, the tryptophan columns with the free amino group (Sepharose-Trp-NH$_2$) were not effective in the adsorption of the HSA digest to any significant extent.

Amino acid analysis of the protein fraction obtained from the Sepharose-Trp-COOH column indicated that the protein had the same amino acid composition as Fragment C, isolated according to McMenamy et al. (12), as can be seen in Table I. The amino acid composition differed significantly from that of Fragments A and B. Further evidence that the protein fraction obtained was identical with Fragment C was obtained from gel electrophoresis. As can be seen from Fig. 3A, Fragment C is the smallest one and should have the lowest isoelectric point as judged from the relatively low content of basic amino acids.

**Table I**

Amino acid content of HSA fragments

The fragments were prepared either by affinity chromatography on Sepharose-Trp-COOH or according to McMenamy et al. (12). The figures given in parentheses were obtained from McMenamy et al. (12).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Fragment obtained by affinity chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fragment A</td>
</tr>
<tr>
<td>Lysine</td>
<td>16.0</td>
</tr>
<tr>
<td>Histradine</td>
<td>4.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.5</td>
</tr>
<tr>
<td>Asparagic acid</td>
<td>13.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.0</td>
</tr>
<tr>
<td>Serine</td>
<td>7.7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>22.6</td>
</tr>
<tr>
<td>Proline</td>
<td>6.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>(6.4)*</td>
</tr>
<tr>
<td>Alanine</td>
<td>20.8</td>
</tr>
<tr>
<td>Cystine</td>
<td>6.5</td>
</tr>
<tr>
<td>Valine</td>
<td>5.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>$\delta$</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>18.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>9.3</td>
</tr>
<tr>
<td>Homoserine</td>
<td>1.0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>n.d. n.d.</td>
</tr>
</tbody>
</table>

* Residual contamination from the elution buffer.

† Methionine is completely transformed to homoserine and homoserine-lactone during CNBr treatment.

‡ n.d., not determined.

‡ Spectrophotometrically determined according to Edelhoch (43).
FIG. 3. Polyacrylamide gel electrophoresis of HSA and HSA fragments obtained after cyanogen bromide digestion. A, an analysis of the affinity chromatography on Sepharose-Trp-COOH shown in Fig. 2. Sample 1 shows the total digest, Sample 2 the proteins which are not adsorbed, and Sample 3 the protein eluted with the glycine buffer. B, the electrophoretic behavior of purified HSA fragments.

Acids. Consequently, it migrates rapidly, almost with the front, while the other fragments migrate somewhat more slowly than the HSA monomer.

The results obtained by the affinity chromatography of the complete CNBr digest of HSA were further confirmed with the isolated HSA Fragments A, B, and C, as shown in Figs. 4 and 5. As can be seen, only Fragment C is adsorbed on Sepharose-Trp-COOH at pH 9.0 in any appreciable amounts. The main parts of Fragments A and B pass straight through the column and only traces are eluted with the glycine and urea buffers (Fig. 4). This adsorption probably reflects the binding to secondary sites of the HSA molecule or may represent an unspecific interaction between the column and the fragments. In contrast to the results obtained with the Sepharose-Trp-COOH column, the Fragments A, B, and C did not show any affinity to the Sepharose-Trp-NH₂ column, where tryptophan is coupled via the carboxyl group to the Sepharose.

Circular Dichroism Studies of HSA Fragments—To obtain an insight into the structure of the residual native HSA after the cyanogen bromide treatment, the circular dichroism spectra were studied for each of the separate Fragments A, B, and C.

The aromatic region (i.e. 250 to 300 nm) gives information about the conformation around the aromatic amino acid side chains. As can be seen in Fig. 6, the HSA monomer and its fragments all have negative ellipticity in the region in question. HSA has negative maxima at 269 and 262 nm, which can most probably be ascribed to the phenylalanine side chains (19). The same characteristic ellipticity is seen in Fragment B. The sum of the molar ellipticity of the fragments is also presented in Fig. 6. As can be seen from the calculated sum, the negative ellipticity below 265 nm is partly lost in the fragments. However, above 265 nm, the calculated ellipticity from the fragments corresponds closely to the experimental ellipticity found for native HSA. Thus, the conformation around the aromatic residues is to a very large extent preserved in the fragments after the CNBr cleavage.

The CD-spectra in the low ultraviolet region relate to the polypeptide backbone structures. A comparison of the spectra of the HSA fragments with that of intact HSA on a mean residue weight basis is shown in Fig. 7. For this comparison, HSA was treated with 70% formic acid under the same conditions as were used in the fragmentation.

The quantitative contribution of the α helical and β structure
referred to the molecular weight of HSA. The calculated values for HSA are obtained from the values of the fragments on molecular basis in relation to the molecular weight of HSA.

**Table II**

Polypeptide backbone structure of HSA and its fragments

The contents of α helical-, β, and random structures are calculated according to Greenfield and Fasman (G. F.) (20) and Chen et al. (C. Y. M.) (21). The calculated values for HSA are obtained from the values of the fragments on molecular basis in relation to the molecular weight of HSA.

<table>
<thead>
<tr>
<th>Fractional content of</th>
<th>M</th>
<th>G. F.</th>
<th>M</th>
<th>G. F.</th>
<th>M</th>
<th>G. F.</th>
<th>M</th>
<th>G. F.</th>
<th>M</th>
<th>G. F.</th>
<th>M</th>
<th>G. F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α helix</td>
<td>0.42</td>
<td>0.42</td>
<td>0.15</td>
<td>0.22</td>
<td>0.43</td>
<td>0.36</td>
<td>33,900</td>
<td>112</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β structure</td>
<td>0.26</td>
<td>0.22</td>
<td>0.14</td>
<td>0.09</td>
<td>0.60</td>
<td>0.69</td>
<td>13,900</td>
<td>113</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Random structure</td>
<td>0.28</td>
<td>0.36</td>
<td>0.32</td>
<td>0.11</td>
<td>0.40</td>
<td>0.54</td>
<td>18,100</td>
<td>114</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molar weight</td>
<td>0.35</td>
<td>0.36</td>
<td>0.10</td>
<td>0.16</td>
<td>0.46</td>
<td>0.48</td>
<td>65,100</td>
<td>113</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean residue weight</td>
<td>0.45</td>
<td>0.50</td>
<td>0.18</td>
<td>0.13</td>
<td>0.36</td>
<td>0.37</td>
<td>65,100</td>
<td>113</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

in proteins can also be calculated from the CD-spectra by different methods (20, 21). In Table II, the figures of the contents of α helix, β structure, and random structures are given for the different fragments, when the ellipticities at 208 nm and 217 nm are used for the calculation, according to Greenfield and Fasman (20). This method is based on the ellipticities obtained from poly(L-lysine) in α helical, β, and random conformation.

The CD values in 1-nm intervals between 205 and 240 nm have also been used for a computerized least-squares fitting according to Chen et al. (21) for the calculation of the secondary structures of fragments and HSA. The CD data of five proteins (myoglobin, lactate dehydrogenase, lysozyme, papain, and bovine ribonuclease) with known structures from x-ray studies are used for these calculations, the results of which are also presented in Table II. The figures presented have also been used for a calculation of the contribution from the fragments of the different structures in the intact HSA molecule. In this summation, the relative weights of the separate fragments in HSA have been used. As can be seen from Table II, the two methods used give corresponding results for the whole HSA molecule, indicating that HSA contains 45 to 50% α helical structure and about 15% β conformation. However, when applied to the fragments, the two methods give corresponding results for the α helical contents, while larger discrepancies are found in the calculation of the β conformation. Large parts of the ordered structures in HSA are preserved in the fragments. Thus, the sum of the amount of β structure in the fragments is the same as present in the intact HSA molecule and the α helix contributions have decreased only from 45 to 50% to 35% in the fragment.

**Binding of L-Tryptophan to Fragments**—CD spectra of the fragments with L-tryptophan added showed that only Fragments A and C gave new Cotton effects. Thus, L-tryptophan binds to these fragments but not to Fragment B. However, the external Cotton effects recorded were too small to allow a reliable determination of the binding constant. Therefore the binding of L-tryptophan to Fragments A and C was quantitatively studied by equilibrium dialysis. The Scatchard plots obtained are seen in Fig. 8. The association constant for the binding of L-[14C]tryptophan to Fragment A was 0.16 x 10^4 M^-1 and to Fragment C 0.17 x 10^4 M^-1. The number of binding sites is about 0.4 to 0.5.

**Binding of Drugs to HSA Fragments**—The binding of propiomazine, phenylbutazon, and chloridiazepoxide to the different HSA fragments was studied by CD measurements from 300 nm and downwards. The difference spectra obtained when the spectra of the respective fragments were subtracted from those obtained with the drug present are shown in Fig. 9. In no case did Fragment B give rise to any new external Cotton effects when the molar drug concentration was five times as high as that of Fragment B. Fragment A and C, in contrast, gave new spectra with all of the drugs, indicating that drug-protein binding has taken place. The difference ellipticity obtained was however in all cases smaller than was observed earlier with intact HSA (5).

**DISCUSSION**

Serum albumin binds many small organic substances of great physiological significance, both endogenous substances and foreign compounds such as drugs (4). In the blood, this binding
more specific proteins such as the corticosteroid-binding globulin transporting macromolecule in contrast to the role played by interactions. The identification of sites on the albumin molecule with complementary properties should, therefore, support codynamic effects and duration of drugs. It has been postulated that a physiological role of albumin is to function as a general transport carrier for hydrophobic compounds. Generally, drugs have mainly hydrophobic properties, but large hydrophobic areas are often combined with more hydrophilic groupings, which can participate in hydrogen bonds, dipole-dipole interactions, or even electrostatic interactions. The identification of sites on the albumin molecule with complementary properties should, therefore, support the idea that albumin also has specific drug-binding sites.

Human serum albumin contains only 1 tryptophan residue. Several reports have shown that binding of sodium dodecyl sulfate (24) and steroids (25) is followed by spectroscopic changes compatible with perturbations of the lone tryptophan. It can thus be concluded that the tryptophan residue is not buried in the interior, but is available for association with smaller compounds. The findings of Swaney and Klotz (26) are also highly supported by the recent work of Gambhir and McMenamy (27). They were able to isolate tryptophan-containing peptides from HSA after trypsin and chymotrypsin digestions, the sequences of which were studied. The structure around the tryptophan was found to be Lys-Ala-Trp-Ala-Val-Ala-Arg. Even if, as is probable, different sections of the peptide chain of HSA form a binding site, a point which will be discussed further, the prerequisite for forming an apolar environment surrounded by positively charged groups can be fulfilled by such a structure.

This paper describes another approach to the study of the drug-binding centre of HSA. It is unreasonable to suppose that extensive degradation of HSA would leave the binding site intact, as the peptide chain in a globular protein most often is highly twisted, and different parts contribute different groups to the active site as has been shown, e.g., for chymotrypsin and other proteolytic enzymes (28). Degradation will, therefore, generally separate binding groups and decrease the binding capacity. Cyanogen bromide digestion of nonreduced HSA, however, gives only three fragments (12), which should retain large parts of the native structure. Affinity chromatography can then easily be applied to determine whether the fragments still contain the binding capacity. In this case, two kinds of agarose-bound tryptophan were prepared. Tryptophan has earlier been shown to bind to HSA (2, 11) and binds to sites where many drugs are bound (9, 10).

Cuatrecasas (14) has shown that the optimum activity of affinity columns is obtained when the active group is not too close to the agarose skeleton, but separated from it by an “arm” containing at least 7 atoms. The two columns prepared had two structurally equivalent 8-atom long arms, to which the tryptophan was attached via either the amino or the carboxyl group. The method used did not permit cross-linking between agarose chains or polymerization of tryptophan at the ends, or both, but enzymatic and chemical hydrolysis showed that both the Sepharose columns contained large amounts of insolubilized tryptophan. Only one cyanogen bromide fragment of HSA, Fragment C, was adsorbed when the total digest was passed through the columns. Fragment C was shown to react with Sepharose-Trp-COOH but not with Sepharose-Trp-NH2. This result strongly supports the findings of McMenamy et al. (2) that the free carboxyl group of tryptophan is necessary for the binding to HSA.

CD spectra and equilibrium dialysis revealed that Fragment A in addition to Fragment C binds free tryptophan, but as none of the columns was able to extract this fragment from the total HSA digest, both the amino and the carboxyl groups evidently are necessary for the binding of tryptophan to the site in A. The lowering of the pH to 6.5 during the affinity chromatography on Sepharose-Trp-NH2 in order to get a positive charge on the column-bound tryptophan gave the same result as that obtained at pH 9.0. Thus, the binding of tryptophan to Fragment A seems to involve mainly strong electrostatic interactions and the indole side chain cannot significantly affect the binding. The present findings as well as those of McMenamy et al. (2) thus indicate that the primary site for tryptophan is situated in Fragment C and a secondary site in Fragment A (3). These findings are also highly supported by the recent work of Gambhir and McMenamy (28). They found that after affinity labeling of HSA with N-bromosuccinyl-l-tryptophan about 70% of the label was found in Fragment C with a subsequent 65% decrease of the binding capacity as found by equilibrium dialysis with N-acetyl-l-tryptophan.

As several drugs compete with tryptophan for the same binding site(s) on the HSA surface, the binding of drugs of different structures was tested by CD measurements. Phenylbutazon (Butazolidine), which has slight acidic properties, binds strongly to at least two sites (29, 30) and the same is true for chlordiazepoxide (Librium), which is a benzodiazepine derivative.

FIG. 9. Difference CD spectra of complexes of Fragment A (○——○) and Fragment C (△——△) with different drugs obtained when the CD spectra of the fragments alone are subtracted. The concentration of the fragment varied from 0.6 to 1.2 mg per ml in 0.1 m KCl, with 0.005 m sodium phosphate, pH 7.4, at 25°. Top, Fragments A and C with phenylbutazon in a 5-fold molar excess; middle, Fragments A and C with chlordiazepoxide in a 4.5-fold molar excess; bottom, Fragments A and C with propiomazine in a 4-fold molar excess.
Propiomazine is a highly hydrophobic phenothiazine derivative, the binding properties of which are not known in greater detail. All of the drugs studied bind to the Fragments A and C but not to R. No quantitative determination of the association constants was made from the CD studies of the drug binding, but the Cotton effects recorded from the difference CD spectra were all smaller than earlier recorded effects with intact HSA (5).

The binding of tryptophan was quantitatively measured at pH 9 and 10° by equilibrium dialysis. The number of sites on the fragments, N, was in both cases low (about 0.5). Similarly, the apparent association constants, $K_a$, were smaller than with intact HSA. Thus, some of the fragment molecules are no longer active with respect to binding and, moreover, the conformation of the binding sites in the remaining active molecules has apparently been modified with a reduced affinity for tryptophan as a consequence. A smaller association constant for the binding of tryptophan was also noted by King and Spencer (31), when bovine serum albumin was digested with trypsin, and the peptide containing the binding site was studied.

Conformational changes of the fragments can conveniently be studied by CD measurements in the ultraviolet region of the spectrum. Above 250 nm, changes around aromatic side chains and S-S bridges can be detected. The main contributions to the ellipticity from tryptophan and tyrosine groups are seen above 250 nm generally, with the largest maxima at 270, 284, and 291 nm for tryptophan (32) and at about 277 nm for tyrosine residues (33, 34). The ellipticity of cystine bridges in proteins has not yet been unequivocally established, but can be significant from 320 nm and downwards to at least 250 nm (34, 35). The contribution from phenylalanine can be most evident at about 255, 261, and 268 nm (36, 37). When the CD spectra of the different fragments from the cyanogen bromide treatment of HSA 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 tryptophan and tyrosines largely remains intact in the fragments. However, the changes below 265 nm can be due to changes in the far ultraviolet region or to conformational changes around the S-S bridges or some phenylalanines, or both. No indications of any profound alterations of the conformation giving rise to new binding sites for tryptophan or other substances, or both, could be seen. Available information thus suggest that the fragments contain the same sites as originally present in native HSA, but that the exact three-dimensional localization of the binding groups could have been slightly changed. Another conclusion is that the primary peptide structure contains enough information to fold the different fragments into secondary and tertiary structures closely approximating the structure occurring in the native HSA after denaturation during the cyanogen bromide treatment in 70% formic acid. The decreased number of active binding sites seen in the dialysis studies possibly means that binding groups, which are not essential for forming the conformation of the fragments, have been modified. Amides might, for instance, have been hydrolyzed by the formic acid treatment. CD studies in the far ultraviolet region, where the contribution from different secondary and tertiary structures mainly determines the ellipticity, further show that the fragments have retained large parts of the native polypeptide backbone structures of HSA. Two different methods were applied; Greenfield and Faasman (20) based their calculations on the ellipticity obtained from a synthetic homopolymer, poly(L-lysine), of known structure, while the method of Chen et al. (21) made use of natural proteins, the structures of which are known from x-ray studies. The latter method, most conveniently used with a computer, can thus take into account effects from aromatic side chains, from different lengths of $\alpha$ helix and from randomized different kinds of "random" structures. Both methods gave similar results for the complete HSA structure, but not for the different fragments. In these fragments, ranging in molecular weight from 13,900 to 32,900, any effects from nonrandomized contributions of amino acid side chains or from different lengths of $\alpha$ helical segments (21) should be more pronounced than in larger proteins. The present results thus indicate that the use of CD data for the determination of different polypeptide backbone structures are reliable only for larger proteins.

HSA contains 1 tryptophan residue, which after cyanogen bromide digestion is found in Fragment C, which is the middle one of the main cyanogen bromide fragments. Earlier spectrophotometric studies in the ultraviolet suggest that the tryptophan can be involved when sodium dodecyl sulfate or some steroids (24, 25) are bound to HSA. These results cannot, however, yield any definite proofs about the participation of the tryptophan chain in the binding of the drugs or L-tryptophan studied, and no other complementary study on the effect of enzymatic degradation on the drug-binding capacity of HSA is available. However, limited trypsin or pepsin digestion of bovine serum albumin can produce fragments which still retain tryptophan or fatty acid-binding properties. Fatty acid, tryptophan, and many drugs compete for the same site on the HSA molecule (11) and it is likely that the situation is similar with bovine albumin. King and Spencer (31) digested bovine serum albumin in free solution, while Peters et al. (38) used albumin adsorbed on a Sepharose column with insolubilized fatty acids. In the first case a fragment of molecular weight about 40,000 was obtained, while Peters et al. (38) were able to isolate two fragments from the Sepharose column after the enzymatic digestion with molecular weights of about 10,000 and 23,000. King (39) digested bovine albumin with pepsin at pH 3.7 in the presence of octanoic acid and obtained two fragments, one of which still showed L-tryptophan-binding capacity. This peptide, as well as the larger one isolated by Peters et al. (38), does not contain tryptophan. Some structural similarities exist between the termini of the bovine and human albums (40–42). If the central parts also show homology and if the primary binding site for tryptophan found in HSA Fragment C is present in the bovine albumin fragments studied by Peters et al. (38) and King (39), their results indicate that the lone tryptophan of HSA should not be an essential part of a binding center. However, as no complete information about the amino acid composition of neither HSA nor bovine albumin is yet available, a more definite discussion about the significance of the tryptophan residue has to await the elucidation of their primary structure.

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