Amino Acid Sequence Adjoining the Lone Tryptophan of Human Serum Albumin. A Binding Site of the Protein*

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ABSTRACT: Serum albumin binds small anions with increasing avidity as their apolar character increases. It has generally been assumed, therefore, that binding sites on the protein contain complementary apolar as well as cationic side chains. Observations described in the literature indicate that tryptophan is in the locus of one of the strong binding sites of serum albumin for certain steroids as well as some detergents. Human albumin contains only one tryptophan residue. The establishment of the primary structure in the neighborhood of this lone residue thus should provide some insight into the molecular nature of one binding site of albumin. Chymotryptic hydrolysis of the protein yielded one tryptophan-containing tripeptide, tryptic digestion gave one tryptophan-containing hexapeptide. Sequence determinations, with overlaps, established the primary structure adjoining the tryptophan as: Lys-Ala-Trp-Ala-Val-Ala-Arg. This cluster of nonpolar residues bracketed by cationic ones offers a very favorable environment for binding of small anions.

Serum albumin, a protein of molecular weight near 65,000, has been extensively studied because, among other reasons, of its exceptional ability to bind small anions and uncharged molecules (Klotz *et al.*, 1946; Klotz, 1949). It has long been evident that the affinity for small anions increases with size of apolar substituents attached thereto, and hence it has been generally assumed that binding sites on the protein provide complementary apolar side chains. Spectroscopic observations in the ultraviolet region (Reynolds *et al.*, 1967; Herskovits and Sorensen, 1968; Ryan, 1968; Ryan and Gibbs, 1970) have suggested that tryptophan may be in the binding area of serum albumin. There is also optical evidence that tryptophan residues are at the binding site of avidin (Green, 1963) and antibody γ-globulins (Little and Eisen, 1967). X-Ray diffraction has established the presence of tryptophan in the cleft in which substrate is held in lysozyme (Blake *et al.*, 1967; Phillips, 1967).

Human serum albumin contains only a single tryptophan per mole of protein. It seemed worthwhile, therefore, to attempt to establish the amino acid sequence in the neighborhood of this lone Trp residue. The determination of the total sequence of serum albumin presents a formidable task since this protein consists of a single polypeptide chain of molecular weight near 65,000. The more limited objective of establishing the sequence around the Trp site can be achieved more readily and should provide information needed for an insight into the binding potential of this region.

Experimental Procedure

Materials. Human serum albumin was obtained from several sources and assessed for purity by disc gel electrophoresis. The sample which was most free of extraneous bands was that (obtained as a gift) from AB Kabi, Stockholm (lot TV 26613). This sample was used for quantitative studies of the tryptophan-containing peptides obtained by enzymic digestion. For preparative-scale isolation of peptides other samples were used including lyophilized human albumin, 96% purity grade (Kabi), crystalline human albumin (lot 23, Pentex, Kankakee, Ill.), and crystalline human serum albumin (lot HP-27, from the Blood Research Laboratories, Jamaica Plain, Mass.).

Chymotrypsin, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin, leucine aminopeptidase, and diisopropyl phosphofluoridate treated carboxypeptidase B were obtained from Worthington Biochemicals and were used without further purification.

This dipeptide, Ala-Trp, was obtained from Cyclo Chemical Co. Technical grade pyridine (Reilly Tar and Chemical Co.) was distilled from phthallic anhydride and the fraction boiling at 115° was collected. Reagents used in the preparation of disc gels were obtained from Canalco. Benzene, trifluoroacetic acid, and phenyl isothiocyanate were purified as described by Edman and Begg (1967). Dioxane was purified according to the procedure of Fieser (1957). Acetic acid and ethyl acetate were redistilled reagent grade materials, and *N*-ethylmorpholine was triply distilled. All other reagents were of the best grade obtainable commercially.

Methods. A. Disc gel electrophoresis. The purity of human serum albumin samples was evaluated by disc gel electrophoresis on 15% acrylamide gel. Protein (200 μg in 100 μl) was layered on the gels and a drop of 40% sucrose was added. Electrophoresis was carried out for about 50 min with a current of 3 mA/tube. The gels were stained with coomassie blue in 12.5% trichloroacetic acid and destained in 12.5% trichloroacetic acid.

B. Chymotryptic digestion. In a typical procedure 1 g of human serum albumin was dissolved in 200 ml of water and heat denatured. The solution was then made 0.1 M in NH_4HCO_3 by the addition of the solid. After the solution had been cooled, 10 mg of chymotrypsin was added and the

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suspension was maintained at 37° with stirring. Another 1% by weight addition of enzyme was made after 3 hr, and digestion was allowed to proceed for a total of 20 hr. The hydrolysate was then lyophilized and redissolved in 0.1 M NH_4HCO_3 in preparation for gel filtration.

C. Tryptic digestion. The procedure for tryptic digestion was similar to that for chymotryptic digestion except that the ketone-treated trypsin was used. The usual addition of calcium was purposely omitted in some cases to avoid complications from the calcium bicarbonate precipitate formed.

D. Gel filtration. The first stage of fractionation of both the tryptic and chymotryptic hydrolysates was chromatography on Bio-Gel P-2 (Bio-Rad, Richmond, Calif.) packed in a 200 × 2.5 cm column. A 0.1 M solution of NH_4HCO_3 was the medium for both packing and elution. The sample (2–5 ml) was layered underneath the buffer with a syringe and eluted at a rate of about 40 ml/hr, by gravity. The effluent was monitored by its absorption either at 254 nm (with an Isco ultraviolet analyzer) or at 280 nm (with a Beckman DB-G grating spectrophotometer). Timed fractions were collected automatically in tubes containing 5 to 10 ml.

E. Ion-exchange chromatography. A few of the peptide fractions from the gel filtration of the chymotryptic hydrolysate were further fractionated on a 120 × 0.9 cm column of AG 50W-X2 (Bio-Rad) by means of a gradient elution procedure. The first chamber of a linear gradient device was filled with 900 ml of a pH 7.0, 0.1 M NH_4OAc solution, and the second chamber contained 900 ml of a pH 10.5, 0.02 M NH_4OAc solution. The sample was dissolved in 1.0 ml of the first buffer, sucrose was added to about a 20% concentration, and the solution was layered underneath the buffer with a syringe. The column was thermostated to maintain a temperature of 37°. The column eluent was monitored continuously by its ultraviolet absorption.

The major tryptophan-containing peptide obtained from gel filtration chromatography of the tryptic digest of human serum albumin was further chromatographed on phospho-cellulose (Cellex-P, Bio-Rad). A linear gradient from pH 4.3, 0.02 M NH_4OAc, to pH 5.1, 0.05 M NH_4OAc was run with a final elution with 0.1 M NH_4OAc, pH 7.0, to ensure that all peptides were removed from the column. The sample was injected with a syringe into a 3-way valve (Hamilton Co., Whittier, Calif.) which was connected to a Bio-Gel chromatographic column with a flow adapter at the top of the cellulose bed. The column was 70 × 1.5 cm and was eluted at a flow rate of 50 ml/hr. Again, the effluent was continuously monitored by ultraviolet absorption.

F. Criteria of identity and purity of the peptides. The identity and purity of the chromatographic fractions and isolated peptides was checked by electrophoresis chromatography (peptide mapping) and by amino acid analysis. Analytical and preparative peptide mapping was carried out as described by Subramanian *et al.* (1968), except that peptides were eluted from the paper twice with 1% acetic acid and three times with 1% ammonium hydroxide.

G. Quantitation of the tryptophan peptides. To evaluate the homogeneity of the protein with respect to the tryptophan locus, the chromatographic fractions from the tryptic hydrolysate were quantitatively analyzed for tryptophan. For this purpose a rapid spectrophotometric method was used (Opiensha-Blauth *et al.*, 1963) in which 1.0 ml of the sample is mixed with 2.0 ml of 0.001 M ferric chloride in glacial acetic acid and this is followed by the addition of 2.0 ml of concentrated sulfuric acid. The optical density is read at 545 nm after careful mixing. Tryptophan content can then be calculated from a calibration curve constructed from readings with standard solutions of this amino acid.

H. Characterization of the peptides. Each peptide was hydrolyzed at 110° for 24 hr with 5.6 M HCl (triply distilled) in a sealed, evacuated tube, flushed three times with nitrogen. The amino acid composition was then determined with a Beckman-Spinco Model 120C analyzer equipped with a digital integrator (Infotronics, Model CRS-12AB).

The sequences of the peptides were established largely through the use of the exopeptidases leucine aminopeptidase and carboxypeptidase. With leucine aminopeptidase an aliquot of the enzyme was added to 0.1 μmole of the peptide in 100 μl of Tris-HCl buffer (pH 8.6) which was made 0.001 M in $MgCl_2$. The tube was then placed in a water bath at 37° for a given time, after which 2.4 ml of pH 2.2 citrate buffer was added and the solution was frozen and stored for later application directly to the amino acid analyzer. The procedure for carboxypeptidase cleavage was the same except that magnesium was omitted from the buffer. It was found that the commercial preparation of carboxypeptidase B which was used had considerable carboxypeptidase A activity. This was satisfactory for our purposes. This preparation will be referred to in the text as carboxypeptidase A + B to avoid confusion.

Results

Determination of Purest Samples of Human Serum Albumin. Since human serum albumin contains only 1 mole of tryptophan per mole of protein (Saifer and Palo, 1969; Heimburger *et al.*, 1964; Spahr and Edsall, 1964), enzymic complete digests should contain one tryptophan-containing peptide if the protein is homogeneous in primary sequence and more than one such peptide if heterogeneity in primary structure exists in this peptide region. Misleading results could be obtained however if preparations of human serum albumin contained even so little as a few per cent impurities, since most other proteins contain a much higher weight per cent of tryptophan. Consequently, a survey was undertaken to find the purest commercial preparation of human serum albumin. Confirming the report of Saifer and Palo (1969) we find that samples from AB Kabi reveal the fewest bands in disc gel electrophoresis. Gels loaded with unusually high quantities of protein (200 μg) and stained with the highly sensitive coomassie blue show only slight impurities. Only Kabi samples of human serum albumin were used, therefore, for quantitative studies of the tryptophan-containing peptides.

Fractionation of the Chymotryptic Hydrolysate. Prior to preparative separation an analytical fingerprint was made of the chymotryptic peptides. Staining with Ehrlich's reagent produced two spots, the basic one being the principal tryptophan-containing peptide, as estimated visually from color intensity.

The first stage of fractionation of the peptides was gel filtration on a 200 × 2.5 cm Bio-Gel P-2 column. A small aliquot of the effluent liquid was removed from each tube, spotted on Whatman No. 3MM paper, dried, and stained

TABLE I: Determination of Sequence of Tryptic Peptide Containing Lone Tryptophan.[a]

Treatment		Ala	Trp	Ala	Val	Ala	Arg
Carboxypeptidase A + B	33 μl, 24 hr	1.05	1.0	1.05	0.94	1.05	0.94
Carboxypeptidase A + B	1 μl, 90 sec					0.14	1.0
Carboxypeptidase A + B	1 μl, 6 min				0.65	1.0	1.0
Carboxypeptidase A + B	33 μl, 5 min			0.52	1.0	1.0	1.0
Sequence derived from carboxypeptidase A + B: (Ala-Trp)-Ala-Val-Ala-Arg							
Leucine aminopeptidase	3 μl, 5 min	1.0	1.0				
Leucine aminopeptidase	3 μl, 20 min	1.0	1.1	0.8	0.5		
Leucine aminopeptidase	25 μl, 5 min	1.0	1.0	1.0	1.0	0.44	0.76[b]
Sequence derived from leucine aminopeptidase: (Ala-Trp)-Ala-Val-(Ala-Arg)							
Subtractive Edman	1st cycle		0.97	1.03	1.00	1.03	0.94
	2nd cycle			1.02	0.93	1.02	1.02
	3rd cycle				0.94	1.03	1.04
	4th cycle				0.10	1.10	0.90

[a] Complete sequence: Ala-Trp-Ala-Val-Ala-Arg. [b] The high value for Arg is attributed to the coelution of the dipeptide Ala-Arg with Arg on the amino acid analyzer.

with Ehrlich reagent. In this manner two tryptophan-containing fractions were recognized. The fraction of lower molecular weight clearly contained the greater amount of tryptophan and in electrophoresis–chromatography appeared in the same position as the major tryptophan peptide in a fingerprint of the entire chymotryptic hydrolysate.

This fraction was, therefore, further fractionated on an AG 50W-X2 cation-exchange column. The six major peaks which were resolved were each fingerprinted; one contained the principal tryptophan peptide. Another peak contained some Ehrlich-positive material but represented only a fraction of tryptophan compared with the principal peak as judged by optical density at 280 nm. The principal tryptophan-containing fraction was then purified further by preparative electrophoresis–chromatography, and eluted as described in the Methods section. The peptide had only traces of impurity as judged by acid hydrolysis and amino acid assay. It was used as such for the sequence determination.

Sequence Determination of the Principal Tryptophan-Containing Peptide Derived from Chymotryptic Hydrolysis. Acid hydrolysis and prolonged digestion with leucine aminopeptidase of this peptide gave the composition: Trp(1) Lys(1.02), Ala(0.98). On the basis of the specificity of chymotrypsin it was assumed that tryptophan was the carboxyl-terminal residue of the tripeptide. Digestion of the peptide with leucine aminopeptidase for short periods released lysine as the first residue.[1] The sequence of this peptide is therefore: Lys-Ala-Trp.

Fractionation of the Tryptic Hydrolysate. Prior to fractionation the entire hydrolysate was fingerprinted to characterize the principal tryptophan-containing peptides. Staining with the Ehrlich reagent showed only two spots, the principal one migrating toward the cathode during clectrophoresis.

The tryptic hydrolysate was applied to a 200 × 2.5 cm Bio-Gel P-2 column. The principal tryptophan-containing fraction was lyophilized and reapplied to the same column in a minimum volume to improve separation of the main component from side bands. The main component was again lyophilized and applied to a 75 × 1.5 cm column of Cellex P cation-exchange cellulose. The large peak contained the tryptophan and was essentially a pure peptide as judged by peptide mapping. It was used as such for sequence determination.

Sequence Determination of the Tryptophan-Containing Peptide Derived from Tryptic Hydrolysis. Acid hydrolysis showed this peptide to have the following composition: Trp(1),[2] Arg(0.8), Ala(3.3), Val(1.0). Leucine aminopeptidase was then used to elucidate the sequence from the amino terminus and carboxypeptidase A + B to do the same from the carboxyl terminus (Table I). One indeterminacy encountered was the release of alanine and tryptophan in equimolar amounts from the amino terminus in short periods of digestion with leucine aminopeptidase. In order to remove this uncertainty and further substantiate the sequence found, four cycles of subtractive Edman degradation were performed (Table I).

TABLE II: Overlap of Chymotryptic and Tryptic Peptides Containing Lone Tryptophan.

Chymotryptic[a]	Lys-Ala-Trp
Tryptic[a]	Ala-Trp-Ala-Val-Ala-Arg
Combined	Lys-Ala-Trp-Ala-Val-Ala-Arg

[a] Successive cleavages with leucine aminopeptidase indicated by ⟶; with carboxypeptidase by ⟵. Subtractive Edman abstraction indicated by ⟵.

[1] Surprisingly high levels of tryptophan were observed in the products of leucine aminopeptidase digestion for short periods. This was shown to be due to the appearance of the dipeptide Ala-Trp at the same elution position as Trp on the amino acid analyzer.

[2] Assumed from positive Ehrlich reaction.

The results confirmed the conclusion derived from the sequence found in the chymotryptic peptide, that alanine was the N-terminal residue of the tryptic peptide.

Combined Sequence. The overlap between the sequence of the chymotryptic peptide and that of the tryptic peptide (Table II) clearly establishes the sequence in the region adjoining the lone tryptophan of human serum albumin as: Lys-Ala-Trp-Ala-Val-Ala-Arg.

Discussion

The fact that a unique sequence has been established for the tryptophan peptide indicates that the heterogeneity of serum albumin found by Foster and his coworkers (Foster *et al.*, 1965; Wong and Foster, 1969; Peterson and Foster, 1965) probably does not reside in differences in primary structure in this region of the polypeptide chain. This conclusion is strengthened by the results obtained in quantitative assessments of the recovery of the tryptophan peptide which show over 90% of the tryptophan in this fraction. The sequence of peptide (1–24) of human albumin has also been established (Bradshaw and Peters, 1969), and the unique order indicates the absence of heterogeneity in primary structure in this amino-terminal region also. Of course using this approach one could never rule out the type of microheterogeneity in which only a minute fraction of the total population differs at any given locus. Also, one cannot rule out heterogeneity in other sections of the primary structure until a complete sequence has been established for the entire polypeptide chain. It may be pertinent to notice in the meantime that the tryptophan and the amino-terminal peptides do not participate in disulfide cross-linkages, 17 of which occur in the entire polypeptide chain.

The sequence in the neighborhood of the lone tryptophan of human serum albumin is striking in having a cluster of apolar residues, Ala-Trp-Ala-Val-Ala, bracketed by two cationic residues, Lys and Arg, respectively. This seems a particularly appropriate environment for interaction with a small organic anion. There is little doubt that Trp is at the locus of a strong binding site, since difference spectra in the ultraviolet region with (charcoal-defatted[3]) human serum albumin in the presence of sodium dodecyl sulfate have been recorded and these show peaks at 292 and 286 nm, regions characteristic of tryptophan perturbations (Herskovits and Laskowski, 1962; Williams *et al.*, 1965).[4]

The physiological importance of this site has been recently shown by ultraviolet difference spectroscopy of certain steroids with human serum albumin (Ryan, 1968; Ryan and Gibbs, 1970). These studies revealed that binding of testosterone, progesterone, and cortisol led to perturbation of the lone tryptophan (whereas androstelanone did not). Spectroscopic considerations led these authors to conclude that hydrogen bonding of the carbonyl to the binding site occurs. This is compatible with the observed amino acid sequence at this site insofar as either Lys or Arg would be a good proton donor for hydrogen bonding. Furthermore, space-filling models show the steroid conformation to be compatible with the linear dimension of the hydrophobic tryptophan region.

Establishment of the one-dimensional structure reveals no precise view of the three-dimensional steric arrangement. Nevertheless, juxtaposition in the linear array places substantial restraints on the residues in the tryptophan heptapeptide. A molecular model illustrates clearly that the sequential side chains of the heptapeptide cannot be far removed from each other and that in concert they must create an apolar environment, bracketed by cationic residues.

From the known three-dimensional structures of proteins it seems almost certain that ionic residues, such as the Lys and Arg of the heptapeptide, project into the aqueous solvent. The complementary assertion that all apolar groups of proteins are buried in the interior, although widely believed, is definitely not true. A substantial number of apolar groups of the proteins examined by X-ray diffraction are accessible to solvent. For subtilisin, for example, almost 50% can be so classified (Wright *et al.*, 1969). For lysozyme some 17% project into the solvent and an additional 50% are at least partially exposed to solvent (Browne *et al.*, 1969). It seems very likely, therefore, that the tryptophan heptapeptide of human serum albumin is also exposed to the solvent, particularly since its N- and C-terminal residues are positively charged ions. Evidently the juxtaposition of a cluster of apolar residues with bracketing cationic ones at the surface of the protein is the molecular basis for binding at the one reasonably defined site of serum albumin.

[3] Charcoal defatting of the human serum albumin was performed as described by Sogami and Foster (1968).

[4] A peak was also found at 280 nm, and is characteristic of tyrosine perturbations. Tyrosine also contributes to the 286-nm peak.

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Conformational Properties of the Isoenzymes of Aspartate Transaminase and the Enzyme–Substrate Complexes*

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ABSTRACT: Possible variations in conformation of two pig heart glutamate aspartate transaminase isozymes, mitochondrial and supernatant, with the elimination of coenzyme or the binding of substrates, were investigated using optical rotatory dispersion, circular dichroism, and microcomplement fixation. The multiple forms, α, β, and γ, within the supernatant isozyme are identical in dichroicity in the ultraviolet region. The multiple forms, A, B, and C, of the mitochondrial isozyme are also indistinguishable from each other and from their apoenzymes. Both enzyme groups have as much as 37–40% α-helix content but none of the subforms in the mitochondrial isozyme react immunologically with subforms in the supernatant isozyme. In the visible region, the circular dichroism patterns for the optically active bands of both isozymes are identical. Although the amplitude of these bands or Cotton effects centered at 430 or 360 mμ vary in absolute magnitude for each subform within an isozyme set, each is proportional to its absorbance. The species of enzyme-bound pyridoxal phosphate absorbing at 340 mμ shows very little optical activity. In the 250–300-mμ range, the apoenzymes of both isozymes show positive dichroic bands. The positions of these bands vary in each isozyme group and some of the optically active transitions change to negative dichroicity upon binding of pyridoxal phosphate. The new bands are centered at 298 and 290 mμ in the supernatant isozyme and at 290 mμ in the mitochondrial isozyme.

The overall circular dichroism pattern in the far-ultraviolet region of the two isozyme groups is unaffected by substrates or changes in pH. L-Aspartate changes the enzyme's absorption spectra to give maxima at 492, 430, and 330 mμ in the supernatant isozyme and 430 and 330 mμ in the mitochondrial isozyme. Only those species with absorbance at 330 mμ show appreciable positive ellipticity but have diverse dissymmetry factors, $\Delta\epsilon/\epsilon$, for each isozyme group. *erythro*-β-Hydroxyaspartate binds to each isozyme forming a characteristic semiquinoid-type complex with absorbance at 492 mμ (supernatant isozyme) or 498 mμ (mitochondrial isozyme) and another maximum in the 330-mμ region. They appear as negative circular dichroism bands at 490–500 mμ and a positive band in the 330-mμ region. The substrate analog, α-methylaspartate, also binds to the active site, producing species absorbing at 430 and 360 mμ. Only the 360-mμ species is optically active and shows a positive dichroic band in both isozymes. Binding of substrates or their analogs alters the optical properties of aromatic chromophores in the supernatant isozyme but not those in the mitochondrial isozyme. An interpretation consistent with the mechanism of transamination is offered in which interactions of the active center with the coenzyme and substrates can be detected and accounted for by conformational changes of the substrate in the covalent pyridoxal phosphate–substrate complex and protein residues at the active site.

The supernatant isozyme and mitochondrial isozyme, although distinct in primary structure (Martinez-Carrion, M., and Tiemeier, D. (1967), *Biochemistry 6*, 1715), have equivalent amounts of ordered structure, mostly as α helix. The active-site topology is very similar in both.

Aspartate transaminase (EC 2.6.1.1) exists as distinctive isozymes with specific cytological localization (Martinez-Carrion *et al.*, 1967). This isozyme system consists of two groups of chemically distinct proteins (Martinez-Carrion and Tiemeier, 1967), each possessing multiple forms with similar structural properties (Martinez-Carrion *et al.*, 1967; Michuda and Martinez-Carrion, 1969a). However, little is known about their conformational properties.

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