Characterization of fragments of human albumin purified by Cibacron Blue F3GA affinity chromatography

David J. LEDDEN,* Richard C. FELDHOFF*‡ and Shung Kai CHAN†

*Department of Biochemistry, School of Medicine, University of Louisville, Louisville, KY 40292, and

†Department of Biochemistry, University of Kentucky Medical Center, Lexington, KY 40506, U.S.A.

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Controlled limited proteolysis of human plasma albumin (0.3 mM; 37°C; 15 min; pH 3.7) with pepsin [pepsin/albumin, 1:1000 (w/w)] in the presence of octanoic acid (4.2 mM) yields at least 14 fragments in the range of 5000–56 000 Da. By utilizing a combination of conventional and affinity-chromatographic procedures, two fragments with mol. wts. 25 000 and 27 000 were purified to more than 99% homogeneity. The larger fragment consists of a continuous polypeptide chain and has been shown to contain the primary bilirubin-binding site. The small fragment contains an internal cleavage site. On the basis of amino acid compositions, *N*-terminal sequences, *C*-terminal sequences, molecular weights and other internal markers the locations of these fragments within the known sequence of human albumin were determined to be residues 49–308 for the 27 000 Da peptide and 309–585 for the 25 000 Da peptide. Peptide 309–585 contains an internal cleavage site and appears to be missing residues 408–423. These non-overlapping fragments should be useful for investigations of individual ligand-binding sites and for the determination of antigenic sites.

Chemical and enzymic fragmentation of proteins has proved to be one of the most useful approaches for investigating structure–function relationships in nearly all proteins. Although much useful information has been obtained by chemical-cleavage procedures, the reaction products are often heterogeneous because of incomplete cleavage and amino-acid side-chain modifications. When used with complex proteins such as plasma albumins, chemical techniques have generally failed to produce fragments which retain native structure. In contrast, under the appropriate conditions, even relatively non-specific proteinases such as pepsin can be controlled in such a manner that a small number of fragments are generated which have unaltered amino-acid side chains.

Owing to the complexity of ligand–albumin interactions and the paucity of X-ray-crystallographic information it has been apparent for several years that the molecular properties of individual ligand-binding sites had to be investigated by the use of site-specific probes or by dissection of the parent molecule. Although many ligand-binding fragments of bovine albumin have been prepared by controlled proteolysis with pepsin or trypsin (Pearlman &

Fong, 1972; King, 1973; Braam *et al.*, 1974; Feldhoff & Peters, 1975; Peters & Feldhoff, 1975), similar studies with human albumin have met with limited success (Peters *et al.*, 1976; Geisow & Beaven, 1977; Kieras & King, 1977; Sjodin *et al.*, 1977). Because studies of drug and metabolite binding to human albumin have much clinical significance, we have been developing new techniques for the purification of ligand-binding fragments of human albumin.

The present paper describes the purification and characterization of two non-overlapping fragments of human albumin based on the utilization of chromatography on Cibacron Blue F3GA–agarose.

Experimental

Materials

Chemicals and biochemicals were generally of the purest grade available unless otherwise noted. Cibacron Blue F3GA–agarose (Affi-Gel Blue) was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. Concanavalin A–Sepharose and Sephadex G-150 were purchased from Pharmacia, Piscataway, NJ, U.S.A. Carboxymethylcellulose (CM-52) was obtained from Whatman, Clifton, NJ, U.S.A. $^{63}\text{NiCl}_2$ (0.5 Ci/mmol) was from New Eng-

Abbreviation used: SDS, sodium dodecyl sulphate.

‡ To whom correspondence should be addressed.

land Nuclear Corp., Boston, MA, U.S.A. and liquid-scintillation cocktail (TT-21) was from Yorktown Research, Miami, FL, U.S.A. Fresh or outdated human plasma was generously donated by the American Red Cross, Louisville, KY, U.S.A.

Albumin purification

We have found it advantageous to purify our own albumin because commercial albumin preparations are derived from a large number of donors and contain variable amounts of albumin polymers, contaminating globulins and crystallizing agents.

Human albumin monomer was prepared from human plasma by our modification of the method of Travis *et al.* (1976). Briefly, an enriched albumin fraction was prepared by precipitation with $(\text{NH}_4)_2\text{SO}_4$, followed by chromatographic purification on Cibacron Blue F3GA-agarose and concanavalin A-Sepharose at pH 7.4. The lone free thiol group (Cys-34) was blocked by the formation of a disulphide bond with half-cystine in order to prevent dimerization of the albumin or its fragments. Albumin concentrations were determined by using $A_{280,1\text{ cm}}^{1\%} = 5.3$.

Digestion considerations

In control experiments we have investigated the effect of endogenous levels of free fatty acids or bilirubin on peptic digestions of human serum albumin. Initially, non-defatted half-cystinyl human albumin monomer was digested in the presence of added palmitate or bilirubin at 1:1, 3:1 and 5:1 molar ratios (palmitate/human serum albumin or bilirubin/human serum albumin) with insignificant or no effect on the peptic digestion pattern. Similarly, when palmitate was added to a defatted half-cystinyl human serum albumin monomer preparation at a 5:1 ratio there was no difference in the digestion pattern from that obtained without octanoic acid. These findings may be a consequence of the extreme insolubility of palmitate and bilirubin under acidic conditions and are consistent with the results of Geisow & Beaven (1977) and Kieras & King (1977).

We have concluded that defatting of albumin is not required for peptic digestions because endogenous levels of fatty acids and bilirubin do not affect the fragmentation pattern at pH 3.7. Additionally, defatting appears to adversely affect the stability of stored albumin preparations. It is essential, however, to utilize a monomer preparation, to block the free thiol group and to include octanoic acid during the digestion in order to limit the number of fragments which are produced and to facilitate purification efforts.

Digestion conditions

Half-cystinyl human albumin monomer (1g) at

20 mg/ml in 0.1 M-ammonium formate, pH 3.7, was digested with pepsin (Sigma, St. Louis, MO, U.S.A.) for 15 min at 37°C in the presence of 4.2 mM-octanoic acid. A pepsin/albumin ratio of 1:1000 (w/w) was utilized. The digestion was terminated by adjusting the reaction mixture to pH 8.0 with 2 M-Tris, pH 10.0, and a portion was taken for polyacrylamide-gel analysis. The digestion mixture was concentrated and the buffer changed to 0.25 M-ammonium formate, pH 3.0, by ultrafiltration with a PM-10 membrane (Amicon Corp., Lexington, MA, U.S.A.) before purification procedures.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis of the digest, column fractions and purified fragments was performed on 7.5% (w/v) gels as described by Weber & Osborn (1969).

Automated Edman degradation

The N-terminal sequences of approx. 100 nmol of each peptide were determined by utilizing a Jeol amino acid sequencer, model JAS-47K (Jeol, Tokyo, Japan). The method of Edman & Begg (1967) for automated phenylisothiocyanate degradation was employed and 0.25 M-Quadrol [1,1',1'',1'''-(ethane-1,2-diyl)dinitrilo)tetrakis(propan-2-ol)] was used as the buffer for the coupling reaction. Fractions, 5 ml each, containing the 2-anilino-5-thioazolinones were obtained after each step of degradation and were evaporated to dryness under a stream of N_2 . Conversion into the corresponding phenylthiohydantoin derivatives was performed as described by Edman & Begg (1967), and the phenylthiohydantoin derivatives were identified by t.l.c. (Cohen-Solal & Bernard, 1973), gas-liquid chromatography (Hermanson *et al.*, 1972), and amino acid analysis after back-hydrolysis in 6 M-HCl for 4 h at 150°C *in vacuo* (Mendez & Lai, 1975). Under these conditions, the initial yield of amino acid derivatives after the first step of Edman degradation is usually around 50%, and the repetitive yield for the next ten subsequent steps is better than 95%.

C-Terminal analyses

Peptides were digested with carboxypeptidases A and B as described previously (Feldhoff & Peters, 1975), except for the following modifications. Portions of the digestion mixture corresponding to 5–10 nmol of peptide were removed at 15, 30, 45, 60, 120, 240 and 480 min. The reaction was terminated by protein precipitation with trichloroacetic acid. After centrifugation the supernatant was extracted twice with ether to remove excess trichloroacetic acid and then freeze-dried. The samples were dissolved with 0.2 M-citric acid, pH 2.2, centrifuged and quantified by automated amino acid

analysis. Norleucine was added to the initial reaction mixture to serve as an internal standard. Buffer and enzyme controls were included.

Hydrazinolysis was performed on 10–20 nmol of peptide as previously described (Feldhoff & Peters, 1975), with quantification by automated amino acid analysis.

Amino-acid composition of fragments

Peptide samples (0.3–0.8 mg) were hydrolysed with 6 M-HCl in sealed evacuated ampoules for 24, 48 and 72 h at 110°C. Amino-acid analysis was performed on a D-300 Dionex amino acid analyser utilizing single-column methodology with sodium citrate eluants. Values for threonine and serine were obtained by back-extrapolation to zero time.

$^{63}\text{Ni}^{2+}$ binding assay

In order to determine the presence of the albumin $\text{Cu}^{2+}/\text{Ni}^{2+}$ -binding site (residues 1–3; Asp-Ala-His) in any given fragment we have developed a rapid screening assay which utilizes $^{63}\text{Ni}^{2+}$, a weak β -radiation emitter. The assay involved incubating fragments with a known amount of $^{63}\text{Ni}^{2+}$ in 25 mM-barbital buffer, pH 8.6, for 10 min at 23°C, followed by application to prewashed (barbital buffer) DEAE-cellulose (DE-81) filter discs. The discs were rinsed with barbital buffer to remove excess unbound $^{63}\text{Ni}^{2+}$. The discs were then placed in scintillation vials containing 1.0 ml of 0.25 M-ammonium formate, pH 3.0, followed by the addition of 9 ml of TT-21 cocktail with subsequent counting of radioactivity in a liquid-scintillation spectrometer.

Determination of tryptophan fluorescence

The presence of the lone tryptophan (Trp-214) and the primary bilirubin-binding site was determined by fluorescence spectroscopy utilizing the bilirubin titration assay described by Levine (1977).

Results

A peptic digest of human albumin was prepared as described in the Experimental section. One half of the digestion mixture was applied to a Sephadex G-150 column (2.5 cm $\times$ 90 cm) equilibrated with 0.25 M-ammonium formate, pH 3.0. As shown in Fig. 1(a), a partial fractionation was achieved on the basis of molecular weight. The shaded fraction from the Sephadex G-150 gel-filtration column contained peptides in the range of 30 000–50 000 Da. This fraction was concentrated by ultrafiltration to 0.025 M-sodium acetate, and adjusted to pH 5.0 before application to a CM-cellulose column (1 cm $\times$ 22 cm) previously equilibrated with 0.05 M-sodium acetate, pH 5.0. The column was eluted with a linear gradient of 0–0.2 M-NaCl (Fig. 1b). The shaded

Fig. 1. Purification of two peptic fragments of human albumin by conventional and affinity chromatography. Human albumin monomer was digested with pepsin as described in the Experimental section. (a) A portion of the digest was separated by Sephadex G-150 gel filtration, with 0.25 M-ammonium formate buffer, pH 3.0, as eluent. (b) CM-cellulose chromatography of the shaded fraction from (a). The column was eluted with a 0–0.2 M-NaCl gradient in 0.05 M-sodium acetate buffer, pH 5.0. (c) Affinity chromatography of the shaded fraction from (b) on Cibacron Blue F3GA-agarose, a 0–4.0 M-NaCl gradient in 0.1 M-sodium phosphate buffer, pH 7.4, being used as eluent. Peaks I and II contained highly purified fragments with molecular masses of 25 000 and 27 000 Da respectively.

fraction eluting between 0.17 and 0.2 M-NaCl was concentrated to 0.1 M-sodium phosphate, and adjusted to pH 7.4 and applied to a 2.5 cm $\times$ 23 cm column of Cibacron Blue F3GA (Fig. 1c). The column was rinsed with 275 ml of 0.1 M-sodium phosphate buffer, pH 7.4, and subsequently eluted with a linear 0–4 M-NaCl gradient. Peaks I and II were concentrated, adjusted to 0.05 M-NaCl and analysed by SDS/polyacrylamide-gel electrophoresis.

Fig. 2 shows the SDS/polyacrylamide gels of a total peptic digest of human serum albumin (gels 1 and 2), peak I (gels 3 and 4) and peak II (gels 5 and 6). Each sample was run in the absence (odd-numbered gels) and presence (even-numbered gels) of 2-mercaptoethanol. As gel 1 shows, peptic digestion of human serum albumin at pH 3.7 in the presence of octanoic acid (4.2 mM) yields a minimum of 12 components, which can be grouped into five molecular-mass classes. These are: (1) 66 000 Da, consisting of component 1 (undigested human serum albumin); (2) 40 000–55 000 Da, consisting of components 2–4; (3) 25 000–40 000 Da, consisting of

Fig. 2. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of a peptic digest and two purified peptic fragments of human albumin

Odd- and even-numbered gels represent sample incubation in the absence and presence of β -mercaptoethanol respectively. 1 and 2, Peptic digests of human albumin at pH 3.7 for 15 min at 37°C; and 3 and 4, peak I from Fig. 1(c); 5 and 6, peak II from Fig. 1(c).

components 5–9; (4) 10 000–20 000, consisting of components 10–11; and (5) <10 000 Da, consisting of component(s) 12. Clearly, the complexity of the digestion pattern is diminished in the presence of reducing agent (gel 2) as evidenced by the fact that eight components (2–9; gel 1) in the molecular-mass range of 30 000–55 000 Da are reduced to two major fractions with molecular masses of about 50 000 and 30 000 Da. This difference in fragmentation pattern in the absence and presence of reducing agent can be explained on the basis of the disulphide-bonded loop structure of albumin. That is, if peptic cleavage occurs within a disulphide-bonded loop, two different hydrolysis patterns are possible: (1) in the absence of reducing agent a single component consisting of two separate polypeptide chains covalently attached by one or more disulphide bonds would be obtained; and (2) in the presence of β -mercaptoethanol, two or more components would be obtained as a result of disulphide-bond reduction. An example of this type of behaviour is demonstrated by peak I, Fig. 1(c) (component 8) in gels 1 and 3. This fraction (peak I), in the absence of β -mercaptoethanol, was shown to contain a single fragment with an apparent molecular mass of 25 000 Da (gel 3). Upon reduction, two peptides with molecular masses of 15 000 and 10 000 Da were detected (gel 4). SDS/polyacrylamide-gel electrophoresis of peak II (gels 5 and 6) showed that this fraction contained a fragment (component 9) with a molecular mass of 27 000 Da. The apparent mole-

cular mass of this fragment increased from 22 000 to 27 000 Da after reduction with mercaptoethanol. This suggests that the disulphide-bonded peptide, even in the presence of SDS, maintains a very compact structure.

In order to make an assignment of the location of these purified peptic fragments within the human albumin sequence, the following properties of each fragment were determined: (1) molecular mass; (2) *N*-terminal sequence; (3) *C*-terminal sequence and (4) the presence or absence of specific amino-acid markers (e.g., the single Trp-214 and the $\text{Cu}^{2+}/\text{Ni}^{2+}$ -binding site, residues 1–3). These data are summarized in Table 1. Briefly, peak I was shown to contain an internal cleavage, as evidenced by its behaviour during SDS/polyacrylamide-gel electrophoresis in the absence and presence of β -mercaptoethanol and by the fact that *N*-terminal sequence analysis yielded two amino acid residues during the first cycle. In addition, this fragment did not contain either internal marker and is therefore believed to be derived from the *C*-terminal portion of the human albumin molecule. In contrast, peak II was shown to be a single polypeptide chain on the basis of its SDS/polyacrylamide-gel-electrophoretic behaviour in the absence and presence of β -mercaptoethanol and its *N*-terminal sequence data. Although this fragment does not possess the $\text{Cu}^{2+}/\text{Ni}^{2+}$ -binding site, the presence of the lone tryptophan at position 214 suggests that it is derived from the *N*-terminal half of the human albumin molecule. In particular, the *N*-terminal sequence data for both fragments is consistent with three of the seven pepsin-sensitive bonds located between residues 48–49, 308–309 and 423–424 (Geisow & Beaven, 1977; Kieras & King, 1977). *C*-terminal residues were determined by hydrazinolysis and carboxypeptidase A and B digestion. Hydrazinolysis yielded two leucine residues for peak I and a leucine/aspartic acid mixture for peak II. Carboxypeptidase digestions of these fragments yielded data (Table 1) consistent with the known specificities and amino acid-release rates for the enzymes (Ambler, 1967). On the basis of the information in Table 1 we tentatively identified peak I as residues 309–585, with an internal cleavage site leaving Val-424 as a second *N*-terminus. The *C*-terminal data indicate that peak I is missing residues 408–423. This fragment, therefore, consists of two peptides, 309–407 and 424–585, which are linked by a single disulphide bond. Similarly, on the basis of the observed *N*-terminal sequence of Phe-Ala-Lys-Thr and the other information in Table 1, we have tentatively assigned peak II to residues 49–308.

In various preparations we have observed a sub-fraction of peak II that possesses a *C*-terminal leucine residue and appears to correspond to residues 49–305. Slight differences in purification

Table 1. *Characterization of albumin and two peptic fragments*

The methods utilized to obtain the data in this Table are described in the Experimental section. The amino acids are expressed in the one-letter code: A, Ala; D, Asp; E, Glu; F, Phe; H, His; K, Lys; L, Leu; N, Asn; Q, Gln; T, Thr; V, Val; Y, Tyr; X, unknown. Abbreviation used: β ME, β -mercaptoethanol.

Sample	Molecular mass (Da)		Internal cleavage	Ni ²⁺ / Cu ²⁺ site	Trp-214	N-Terminal sequence cycle				C-Terminal residues		Apparent residues
	$-\beta$ ME	$+\beta$ ME				1	2	3	4	Hydrazinolysis	Carboxypeptidase	
Human serum albumin	66 000	66 000	No	Yes	Yes	D	A	H	K*	L*	L*	1-585
Peak I	25 000	15 000 10 000	Yes	No	No	F	V	E	S*	L†	L†	309-407
						F	V	E	S†	L†	L*	
						V	E	V	S*	L*	YKFQNAL*	
						V	V	V	S†	L†	YKF(X)AL†	
Peak II	22 000	27 000	No	No	Yes	F	A	K	T*	D*	D*	424-585
						F	A	K	E/T†	D,L†	L,A†	

* Expected sequence based on the data contained in references (Behrens *et al.*, 1975; Meloun *et al.*, 1975; Walker, 1976).

† Experimental sequence determined as described in the Experimental section.

Table 2. *Amino-acid composition of peptic fragments of human serum albumin*

Samples of human albumin and fragments were hydrolysed in 6 M-HCl for 24, 48 and 72 h as described in the Experimental section. The values represent the mean $\pm$ S.D. of three analyses with threonine and serine corrected for hydrolysis loss by back-extrapolation to zero time. The lone Trp-214 residue of human albumin was determined qualitatively by fluorescence spectroscopy as described in the text.

Amino acid	Composition (residues/molecule)					
	Human serum albumin	Residues 1-585	Peak I	Residues 309-585	Peak II	Residues 49-308
Asp	51.3 $\pm$ 1.0	53	20.6 $\pm$ 0.3	21	25.8 $\pm$ 0.7	27
Thr	27.4 $\pm$ 0.3	28	12.3 $\pm$ 0.7	13	9.9 $\pm$ 0.2	11
Ser	23.9 $\pm$ 0.2	24	10.0 $\pm$ 0.9	10	10.2 $\pm$ 0.8	12
Glu	82.0 $\pm$ 0.9	82	38.1 $\pm$ 0.2	38	34.0 $\pm$ 0.7	34
Pro	26.2 $\pm$ 0.1	24	10.6 $\pm$ 0.8	10	12.4 $\pm$ 0.4	11
Gly	11.8 $\pm$ 0.4	12	5.6 $\pm$ 0.1	6	4.8 $\pm$ 0.1	5
Ala	60.0 $\pm$ 0.6	62	24.5 $\pm$ 0.3	25	25.9 $\pm$ 3.5	26
$\frac{1}{2}$ -Cys	33.8 $\pm$ 1.6	35	15.5 $\pm$ 0.2	16	14.2 $\pm$ 0.7	18
Val	39.1 $\pm$ 2.8	41	21.2 $\pm$ 0.7	23	10.9 $\pm$ 1.2	10
Met	5.0 $\pm$ 0.2	6	2.2 $\pm$ 0.2	3	1.9 $\pm$ 0.3	3
Ile	7.1 $\pm$ 0.3	8	2.8 $\pm$ 0.1	3	3.9 $\pm$ 0.1	4
Leu	61.4 $\pm$ 0.5	61	26.9 $\pm$ 0.2	26	28.1 $\pm$ 0.6	28
Tyr	16.5 $\pm$ 0.4	18	8.8 $\pm$ 0.3	9	6.5 $\pm$ 0.4	7
Phe	32.5 $\pm$ 0.4	31	14.5 $\pm$ 0.2	14	13.5 $\pm$ 0.2	13
Lys	60.2 $\pm$ 1.4	59	29.5 $\pm$ 0.5	29	24.0 $\pm$ 0.5	24
His	16.1 $\pm$ 0.7	16	6.0 $\pm$ 0.8	6	8.2 $\pm$ 0.2	7
Arg	23.9 $\pm$ 0.2	24	9.3 $\pm$ 0.1	9	12.7 $\pm$ 0.3	13
Trp	+	1	(-)	0	+	1

schemes can result in a mixture containing variable amounts of these very similar peptides. Kieras & King (1977) also noted a microheterogeneity in their purified peptic fragments of human serum albumin.

In order to confirm our preliminary assignments, multiple samples of human serum albumin, peak I and peak II were hydrolysed with 6 M-HCl *in vacuo*

for 24, 48 and 72 h with subsequent quantification by automated amino acid analysis. These data are summarized in Table 2 and clearly show that the observed amino-acid compositions of peaks I and II are consistent with theoretical compositions determined from the known sequence of human serum albumin. The utility and significance of these peptic fragments of human albumin is discussed below.

Discussion

Enzymic-fragmentation studies involving bovine albumin have proved to be quite productive, resulting in a better understanding of the relative locations and properties of several ligand-binding sites (Pearlman & Fong, 1972; King, 1973; Braam *et al.*, 1974; Feldhoff & Peters, 1975; Peters & Feldhoff, 1975). Similar studies with human serum albumin have met with limited success, probably owing to the numerous differences in the amino-acid sequences of human and bovine albumins, which have resulted in an increased number of pepsin-sensitive bonds. Consequently, hydrolysates of human serum albumin are more complex, consisting of an increased number of components. Apparently this increase in digestion products has hampered purification efforts of peptic fragments of human albumin, since only five fragments have been purified and characterized (Peters *et al.*, 1976; Geisow & Beaven, 1977; Kieras & King, 1977).

By utilizing conventional techniques we have partially purified eight peptic fragments of human serum albumin (Ledden *et al.*, 1980). However, it is both technically difficult and time-consuming to purify human peptic fragments by conventional procedures alone. Therefore alternative purification procedures employing a combination of affinity and conventional chromatographic techniques were developed.

Although human albumin can be purified by affinity chromatography on Cibacron Blue F3GA-agarose (Travis *et al.*, 1976), this support has not been previously utilized for fragment purification. In preliminary experiments we applied a peptic digest of human serum albumin directly to a Cibacron Blue F3GA column in 0.1 M-sodium phosphate, pH 7.4, followed by elution with a linear NaCl gradient (0–4 M). Unfortunately this approach met with only limited success, since several of the fragments in the 25 000–55 000 Da molecular-mass range could only be eluted from the column with 8 M-urea. This may be due to fragment aggregation, which can occur at neutral pH. Additionally, fragment peaks eluted by NaCl gradient were heterogeneous. We have therefore found it necessary to employ conventional chromatographic procedures before the affinity chromatography step. As described in the Results section, we have utilized this approach (Fig. 1) to purify two peptic fragments of human serum albumin to >99% homogeneity (Fig. 2). We have partially characterized these fragments, utilizing molecular mass, *N*-terminal sequences, *C*-terminal sequences and specific amino-acid markers, and have shown that these fragments appear to correspond to residues 49–308 and 309–585 (Tables 1 and 2). On the basis of fluorescence-quenching titrations of Trp-214 after bilirubin addition, the former fragment has also been shown to contain the

primary bilirubin binding site and may be identical with the human peptic fragment P29 isolated by Geisow & Beaven (1977). To date, this fragment represents the smallest bilirubin-binding peptide of human albumin that has been isolated and should prove invaluable in studies directed toward the localization and characterization of the primary bilirubin-binding site.

We have prepared several different batches of peak II which have appeared to be homogeneous, but have shown variable amounts of *C*-terminal leucine or aspartic acid. The amount of *C*-terminal Asp-308 has varied from 30 to 80% and appears to result from the close proximity of two pepsin-sensitive bonds at Leu-305 and Asp-308. During the ion-exchange-chromatography steps, slight modifications in procedure or in fraction selection can alter the ratio of these nearly identical peptides. The amount of each peptide (residues 49–305 and 49–308) can only be determined by *C*-terminal analysis. It appears that other peptic fragments may also possess such ragged ends which may account for some of the technical problems encountered during purification efforts.

Peak I contains the *C*-terminus of human serum albumin and an internal cleavage site. A similar fragment has been identified by other researchers; however, the amount of internal cleavage appears to vary depending on the digest conditions. Our fragment preparation, which is entirely cleaved at residue 424, is also missing residues 408–423, which helps explain the experimental molecular masses (Table 1) being less than those calculated from the sequence data. Peak I therefore corresponds to two peptides 309–407 and 424–585 covalently linked by a disulphide bond. It is presently unclear whether this fragment is the same as, or different from, similar peptides previously purified (Geisow & Beaven, 1977; Kieras & King, 1977). In any event the internal-cleavage site limits the usefulness of this fragment.

In contrast, the potential usefulness of fragments such as peptide 49–308 is just starting to be realized in studies of human albumin. Recently we have shown that peptide 49–308 will reassociate with the bovine *C*-terminal fragment 308–582 to form an albumin-like interspecies hybrid molecule (Ledden *et al.*, 1981). Such fragments might be utilized to probe both metabolite- and drug-binding sites and to further investigate domain-domain interactions. Therefore new techniques, such as those described in the present paper, for the preparation and purification of overlapping and non-overlapping fragments of human serum albumin obtained by controlled limited proteolysis, need to be pursued.

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