

Limited Pepsin Digestion of Bovine Plasma Albumin

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Limited pepsin digestion of bovine plasma albumin at pH 3.7 and 25°C in the presence of octanoic acid gave two fragments, A and B, each in about 16% yield. In the absence of octanoic acid fragment A was rapidly degraded further into smaller fragments. Sodium dodecylsulfate gel electrophoreses and amino acid analyses of fragments A and B indicated their molecular weights to be about 29,000 and 34,000, respectively. Comparative studies of the cyanogen bromide peptides of fragments A and B with those of intact albumin established that fragments A and B represent, respectively, the carboxyl and the amino terminal portions of the albumin molecule.

In Tris-HCl buffer (pH 7.95) at 25°C, fragment A has one primary binding site for octanoic acid with a binding constant about one-eighth of that of albumin. This binding constant is doubled in the presence of an equimolar amount of fragment B, although fragment B itself shows very weak activity, less than one three-hundredth of that of albumin. L- and D-tryptophans competitively bind at the same primary octanoate binding site of fragment A, just as is the case with albumin.

These findings together with those of other studies suggest that the albumin molecule might consist of several compact regions and that the interactions of these regions within the molecule vary with the solvent environment and upon binding of organic ligands.

INTRODUCTION

One of the interesting properties of plasma albumins is their propensity to bind a wide variety of organic compounds (1-3). The list of these organic compounds includes various dyes, normal metabolites and commonly used drugs. Many of these compounds apparently bind competitively at the same primary site of the molecule. This binding property of albumin is believed to be of biological importance as it can serve as a plasma transport protein, thereby regulating the plasma level of the various metabolites and drugs.

We have reported (4) that the primary organic ligand binding site of bovine plasma albumin resides in the carboxyl terminal two-thirds of the albumin molecule. A tryptic fragment with a molecular weight about 40,000 was isolated which retained the bind-

ing activity of the intact molecule. With the purpose of obtaining smaller fragments which retain the binding activity of albumin, I have studied the limited pepsin digestion of this protein.

Plasma albumins are known to undergo several conformational changes in the pH region of 2-5 (5). These conformers differed markedly in their susceptibilities toward pepsin digestion (6-11). Below pH 3.6, there was rapid digestion of albumin to yield complex mixtures, while above pH 3.8 there was slow digestion to yield less complex mixtures. The rate of pepsin digestion of albumin was decreased when carried out in the presence of octanoic acid (7), suggesting that the ligand stabilizes the albumin (cf. 12) and/or the released fragments against digestion.

The present work describes digestion conditions under which albumin is cleaved by pepsin into two fragments of about equal size. The isolation and characterization of these

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two fragments as well as their organic ligand binding properties will be presented.

EXPERIMENTAL

Materials

Crystalline bovine plasma albumin samples (Lots B-70411 and T-68204) were from Armour Company. The commercial albumin samples were used directly in this work after first converting the single sulfhydryl group to a mixed disulfide by reaction with L-cystine (4). Pepsin (Lot PM 8BE) was from Worthington Biochemical Corporation. The samples of sodium [1-¹⁴C]octanoate, L-[1-¹⁴C]-tryptophan and D-tryptophan used for binding studies were the same as those used in the previous report (4). Rabbit anti-bovine plasma albumin sera was obtained from Nutritional Biochemical Corporation.

Methods

Pepsin digestion of albumin. This was carried out using half cystinyl albumin (530 mg) and pepsin (1.0 mg) dissolved in 21 ml of 0.1 M ammonium formate buffer (pH 3.7) containing 3.2 mM octanoic acid. After 20 min at 25°, the digestion was terminated by adjusting the pH of the solution to 7.9 with 2 M Tris (3 ml). The digest was dialyzed in Visking ²⁰/₃₂ tubing against three changes of 200 ml of 0.1 M Tris-HCl buffer (pH 7.95) for about 5 hr. The dialyzed solution was next applied to a column (0.9 × 25 cm) of DEAE-cellulose (Whatman DE-32) which was equilibrated with a pH 7.95 buffer of 0.1 M Tris and 0.06 M HCl. The column was eluted at 25°C and at 40 ml/hr with a linear salt gradient formed with 300 ml each of 0.1 M Tris-HCl buffer (pH 7.95) and of 0.1 M Tris-HCl buffer (pH 7.95) containing 0.1 M NaCl. Fractions of 10 ml volume were collected. The desired fractions were concentrated by ultrafiltration through Visking ⁹/₃₂ tubing (4), dialyzed against water and then lyophilized.

Cyanogen bromide cleavages of the pepsin fragments of albumin, and the reduction followed by carboxymethylation or carboxamidomethylation, were carried out as described previously (4).

Isoelectric focusing was carried out on

polyacrylamide gels containing 6 M urea (13). Disc electrophoresis was carried out in pH 4.3 gels containing 6 M urea and acetate buffer (14). For molecular weight determinations electrophoreses were carried out in gels containing sodium dodecylsulfate (15). The procedure used for immunodiffusion has been described previously (16).

Amino acid analyses (17) were performed as described previously. For samples containing carboxymethylcysteine, hydrolysis was carried out in 6 N HCl containing 1% mercaptoethanol (18). For samples containing tryptophan, hydrolysis was carried out in methanesulfonic acid (19). Edman degradation was carried out using the Beckman Sequenator (20) with the Quadrol program. The phenylthiazolinone released after each cycle of degradation was determined by amino acid analysis after hydrolysis in hydrochloric acid (21).

Equilibrium dialysis experiments to determine the binding of the fragments for different ligands were carried out in the apparatus described previously (4) employing 300 μ l of solution in each compartment and also in a smaller apparatus of 100 μ l volume (22). The experiments were done at $24 \pm 1^\circ$ in a buffer of 0.05 M Tris, 0.03 M HCl, and 0.10 M NaCl (pH 7.95). The concentration of the fragments was about $1-3 \times 10^{-4}$ M, and the concentration of the ligands varied from 0.3 to 30×10^{-4} M.

RESULTS

Commercial crystalline samples of bovine plasma albumin are known to contain several components which differ in their isoelectric points. The two major components, differing by one unit charge, account for 96% of the albumin sample (13). In this work commercial samples were used directly after converting the single sulfhydryl group of albumin to a half cystinyl group (4).

Pepsin digestions of half cystinyl bovine plasma albumin were carried out at pH 3.7 and 25°C both in the presence and in the absence of added octanoic acid. The digests obtained at different time intervals were analyzed by isoelectric focusing in polyacrylamide gels containing 6 M urea. Bands 1 and 2 of Fig. 1 represent the two major

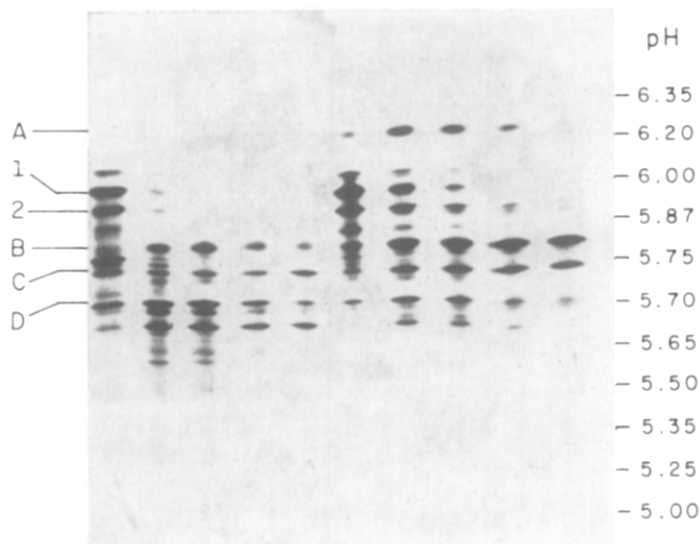


FIG. 1. Isoelectric focusing of pepsin digests of bovine plasma albumin on polyacrylamide gel containing 6 M urea. Digestions of albumin were carried out at pH 3.7 and 25°C in the absence and in the presence of added octanoic acid. Left to right, lanes 1 through 5 are for 1-, 10-, 20-, 40-, and 80-min digests in the absence of octanoic acid, and lanes 6 through 10 are for similar digests in the presence of added octanoic acid. About 220 μ g of albumin were used for each lane.

isoelectric components of intact albumin, while bands A, B, C, and D as well as the other unlabeled ones are the fragments released from albumin. A comparison of these electrofocusing patterns shows that rates of pepsin digestion of both intact albumin and of the released fragments were reduced in the presence of octanoic acid, when compared to those rates obtained in the absence of added acid. This is particularly apparent for fragment A: in the presence of octanoic acid its concentration reached a maximum after 10–20 min of digestion, while in the absence of octanoic acid its concentration was barely detectable after a 1-min digestion.

Crystalline samples of albumin usually contains about one mole of bound fatty acid which can be removed by treatment with charcoal in acid solution (23). The defatted samples were digested more rapidly than the nondefatted samples as indicated by the rapid release of the fragments on electrofocusing. However, in the presence of added octanoic acid, there was no observable difference between the rates of digestion of defatted and nondefatted samples.

The digestion of albumin in the presence of octanoic acid was also studied at pH values other than 3.7. The electrofocusing patterns of these digests are in agreement with the findings of other workers (6–8, 10). There was very slow digestion above pH 3.7, but rapid digestion below pH 3.7 yielding complex mixtures.

Fragments A, B, C, and D can be isolated from a 20-min digest of albumin by chromatography on DEAE-cellulose (top of Fig. 2). Under the chromatographic conditions used intact albumin was eluted at a position between those of fragments A and B. To reduce cross contamination fractions A, B and C were rechromatographed as shown in the bottom of Fig. 2. The rechromatography of fraction B greatly reduces the cross contamination of fragments A and C as shown in the lower graph of Fig. 2. The presence of fragment A in fraction B was not solely a result of imperfect separations but it was found later to be a result of specific association of fragments A and B.

Figure 3 shows the electrofocusing pattern of the rechromatographed fragments. Only fragment A was homogeneous by this test;

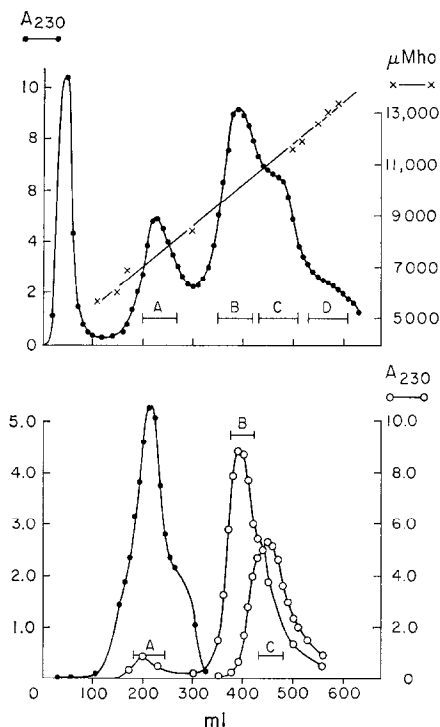


FIG. 2. Chromatography of pepsin digest of bovine plasma albumin on DEAE-cellulose. The upper graph is for the separation of a 20-min digest of albumin (530 mg) obtained in the presence of octanoic acid. The lower graph shows the composite rechromatographies of the cuts from the upper graph, which were pools of two digests of albumin (1.06 g). The columns (0.9×25 cm) were eluted with a sodium chloride gradient in Tris-HCl buffer (pH 7.95).

fragments B and C were cross-contaminated with each other as well as with a small amount of albumin, probably less than 1% (the gel was purposely overloaded with the samples to detect contaminants). Fragments A, B and C were obtained in the following approximate amounts: 70, 90, and 30 mg, respectively, starting from 1.06 g of albumin.

On disc electrophoresis at pH 4.3 in acetate-urea gel fragment A traveled as a single band. Fragment B showed two trace components, one with a mobility slightly faster than the main component and the other slightly slower. The molecular weights of these fragments were estimated by sodium dodecylsulfate gel electrophoresis to be about 28,000 for fragment A and 34,000 for both fragments B and C; reduced and carboxymethylated fragments A and B had the same molecular weights as those of the unmodified fragments. The amino acid compositions of these three fragments are shown in Table I. The sum of the compositions of fragments A and B accounted for nearly all the albumin molecule (97%). Fragments B and C have very similar if not identical compositions. The molar extinction coefficients at 280 nm of fragments A and B were found to be 1.54×10^4 and 2.56×10^4 , respectively. The corresponding values, calculated from their compositions (Table I), were 1.26×10^4 and 2.53×10^4 ; the extinction coefficients of tryptophan, tyrosine and

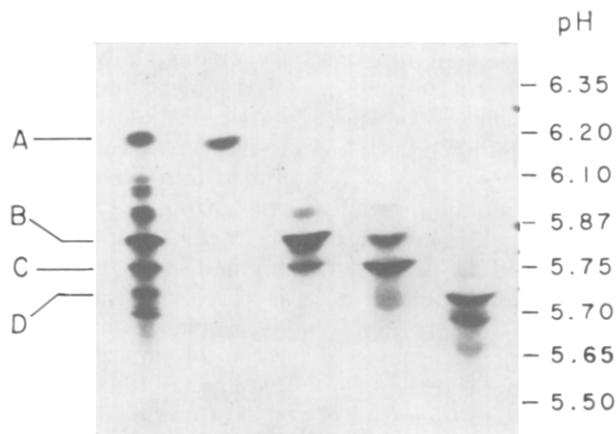


FIG. 3. Isoelectric focusing of the isolated pepsin fragments of albumin. From left to right, 20-min digest of albumin (220 μ g), cut A (60 μ g), cut B (140 μ g), cut C (140 μ g), and cut D (140 μ g).

TABLE I
AMINO ACID COMPOSITIONS OF THE PEPTIC FRAGMENTS OF BOVINE PLASMA ALBUMIN

Amino acid ^a	Fragment A		Fragment B		Fragment C		Sum of Fragments A and B	Bovine plasma albumin
	Residues found	Nearest integer	Residues found	Nearest integer	Residues found	Nearest integer		
Lysine	24.7	25	30.4	30	30.0	30	55	58
Histidine	6.0	6	10.1	10	11.0	11	16	17
Arginine	10.0	10	12.0	12	11.2	11	22	23
Aspartic acid	22.2	22	31.3	31	32.4	32	53	54
Threonine	19.1	19	13.1	13	12.2	12	32	32
Serine	13.0	13	14.3	14	15.3	15	27	26
Glutamic acid	35.2	35	41.8	42	42.4	42	77	80
Proline	13.8	14	14.7	15	13.7	14	29	28
Glycine	6.0	6	10.1	10	8.8	9	16	15
Alanine	19.4	19	24.9	25	25.1	25	44	44
Half cystine ^b	15.6	16	18.2	18	18.2	18	34	36
Valine	19.7	20	12.6	13	13.8	14	33	35
Methionine	1.7	2	1.9	2	1.4	2	4	4
Isoleucine	4.0	4	8.7	9	6.2	6	13	14
Leucine	25.1	25	31.2	31	30.7	31	56	58
Tyrosine	8.7	9	9.6	10	11.4	11	19	19
Phenylalanine	10.7	11	11.8	12	12.6	13	23	26
Tryptophan	0.1	0	1.9	2	n.d.	(2)	2	2
Total		256		297		296	553	571
Molecular weight		29,013		34,125		34,123	63,138	65,243

^a The number of residues per mole of fragment was calculated by taking the lysine, the aspartic acid, the glutamic acid, the glycine and the alanine contents to be 25, 22, 35, 6, and 19, respectively, for fragment A, and 30, 32, 42, 10, and 25, respectively, for fragments B and C. The composition of bovine plasma albumin is taken from Ref. (4).

^b Half cystine residues were determined as carboxymethyl-cysteine residues using reduced and carboxymethylated fragments.

cystine were taken to be 5690, 1280, and 120, respectively (24), for the calculation.

Edman degradation of fragment A was carried out for seven cycles, and the phenylthiazolinones released were quantitated as amino acids following acid hydrolysis. The results in Table II indicate that fragment A has the following partial sequence of Phe Ala Glu Asp Lys Asp Val. . . . The results also indicate that the sequenced portion of fragment A was a mixture of two closely related components in a molar ratio of about 4 to 1, with the minor component being one residue shorter than the major component. Cyanogen bromide cleavage of the two methionyl bonds of fragment A gave a product with the same molecular weight as that of the starting material. After reduction of the cross-linking disulfide bonds fol-

lowed with carboxamidomethylation, the three expected cyanogen bromide peptides were separated into two cuts in the yield of about 60% each by chromatography on Sephadex G-75 (Fig. 4). The sum of the amino acid compositions of these two cuts agreed satisfactorily with that of fragment A (Table III). The absence of homoserine from the composition of cut 2 indicated it to be the carboxyl terminal cyanogen bromide peptide of fragment A. This composition also is in exact agreement with that of the carboxyl terminal cyanogen bromide peptide of albumin. Cut 1 was shown by sodium dodecylsulfate gel electrophoresis to be a mixture of two peptides, one with a molecular weight of about 16,000 and the other of less than 10,000. Unsuccessful attempts were also made to separate these

TABLE II
 EDMAN DEGRADATION OF FRAGMENT A^a

Amino acid	Cycle (residue per mole of fragment)						
	1	2	3	4	5	6	7
Lysine				0.14	<i>0.59</i>		
Aspartic acid			0.16	<i>0.75</i>	<i>0.24</i>	<i>0.64</i>	
Glutamic acid	0.03	0.21	<i>0.73</i>	0.05			
Glycine	0.05	0.03		0.04		0.04	0.03
Alanine	0.16	<i>0.69</i>					
Valine	0.03		0.04	0.04		0.14	<i>0.56</i>
Leucine					0.04		0.04
Phenylalanine	<i>0.73</i>						

^a Degradation was carried out with 170 nmole of fragment A. The phenylthiazolinones released after each cycle of degradation were determined by amino acid analysis following hydrolysis in 6 N HCl at 140° for 17 hr. Recovery of amino acids from phenylthiohydantoins ranged from 70 to 90% in agreement with the published values (21). The above values were corrected for a standard recovery factor of 80%. Values less than 0.03 residue per mole are not reported. The assignment of the aspartyl and the glutamyl residues followed from the findings that there were no corresponding increases of the ammonia values. The italicized values indicate the partial sequence.

two peptides in the form of carboxymethylated derivatives by chromatography on Sephadex G-100 with 0.1 M ammonium bicarbonate containing 0.1% sodium dodecylsulfate as the solvent. The peptide with molecular weight of 16,000 probably corresponds to the penultimate carboxyl terminal cyanogen bromide peptide of albumin which as a molecular weight of 17,000 (4). It follows then that the peptide with molecular weight of less than 10,000 occupies the amino terminal position of fragment A. These results are consistent with the conclusion that fragment A represents

the carboxyl terminal half of the albumin molecule (Fig. 5).

Cyanogen bromide cleavage of fragment B gave two products which were separated by chromatography on Sephadex G-100 with 0.1 M ammonium bicarbonate containing 0.1% sodium dodecylsulfate as the solvent (Fig. 6). Apparently there is strong interaction between these two products as no separation occurred in the absence of sodium dodecylsulfate in the solvent. To reduce cross contamination, cut 1 was rechromatographed on a column (60 × 0.9 cm) of Sepharose 6B in 0.05 M phosphate buffer (pH 6.7) containing 6 M urea and its elution position was the same as that of native albumin. Cut 2 was rechromatographed under the same conditions of Fig. 6. The rechromatographed cuts were obtained in yields of about 50% and they each showed one band on sodium dodecylsulfate gel electrophoresis with molecular weights of about 13,000 and 17,000 for cuts 1 and 2, respectively. The sum of their amino acid compositions is in close agreement with that of fragment B (Table IV). The absence of homoserine in the composition of cut 1 indicated it to occupy the carboxyl terminal portion of fragment B. Cut 2 has two homoserine residues indicating that it is a complex of two cyanogen bromide peptides

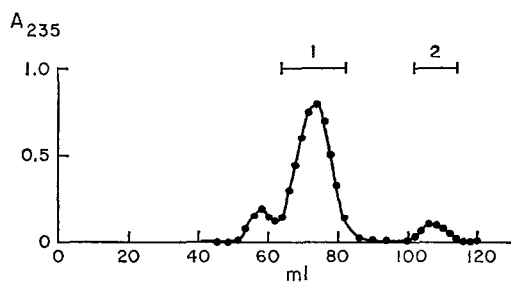


FIG. 4. Separation of cyanogen bromide cleaved fragment A followed with reduction and carboxyamidomethylation on Sephadex G-75. The column (200 × 0.9 cm) was eluted with 2 N acetic acid. About 230 nmole of the fragment were used.

TABLE III
AMINO ACID COMPOSITIONS OF THE CYANOGEN BROMIDE PEPTIDES OF FRAGMENT A^a

Amino acid	Cut 1		Cut 2		Sum of cuts 1 and 2	Fragment A
	Residues found	Nearest integer	Residues found	Nearest integer		
Lysine	21.7	22	3.0	3 (3)	25	25
Histidine	5.2	5			5	6
Arginine	9.6	10			10	10
Aspartic acid	17.1	17	3.8	4 (4)	21	22
Threonine	16.0	16	2.0	2 (2)	18	19
Serine	10.9	11	1.0	1 (1)	12	13
Glutamic acid	31.0	31	4.0	4 (4)	35	35
Proline	12.0	12	0.9	1 (1)	13	14
Glycine	5.0	5	1.1	1 (1)	6	6
Alanine	12.4	12	6.5	7 (7)	19	19
Half cystine						16
Valine	16.0	16	4.6	5 (5)	21	20
Methionine						2
Isoleucine	3.8	4			4	4
Leucine	22.8	23	1.8	2 (2)	25	25
Tyrosine	8.2	8			8	9
Phenylalanine	8.0	8	2.6	3 (3)	11	11
Homoserine	1.7	2			2	
Carboxy- methyl-cysteine	12.9	13	3.0	3 (3)	16	
Total		215		36	251	256
Molecular weight		25,347		3,967	29,314	29,013

^a The number of residues per mole of peptide was calculated by taking the lysine, the glutamic acid, the glycine and the alanine contents to be 22, 31, 5, and 13 for cut A, and 3, 4, 1 and 7 for cut B. The values in parentheses are those of the carboxyl terminal cyanogen bromide peptide of albumin (4, 25).

cross linked by disulfide bonds. Its composition is identical with that of the amino terminal complex obtained on cyanogen bromide cleavage of albumin (4). These findings establish that fragment B originated from the amino terminal half of the albumin molecule as shown in Fig. 5.

Immunodiffusion of fragments B and C against rabbit anti-bovine plasma albumin showed a complete fusion of their precipitin lines. This indicates that these two fragments represent the same region of the albumin molecule in agreement with the similarity of their amino acid compositions. Studies of fragments A and B showed that there was crossing of their precipitin lines indicating their nonidentity.

Fragment A and B as well as admixtures of the two fragments in varying concentra-

tion ratios were tested for their binding of octanoate at 25°C in Tris HCl buffer (pH 7.95) by equilibrium dialysis. Fragment A was also tested for its binding of L- and D-tryptophans; the binding of D-tryptophan was studied indirectly by competitive binding with the L-isomer and with octanoate. These data are given in Figs. 7 and 8 in the form of Scatchard plots (25) where $\bar{v}$ represents the fraction of total protein molecules with bound ligand and c represents the concentration of free ligand. The number of binding sites and their binding constants of the fragments and of the admixture of two fragments (Table V) were extrapolated from the binding data by curve fitting on the assumption of the presence of only two classes of binding sites as assumed previously for albumin (4). With the competi-

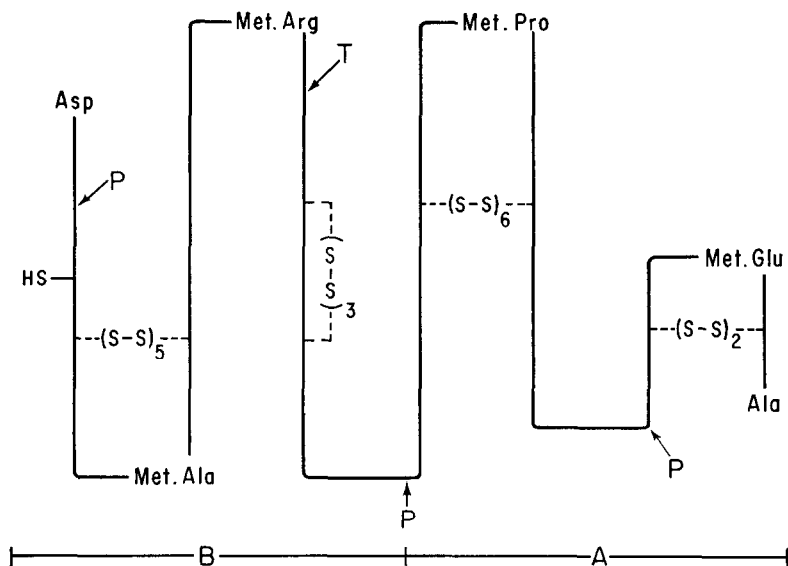


FIG. 5. A schematic structure of bovine plasma albumin [adapted from Ref. (4)]. P and T indicate the pepsin and the trypsin sensitive sites. Two of the three pepsin sensitive sites shown were established by Peters and Hawn (9). The amino acid sequences of the amino and the carboxyl terminal cyanogen bromide peptides have been determined (25).

tive binding data, it was assumed that only one of the two classes of binding sites was involved.

The binding data previously obtained for bovine albumin and its tryptic fragment (4) are given in Table V for comparison.

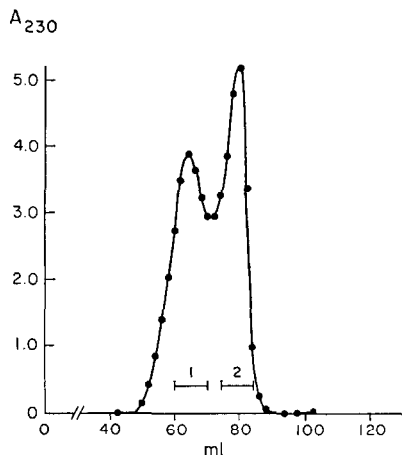


FIG. 6. Separation of cyanogen bromide cleaved fragment B on Sephadex G-100. The column was eluted with 0.1 M ammonium bicarbonate containing 0.1% sodium dodecylsulfate. About 500 nmole of the fragment were used.

Fragment A behaved like albumin and the tryptic fragment in that all three test ligands competitively bind at the same primary site of the protein. The binding constants of fragment A for the three ligands were about $\frac{1}{8}$ to $\frac{1}{6}$ of those for albumin. These decreases are about the same considering that the experimental and the extrapolation errors taken together are probably about 10–20%. They suggest that the primary binding sites of fragment A and albumin share the same structural features; therefore, these sites are the same.

Fragment A is like the tryptic fragment in that the secondary sites made only minor contributions to the octanoate anion binding process (4), hence it differs from albumin in this respect. An admixture of equimolar concentrations (1×10^{-4} M) of fragments A and B showed, in addition to an increased primary binding constant, an increased contribution of the secondary sites to the binding process though fragment B alone shows only weak binding. Identical results were obtained when the binding studies were done with a mixture of fragments A and B having concentrations of $1 \times$

TABLE IV
AMINO ACID COMPOSITIONS OF THE CYANOGEN BROMIDE PEPTIDES OF FRAGMENT B^a

Amino acid	Cut 1		Cut 2		Sum of cuts 1 and 2	Fragment B
	Residues found	Nearest integer	Residues found	Nearest integer		
Lysine	11.9	12	18.2	18 (18)	30	30
Histidine	3.0	3	7.5	8 (8)	11	10
Arginine	4.9	5	4.8	5 (5)	10	12
Aspartic acid	12.1	12	18.8	19 (19)	31	31
Threonine	5.1	5	7.1	7 (7)	12	13
Serine	6.1	6	6.5	7 (7)	13	14
Glutamic acid	15.4	15	28.0	28 (28)	43	42
Proline	4.9	5	9.0	9 (9)	14	15
Glycine	3.1	3	7.5	8 (8)	11	10
Alanine	10.1	10	13.1	13 (13)	23	25
Half cystine	5.7	6	12.1	12 (12)	18	18
Valine	5.2	5	5.8	6 (6)	11	13
Methionine	0		0			2
Isoleucine	3.1	3	3.5	4 (4)	7	9
Leucine	11.5	12	17.2	17 (18)	29	31
Tyrosine	1.9	2	7.6	8 (8)	10	10
Phenylalanine	3.3	3	10.5	11 (11)	14	12
Tryptophan	0.8		n.d.	1 (1)	2	2
Homoserine			1.5	2 (2)	2	
Total		108		184	291	297
Molecular weight		12,223		21,088	33,311	34,125

^a The number of residues per mole of peptide was calculated by taking the lysine, the aspartic acid, the glutamic acid, and the leucine contents to be 12, 12, 15, and 12, respectively, for cut 1, and 18, 19, 28 and 18, respectively, for cut 2. The values in parentheses are those of the amino terminal complex of two cyanogen bromide peptides of albumin (4).

10^{-4} M and 3×10^{-4} M, respectively. These results indicate that the association between fragments A and B was nearly complete when their initial concentrations were 1×10^{-4} M. The association constant of the two fragments can be estimated to be at least 10^5 if one assumes that the association involves only a complex containing one mole each of the two fragments. This is probably the case as indicated by the findings that the elution positions of fragments A and B, and their mixtures from a column (200×0.9 cm) of Sephadex G-100, which was equilibrated with 0.1 M ammonium bicarbonate, were all about the same but slightly behind the position of albumin.

DISCUSSION

The above results show that limited pepsin digestion of bovine plasma albumin at

pH 3.7 gives a number of fragments. Two of these fragments, A and B, have been purified and characterized. Fragments A and B were found to represent the carboxyl and the amino terminal halves of the albumin molecule (Fig. 5). The sum of the amino acid compositions of these two fragments was only slightly less than the composition of albumin. This difference may be the result of incorrectly assumed molecular weights of the fragments, or it may indicate that small peptides were released simultaneously with the fragments.

The best yields of these two fragments were obtained by digestion at pH 3.7 in the presence of octanoic acid. In the absence of octanoic acid and/or at pH values more acidic than 3.7, both fragments were rapidly digested further and this was particularly so for fragment A. These findings are consistent

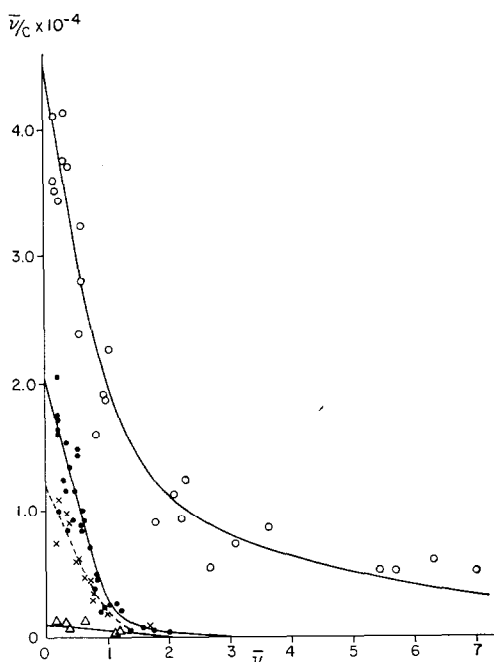


FIG. 7. Octanoate binding by the pepsin fragments of albumin. Open circles, data for admixtures of fragments A (1×10^{-4} M) and B (1×10^{-4} M or 3×10^{-4} M); filled circles, data for fragment A (1.4×10^{-4} M); crosses, data for fragment A (0.7×10^{-4} M) in the presence of 50×10^{-4} M D-tryptophan; open triangles, data for fragment B (3.2×10^{-4} M). The curves were calculated with the constants given in table V as described previously (4).

with a model that the polypeptide chain of albumin molecule is folded into two globular regions connected by a short peptide segment. At pH 3.7 or greater there is tight intramolecular association of these two globular regions. At pH 3.7 or less the association between these regions is decreased, thereby increasing the accessibility of the connecting segment to proteolytic digestion (8, 27). Also the two globular regions of albumin as well as the released fragments both show a pH-dependent conformational motility between states of compact and loose structures. The protein in the state of loose structures offers additional proteolytically sensitive bonds to give complex products. Binding of octanoic acid by albumin or fragment A decreases the conformational motility by stabilizing the state of compact

structures thus reducing their susceptibility to digestion.

The above suggested model of albumin is in agreement with the observations of other workers. (a) The conformational change of albumin accompanying the lowering of pH from 4.5 to 3.5 (28) is reflected by an increase in the intrinsic viscosity of the molecule (29). (b) Measurement of the depolarization of fluorescence of albumin conjugated with dyes showed that there was a decrease in the rotational relaxation time with decrease in pH below 4. This change was interpreted as resulting from that the albumin molecule might consist of two globular regions which were held together tightly in neutral pH and not so below pH 4, and that the decrease in rotational relaxation time was related to the increasing rotational freedom of the globular regions within the molecule (30). (c) Subtilisin digestion of bovine albumin in the presence of 100 mole of sodium dodecyl sulfate at pH 8.9 was reported to give two half molecules (31). The amino acid compositions of the two subtilisin fragments are very similar to those of fragments A and B. The two subtilisin fragments were found to exhibit pH dependent association being maximal near neutrality with an association constant of about 10^6 .

It is of interest to note that the major

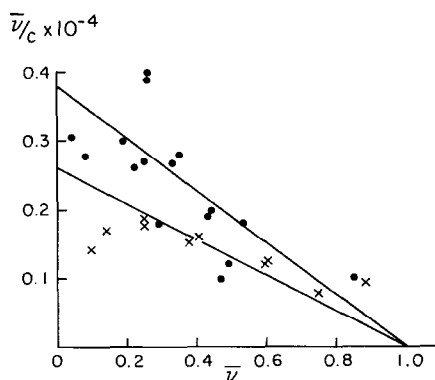


FIG. 8. L-Tryptophan binding by fragment A. Filled circles, the data for L-tryptophan binding; crosses, the data for competitive binding of L-tryptophan in the presence of 33×10^{-4} M D-isomer. The concentration of fragment A was 1.2×10^{-4} M in both studies.

TABLE V
SUMMARY OF THE ORGANIC LIGAND BINDING CONSTANTS OF FRAGMENTS A AND B^a

	Octanoate	L-Tryptophan	D-Tryptophan
Bovine plasma albumin	$n_1 = 1, K_1 = 16 \times 10^4$ $n_2 = 10, K_2 = 0.1 \times 10^4$	$n_1 = 1, K_1 = 2.1 \times 10^4$ $n_2 = 2, K_2 = 0.05 \times 10^4$	$n_1 = 1, K_1 = 0.09 \times 10^4$
Tryptic fragment	$n_1 = 1, K_1 = 5 \times 10^4$ $n_2 = 3, K_2 = 0.02 \times 10^4$	$n_1 = 1, K_1 = 0.84 \times 10^4$	$n_1 = 1, K_1 = 0.028 \times 10^4$
Peptic fragment A	$n_1 = 1, K_1 = 2 \times 10^4$ $n_2 = 2, K_2 = 0.02 \times 10^4$	$n_1 = 1, K_1 = 0.38 \times 10^4$	$n_1 = 1, K_1 = 0.014 \times 10^4$
Peptic fragment B	$n_1 = 2, K_1 = 0.05 \times 10^4$		
Peptic fragments A and B	$n_1 = 1, K_1 = 3.8 \times 10^4$ $n_2 = 10, K_2 = 0.07 \times 10^4$		

^a The values for albumin and its tryptic fragment are taken from Ref. (4). The other values were extrapolated from the data of Figs. 7 and 8 by curve fitting on the assumptions that fragment A, or an admixture of fragments A and B, contains only two classes of binding sites, and that the number of sites of the primary class is one. The extrapolated binding constants of the primary site is estimated to have an error of about 20%. The number of the secondary sites and their binding constants are only approximate due to insufficient data for accurate extrapolations.

point of cleavage of defatted bovine albumin by trypsin at pH 8.8 (4) is different from that by pepsin at pH 3.7 (Fig. 5). This difference can be rationalized on the assumption that the globular region represented by fragment B consists of two smaller subregions of compact structures with unequal sizes. At pH 3.7 there is strong association of the B subregions of the molecule but there is weak association of A and B regions. At pH 8.8 the association between the two subregions of B is weakened, but there is strong association of one of the B subregions with the A region. These changes may correspond to the known alkaline conformational change of albumin in the pH region of 8 to 9 (32). The subtilisin digestion pattern of the albumin-detergent complex (31) discussed above suggests that upon ligand binding, the association of the B subregions of the albumin molecule is enhanced so that subtilisin digestion at pH 8.8 gives the same products as those obtained at pH 3.7.

Probably the A region of the albumin molecule also consists of two or more smaller subregions of compact structures. This is suggested by the reported isolation of a pepsin fragment of 76 amino acid residues from the carboxyl terminal end of albumin as shown in Fig. 5 (9). On the basis of other physical and chemical data other workers have proposed earlier that the albumin

molecule might consist of four compact regions of different sizes (2, 5).

The organic ligand binding properties of fragment A at its single primary site are very similar to those of albumin suggesting that it is the same primary site of albumin. This conclusion is supported by the weak binding of fragment B for octanoate which is less than $1/40$ of that of fragment A. When the binding studies of fragment A for octanoate were carried out in the presence of fragment B, the binding constants of the primary and the secondary sites were doubled; the number of primary site remained the same but the number of secondary sites increased from 2 to 10 which is the same number for albumin. These differences in the binding properties of fragment A in the presence of fragment B are probably the result of formation of a one to one complex of the fragments.

The decrease in the binding constants of the primary and the secondary sites of fragment A may reflect that the fragment contains only major portions of the binding sites of albumin and/or that the fragment has undergone conformational changes to give less perfect binding sites than those present in albumin. Upon forming the complex of fragments A and B these changes can be restored partially to give increased binding activities. The increase in the num-

ber of secondary binding sites may be explained in the same manner.

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