Fragments of Bovine Serum Albumin Produced by Limited Proteolysis. Isolation and Characterization of Peptic Fragments†

Richard C. Feldhoff‡ and Theodore Peters, Jr.*

ABSTRACT: Five new fragments of bovine serum albumin have been isolated following limited peptic hydrolysis. These fragments, and the two peptic fragments previously described by King (King, T. P. (1973), *Arch. Biochem. Biophys. 156,* 509), were positioned within the albumin sequence published by Brown (Brown, J. R. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 591) on the basis of molecular weight, amino acid composition, and amino- and carboxyl-terminal sequences. The fragments correspond to residues 1–385, 1–306, 307–581, 49–185, 186–306, 307–385, and 453–503 in the albumin sequence. These peptides are likely to be native in structure since disulfide bonds were not cleaved during their preparation. In each case the amino acid composition and terminal sequences have confirmed the bovine serum albumin sequence and disulfide bridging pattern proposed by Brown, and offer further proof that bovine albumin is composed of a series of nine independent loops. These fragments should be useful in elucidating the structure–function relationships of albumin.

Serum albumin is known to bind a number of ligands, including fatty acids, bilirubin, amino acids, hormones, drugs, dyes, and metal ions. Fragments of albumin which are large enough to show affinity sites for these ligands are of use in the study of the location and properties of binding sites as well as for the determination of other structural, functional, and immunological features of the molecule.

Using limited peptic digestion, four fragments of bovine serum albumin have previously been isolated and their positions in the parent molecule identified. Peters and Hawn (1967) characterized two peptic pieces, termed P-Asp and P-Phe on the basis of their amino-terminal residues. These fragments correspond to residues 1–24 and 504–581, respectively, in the sequence of albumin recently elucidated by Brown (1975). Brown used the P-Phe peptide as a model from which he deduced the entire disulfide bridging pattern for bovine and human albumins. More recently, King (1973) has obtained two large peptic fragments of bovine albumin termed P-A and P-B. The carboxyl half of the molecule, P-A, could be obtained only when octanoic acid was present during the digestion.

In the present study we have employed the conditions for peptic digestion devised by King (1973) in an effort to obtain additional peptides of bovine albumin. Four new nonoverlapping fragments have been isolated. Their molecular weights range from 6000 to 16,000. In addition, a fifth fragment of mol wt 44,000 was isolated from digests in which octanoic acid was omitted. The latter fragment corresponds to the amino-terminal two-thirds of the albumin molecule and is the largest peptide which has been prepared from bovine albumin by limited proteolysis. The P-A and P-B fragments described by King (1973) were also isolated. The location of all seven of these peptic fragments within the parent molecule has been determined in accordance with the published sequence of albumin (Brown, 1975). The ability to isolate these seven peptic fragments, and the three tryptic fragments previously described (Peters and Feldhoff, 1975), without cleaving disulfide bridges supports the proposed model of albumin as a series of nine independent disulfide loops (Brown, 1974, 1975).

Experimental Section

Materials. Commercial materials used were crystalline bovine albumin, Armour Pharmaceutical Co., Kankakee, Ill.; crystalline swine pepsin and crystalline swine carboxypeptidases A and B, both diisopropyl fluorophosphate treated, Worthington Biochemical Corp., Freehold, N.J.; Sephadex G-50, G-75, G-100, and G-150, Pharmacia Fine Chemicals, Piscataway, N.J.; reagents for Edman degradation and automated amino acid analysis, Pierce Chemical Co., Rockford, Ill.; DEAE-cellulose (DE-52) and CM-cellulose (CM-52), Reeve-Angel Co., Clifton, N.J.

Half-cystinyl albumin, with a half-cystine residue coupled to the lone thiol group of the albumin to minimize dimerization of the albumin or its fragments, was prepared as previously described (Peters et al., 1973).

Methods. Details of the analytical techniques employed in these studies were described previously (Peters and Feldhoff, 1975). Amino-terminal sequences were determined by the Edman procedure; the thiazolinone derivatives were hydrolyzed with hydriodic acid and the resulting free amino acids quantitated on an amino acid analyzer. Carboxyl-terminal residues were determined by hydrazinolysis. Carboxyl-terminal sequences were detected by treatment with a mixture of carboxypeptidases A and B. In some instances half of the carboxypeptidase sample was hydrolyzed with 5.8 *N* HCl for 2 hr at 100° prior to assay on the amino acid analyzer in order to resolve ambiguities relating to glutamine or asparagine content. Amino acid analyses were conducted on a single-column system. Electrophoresis in cellulose acetate was performed at pH 8.6 in sodium–barbital

† From The Mary Imogene Bassett Hospital (affiliated with Columbia University), Cooperstown, New York 13326. *Received May 27, 1975.* This work was supported by U.S. Public Health Service Research Grant No. HL-02751 and the Stephen C. Clark Research Fund of The Mary Imogene Bassett Hospital.

‡ Present address: Department of Biological Chemistry, The Milton S. Hershey Medical Center, Hershey, Pa. 17033.

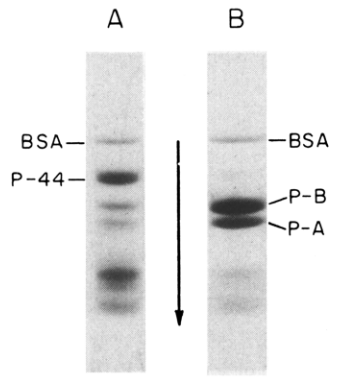

FIGURE 1: Dodecyl sulfate–polyacrylamide gel electrophoresis of peptic digestion mixtures of bovine albumin, after treatment with β-mercaptoethanol: (A) digestion for 20 min at pH 3.70; (B) digestion for 20 min at pH 3.70 in the presence of 3.2 m*M* octanoic acid. Approximately 16 μg of protein was applied per gel. The direction of migration is indicated by the arrow; BSA = bovine albumin; P-44, P-B, and P-A are peptides described in the text.

buffer or at pH 5.0 in pyridine–acetic acid buffer; the peptides were stained with Ponceau S in 10% trichloroacetic acid. Polyacrylamide gel electrophoresis with dodecyl sulfate was conducted in 6% gels according to Weber et al. (1972). Thin-layer gel exclusion chromatography was performed according to the procedure of Andrews (1970).

Peptide fractionation procedures were conducted at 20–25° unless otherwise indicated. Column effluents were monitored at 280 nm and fractions of 6–10 ml collected. Pooled fractions were concentrated under nitrogen pressure on an ultrafilter using a membrane appropriate for the molecular size of the fraction. Lyophilization was avoided because it often led to insoluble products, as noted by King and Spencer (1970). Peptides in solution were stored frozen.

Fragment Nomenclature. The five new fragments described in this manuscript are designated P-44, P-16, P-14, P-9, and P-6. The letter P indicates that the fragment was obtained from a peptic digest and the numeral indicates approximate molecular weight in thousands. Fragments P-A and P-B have been previously described by King (1973) and his designations have been retained.

Results

Digestion of Albumin by Pepsin. In a typical digestion, 0.5 to 1.5 g of half-cystinyl albumin was brought to pH 3.70 ± 0.02 at 25° as a 2.5% solution (w/v) in 0.12 *M* ammonium formate buffer. Freshly dissolved pepsin at 0.05% (w/v) in 1 m*M* HCl was added at a pepsin:albumin ratio of 1:1000 and the solution gently stirred at 25 ± 0.5° for 20 min. The digestion was stopped and the pepsin denatured by the addition of 2 *M* Tris to a final pH of 8.2 ± 0.2. The preparation was stirred for at least 1 hr at room temperature and then concentrated using an ultrafilter. The concentrated digest was adjusted to pH 3.3 ± 0.2 with 2.3 *M* ammonium formate (pH 3.0) and loaded (in a volume equal to 2% of the column volume) on a gel filtration column which had been previously equilibrated with 0.23 *M* ammonium formate buffer (pH 3.0). Octanoic acid at 3.2 m*M* was present during all digestions with the exception of those from which fragment P-44 was prepared. Electrophoretic separations of 20-min peptic digests in the presence and absence of octanoic acid are shown in Figure 1.

Preparation of Fragment P-44. Fragment P-44 was the principal product resulting from a 20-min digestion of half-cystinyl albumin in the absence of octanoic acid (Figure 1A). The purification scheme for fragment P-44 is presented in Figure 2. The elution profile of the digest (0.5 g) following gel filtration on a Sephadex G-100 column (2.5 × 95 cm) at pH 3.0 is shown in Figure 2A. The eluent was 0.23 *M* ammonium formate buffer at a flow rate of 20 ml/hr. The shaded fraction corresponding to peptides of molecular weight range 30,000–55,000 was pooled, concentrated, and applied to a CM-cellulose column (1.1 × 22 cm) which had been equilibrated with 0.05 *M* sodium acetate buffer (pH 5.0). Following sample loading, the column was rinsed with 100 ml of this buffer and then the remaining peptides were eluted using a linear gradient formed with 200 ml of 0.05 *M* sodium acetate buffer (pH 5.0) and 200 ml of 0.1 *M* NaCl in 0.2 *M* sodium acetate buffer (pH 5.0) (Figure 2B). The flow rate was 40 ml/hr. The shaded fraction from the CM-cellulose column was again pooled, concentrated, and loaded onto a DEAE-cellulose column (1.1 × 25 cm) which had been equilibrated with 0.1 *M* Tris-HCl buffer (pH 8.0). Following sample loading, the column was rinsed with 100 ml of this buffer and the peptides were eluted using a linear gradient formed with 200 ml of 0.1 *M* Tris-HCl buffer (pH 8.0) and 200 ml of 0.2 *M* NaCl in the same buffer (Figure 2C). The flow rate was 25 ml/hr. The shaded fraction from the DEAE-cellulose column corresponded to fragment P-44.

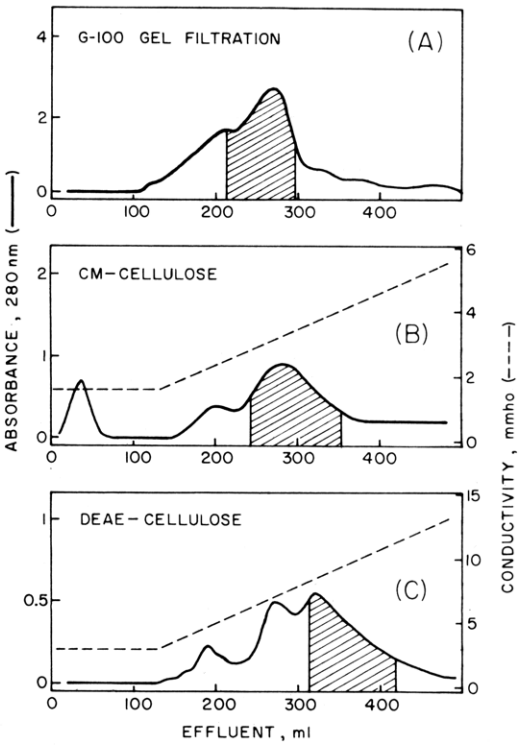

FIGURE 2: Purification of fragment P-44. Experimental procedures are described in the text. (A) Sephadex G-100 gel filtration at pH 3.0 of 0.5 g of a peptic digest without octanoic acid (as in Figure 1A). (B) CM-cellulose chromatography of the shaded fraction from A. The column was eluted by using a gradient formed in sodium acetate buffer (pH 5.0). (C) DEAE-cellulose chromatography of the shaded fraction from B. The column was eluted by using a NaCl gradient in 0.1 *M* Tris-HCl buffer (pH 8.0). The shaded area in C corresponds to fragment P-44.

Preparation of Fragments P-A and P-B. Fragments P-A and P-B were the principal products resulting from a 20-min digestion of half-cystinyl albumin in the presence of octanoic acid (Figure 1B). The purification scheme for frag-

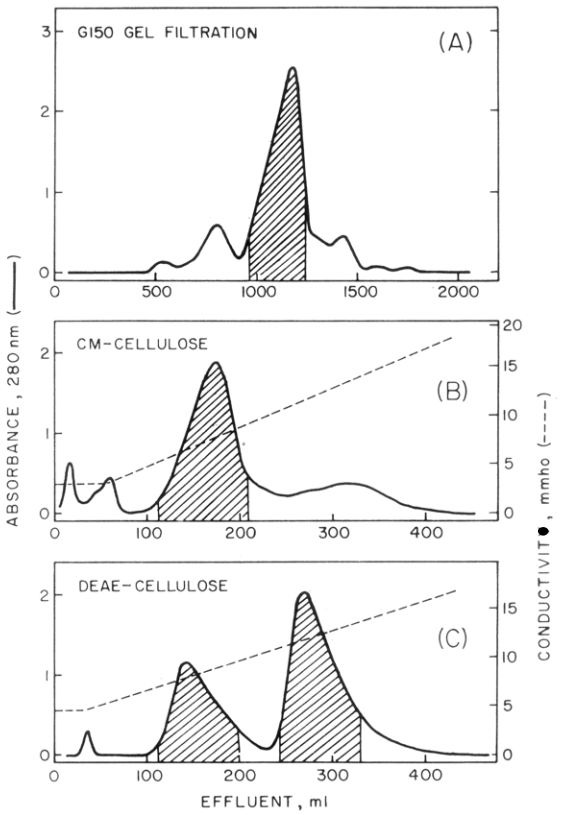

FIGURE 3: Purification of fragments P-A and P-B. Experimental procedures are described in the text. (A) Sephadex G-150 gel filtration at pH 3.0 of 1.3 g of a peptic digest (as in Figure 1B). (B) CM-cellulose chromatography of the shaded fraction from A. The column was eluted with a NaCl gradient in 0.05 *M* sodium acetate buffer (pH 5.0). (C) DEAE-cellulose chromatography of the shaded fraction from B. The column was eluted with a NaCl gradient in 0.1 *M* Tris-HCl buffer (pH 8.0). The first eluted peak in C corresponds to P-A, the second peak to P-B.

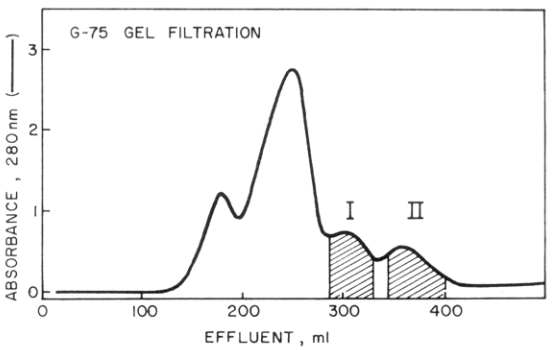

FIGURE 4: Purification of fragments P-16, P-14, P-9, and P-6; Sephadex G-75 gel filtration at pH 3.0 of 0.5 g of a peptic digest performed in the presence of octanoic acid (as in Figure 1B). Fragments P-16 and P-14 were isolated from fraction I by zone electrophoresis in a bed of Sephadex G-25 at pH 5.0. Fragments P-9 and P-6 were similarly isolated from fraction II. Experimental procedures are described in the text.

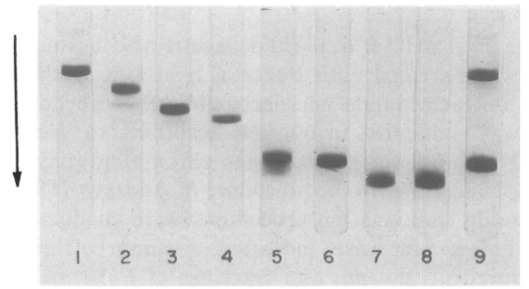

FIGURE 5: Dodecyl sulfate–polyacrylamide gel electrophoresis of peptic fragments of bovine albumin, after treatment with β-mercaptoethanol: (1) bovine albumin; (2) P-44; (3) P-B; (4) P-A; (5) P-16; (6) P-14; (7) P-9; (8) for reference P-Phe, mol wt 8600; (9) for reference bovine albumin and cytochrome *c*, mol wt 66,200 and 12,300, respectively. The direction of migration is indicated by the arrow.

ments P-A and P-B is presented in Figure 3. The digest (1.3 g) was fractionated on a Sephadex G-150 column (5 × 90 cm) as shown in Figure 3A. The buffer was 0.23 *M* ammonium formate (pH 3.0) pumped at a flow rate of 22 ml/hr. The shaded fraction corresponding to peptides of molecular weight range 25,000–40,000 was pooled, concentrated, and chromatographed as two portions on a CM-cellulose column (1.1 × 22 cm) which had been equilibrated with 0.05 *M* sodium acetate buffer (pH 5.0). Each sample was eluted at 70 ml/hr by using a linear gradient formed with 300 ml of 0.05 *M* sodium acetate buffer (pH 5.0) and 300 ml of 0.25 *M* NaCl in this buffer (Figure 3B). The shaded fraction from the CM-cellulose column was pooled, concentrated, and loaded onto a DEAE-cellulose column (1.1 × 25 cm) which had been equilibrated with 0.1 *M* Tris-HCl buffer (pH 8.0). The peptides were eluted from the DEAE-cellulose column at 70 ml/hr using a linear gradient formed with 300 ml of 0.1 *M* Tris-HCl buffer (pH 8.0) and 300 ml of 0.2 *M* NaCl in this buffer (Figure 3C). The first shaded peak which was eluted between 100 and 200 ml of effluent corresponds to P-A and the second shaded peak to P-B.

Preparation of Fragments P-16, P-14, P-9, and P-6. The smaller fragments were also prepared from 20-min digestions in the presence of octanoic acid. These digests were initially fractionated on a Sephadex G-75 column (2.5 × 95 cm) as shown in Figure 4. The buffer was 0.23 *M* ammonium formate (pH 3.0), pumped at a flow rate of 50 ml/hr. Fraction I contained at least four peptides, each with a mol wt of approximately 15,000. Preliminary tests of this fraction by zone electrophoresis on cellulose acetate at pH 5.0 indicated that two of these peptides, P-16 and P-14, could be cleanly separated from the others. Therefore, a pooled fraction I from several digests was purified by zone electrophoresis at pH 5.0 in a bed of Sephadex G-25 at 3° as previously described by Peters and Hawn (1967). Electrophoresis for 20 hr at 4 V/cm sufficed to separate P-16 and P-14 from the other peptides. At pH 5, P-14 is the most positively charged peptide in the mixture and P-16 is the least positively charged.

Fragments P-9 and P-6 were similarly obtained from pooled fraction II (Figure 4) by preparative zone electrophoresis in a bed of Sephadex G-25 at pH 5.0 with 4 V/cm for 20 hr. Under these conditions fragment P-6 is the most positively charged peptide and P-9 the least. A substantial amount of peptide P-Phe (Peters and Hawn, 1967) was also present in fraction II, migrating between fragments P-6 and P-9.

Characterization of Fragments. The homogeneity of peptides P-44, P-B, P-A, P-16, P-14, and P-9 on gel electrophoresis, run following disulfide bond reduction, is shown in Figure 5. Each of these six peptides also migrated as a single band on gels run without disulfide bond reduction. Fragment P-6 had a molecular weight below the limits of resolution of the gel system, but was greater than 95% homogeneous by Sephadex G-50 thin-layer gel filtration at pH 3.0. All seven peptides migrated as single bands during electrophoresis on cellulose acetate at pH 5.0 and 8.6.

Table 1: Amino Acid Composition of Peptic Fragments of Bovine Albumin.

Amino Acid	P-44[a]	Res.[b] 1–385	P-B[c]	Res.[b] 1–306	P-A[d]	Res.[b] 307–581	P-16[e]	Res.[b] 49–185	P-14[e]	Res.[b] 186–306	P-9[f]	Res.[b] 307–385	P-6[g]	Res.[b] 453–503
Asp	42.7 ± 2.7	40	32.3 ± 3.5	31	23.3 ± 2.2	23	13.1 ± 1.9	14	11.1 ± 0.8	12	8.6 ± 0.5	9	4.1 ± 0.6	4
Thr[h]	12.7 ± 1.2	15	13.7 ± 1.2	13	22.3 ± 2.6	21	5.2 ± 0.3	5	6.3 ± 0.1	6	2.0 ± 0.6	2	4.5 ± 0.2	5
Ser[i]	21.0 ± 1.7	19	14.8 ± 2.4	15	15.9 ± 1.2	13	5.6 ± 0.8	6	8.7 ± 0.2	8	4.1 ± 0.4	4	4.2 ± 0.1	3
Glu	50.4 ± 2.6	53	41.3 ± 1.4	42	36.2 ± 4.7	37	17.2 ± 4.2	19	13.4 ± 1.3	14	10.1 ± 0.6	11	5.3 ± 0.5	5
Pro	19.2 ± 2.4	17	15.1 ± 1.2	14	13.8 ± 1.1	14	7.8 ± 0.8	8	6.3 ± 0.5	5	3.6 ± 0.4	3	3.9 ± 0.5	4
Gly	11.6 ± 0.7	11	9.8 ± 0.7	10	6.1 ± 0.3	5	5.7 ± 0.4	6	2.1 ± 0.1	2	1.1 ± 0.2	1	0.8 ± 0.0	0
Ala	30.9 ± 1.3	33	23.6 ± 1.2	25	20.5 ± 2.0	21	10.7 ± 0.7	11	11.7 ± 0.2	12	7.5 ± 0.4	8	2.2 ± 0.1	2
Half-Cys	22.9 ± 1.1	23	19.6 ± 2.3	19	16.6 ± 1.1	16	9.6 ± 0.2	10	7.9 ± 1.0	8	4.0 ± 0.7	4	3.4 ± 0.3	4
Val	17.3 ± 1.4	18	12.6 ± 1.3	13	22.4 ± 2.1	23	4.7 ± 0.2	3	7.1 ± 1.0	7	5.2 ± 1.4	5	4.5 ± 0.4	5
Met	1.7 ± 0.2	2	1.6 ± 0.4	2	1.7 ± 0.5	2	1.1 ± 0.1	2	0.5 ± 0.1	0	0.4 ± 0.6	0	0.1 ± 0.1	0
Ile	7.3 ± 1.4	9	7.7 ± 1.0	9	4.2 ± 0.8	5	2.1 ± 0.1	2	3.5 ± 0.4	5	0.1 ± 0.2	0	0.9 ± 0.2	1
Leu	41.4 ± 2.8	42	33.2 ± 4.6	33	26.9 ± 1.9	28	13.5 ± 1.7	13	14.6 ± 2.0	14	9.0 ± 1.3	9	5.3 ± 0.3	6
Tyr	15.6 ± 1.1	15	8.1 ± 1.9	9	9.3 ± 0.8	10	7.6 ± 0.4	7	0.9 ± 0.1	1	5.7 ± 0.5	6	0.9 ± 0.3	1
Phe	21.0 ± 0.8	18	12.8 ± 2.1	14	13.4 ± 1.7	13	7.8 ± 0.4	7	3.3 ± 0.2	3	4.8 ± 0.6	4	2.2 ± 0.2	2
His	12.1 ± 1.4	14	11.0 ± 2.4	11	5.0 ± 0.4	6	4.4 ± 0.6	4	2.7 ± 0.3	3	2.3 ± 0.5	3	1.3 ± 0.2	1
Lys	39.5 ± 1.7	39	34.4 ± 3.3	32	26.3 ± 2.4	27	15.0 ± 0.7	14	12.7 ± 1.3	14	7.3 ± 0.4	7	4.5 ± 0.1	5
Arg	15.7 ± 0.7	15	12.9 ± 1.1	12	11.1 ± 1.4	11	4.9 ± 0.3	5	7.2 ± 0.2	6	3.3 ± 0.5	3	3.2 ± 0.2	3
Trp[j]	2	2	2	2	0	0	1	1	1	1	0	0	0	0
NH_3		19		15		18		7		4		4		2
Total residues	385	385	306	306	275	275	137	137	121	121	79	79	51	51
Mol wt		44,140		34,953		31,275		15,766		13,554		9,205		5,820

[a] Mean ± standard deviation of 9 analyses of 2 preparations. [b] Calculated from the bovine albumin sequence reported by Brown (1975). Residues 400–402, which are blank in the albumin sequence, were assumed to be Phe-Gln-Asn by homology with the sequence of human albumin (Behrens et al., 1975). [c] Mean ± standard deviation (SD) of 12 analyses of 3 preparations. [d] Mean ± SD of 7 analyses of 2 preparations. [e] Mean ± SD of 3 analyses of 1 preparation. [f] Mean ± SD of 8 analyses of 3 preparations. [g] Mean ± SD of 4 analyses of 1 preparation. [h] Corrected for 4% loss in 24-hr hydrolysis. [i] Corrected for 10% loss in 24-hr hydrolysis. [j] Determined semiquantitatively by staining with *p*-dimethylaminobenzaldehyde on filter paper (Smith, 1953).

Table II: Molecular Weights, Absorptivities, and Carboxyl-Terminal Sequences of Peptic Fragments of Bovine Albumin.

Fragment	Mol Wt Range ($\times 10^{-3}$)[a]	$A_{280nm}^{1g/l.}$	Carboxyl-Terminal Residue[b]	Carboxyl-Terminal Sequence[c]
P-44	44–46	0.8	Leu	-(Gln-Asn)-Leu
P-B	32–34	0.74	Asp,Phe	-(Leu,Thr,Ala,Asp)
P-A	28–30	0.53	Ala	-Leu-Ala
P-16	12–14	1.23	Glu,Leu	-(Ala,Lys,Tyr,Leu,Glu)
P-14	12–14	0.63	Asp	(Not determined)
P-9	8–10	0.85	Leu	-Gln-Asn-Leu
P-6	6–8		Leu	-(Lys,Ala,Phe,Val)-Leu

[a] Experimental values obtained by dodecyl sulfate gel electrophoresis, except P-6 by thin-layer gel filtration. [b] By hydrazinolysis. [c] By carboxypeptidase treatment.

Table III: Summarized Results of Edman Degradation of Peptic Fragments of Bovine Albumin.[a]

Fragment	Cycle 1	2	3	4
P-44	Asp-	Thr-	His-	Lys
P-B	Asp-	Thr-	His	
P-A	Phe-	Ala-	Glu	
P-16	Phe-	Ala-	Lys-	Thr
P-14	Lys-	Val-	Leu-	Thr
P-9	Phe-	Ala-	Glu-	Asp
P-6	Ile-	Leu-	Asn-	Arg

[a] The residue obtained in highest yield at each cycle is shown. Under the conditions employed, serine, cystine, and alanine all appear as alanine, threonine appears as α-amino-*n*-butyric acid, and glutamine and asparagine as glutamic and aspartic acids. Complete results are available in the microfilm version of this article (see Supplementary Material Available paragraph).

The amino acid compositions of the peptides are given in Table I. The calculated composition of the corresponding segment from the albumin sequence is listed in the column adjacent to the experimental value. Molecular weights (Table II) plus amino- and carboxyl-terminal sequences (Tables II and III) helped to locate each peptide in the amino acid sequence of albumin derived by Brown (1975). (A complete version of Table III will appear in the microfilm edition; see paragraph at end of paper regarding supplementary material.)

Fragment P-44 is seen by composition, size, and terminal sequences to comprise the amino-terminal two-thirds of the albumin molecule. The terminal sequences of this peptide are (Tables II and III): H_2N-Asp-Thr-His-Lys···(Gln-Asn)-Leu-COOH. These terminal sequences correspond to residues 1–4 and 383–385. Fragment P-44 can be more accurately designated P_{1-385}, indicating that it is a fragment obtained by peptic cleavage and consisting of residues 1–385.

Fragments P-B and P-A have been previously purified and characterized by King (1973), who on the basis of comparative studies of the cyanogen bromide peptides of these fragments showed that P-B and P-A correspond respectively to the amino and carboxyl halves of the albumin molecule. We have found the terminal sequences of P-B to be (Tables II and III): H_2N-Asp-Thr-His···(Leu,Thr,Ala)-Asp-COOH. This corresponds to residues 1–3 and 303–306. Fragment P-A has terminal sequences of H_2N-Phe-Ala-Glu···Leu-Ala-COOH, corresponding to residues 307–309 and 580–581. The composition and molecular weight data (Tables I and II) agree with King's data and Brown's sequence. Fragments P-B and P-A can be designated P_{1-306} and $P_{307-581}$, respectively.

The data for fragments P-16 and P-14 indicate that they are derived from adjacent areas in the amino half of the parent molecule. The presence of one tryptophan in each of these fragments was important in establishing their approximate locations since there are only two tryptophan residues in bovine albumin (Trp_{134} and Trp_{212}), each located in a different disulfide loop in the amino-terminal half of the molecule. Peptide P-16 was found to have an amino-terminal sequence of H_2N-Phe-Ala-Lys-Thr- (Table III). This corresponds to residues 49–52 in the albumin sequence. The amino acid composition and molecular weight data (Tables I and II) suggested that the carboxyl terminus for P-16 should be in the link between disulfide loops 3 and 4 of Brown's model, but the carboxyl-terminal sequence data (Table II) were inconclusive. A likely explanation for the ambiguity of these data is that a certain portion of P-16 peptides may possess a peptic split within a disulfide loop. The amino-terminal sequence of fragment P-14 was found to be H_2N-Lys-Val-Leu-Thr- (Table III), which corresponds to residues 186–189 in the albumin sequence. The glutamic acid at position 185, adjacent to the proposed amino terminus of P-14, is proposed as the carboxyl terminus of P-16. This C terminus for P-16 (Glu_{185}) is compatible with the hydrazinolysis results for P-16 (Table II), and also fits best with its amino acid composition (Table I).

The absorptivity of P-16 was the highest of the peptic fragments (Table II), and was nearly twice that of bovine albumin. This is consistent with the high aromatic content of this portion of the molecule, which contains more than one-third of the tyrosines and one of the two tryptophans present in the whole molecule.

The composition and molecular weight data for P-14 (Tables I and II) suggest that its carboxyl terminus lies in the link between disulfide loops 5 and 6. The aspartic acid found to be the carboxyl-terminal residue in P-14 (Table II) probably corresponds to residue 306 since this is the site where fragment P-B ends. The proposed C terminus also correlates most closely with the amino acid composition data (Table I). Gel electrophoretic studies of the peptic cleavage products in the presence of octanoic acid indicate that the hydrolysis of peptide bond 306–307 occurs at a rate which is at least an order of magnitude greater than that of any other peptide bond in the molecule (Feldhoff and Peters, 1974[1]). This suggests that the smaller fragments result primarily from the breakdown of fragments P-B (P_{1-306}) and P-A ($P_{307-581}$). We have concluded, therefore, that fragments P-16 and P-14 derive from adjacent, but nonoverlapping, regions of P-B (P_{1-306}) and can more specifically be designated P_{49-185} and $P_{186-306}$, respectively.

Fragments P-9 and P-6 similarly result from the partial degradation of fragment P-A ($P_{307-581}$). The terminal sequences of P-9 are H_2N-Phe-Ala-Glu-Asp···Gln-Asn-Leu-COOH (Tables II and III). These correspond to residues 307–310 and 383–385, respectively. The amino acid composition and molecular weights (Tables I and II) also agree with the calculated values shown in Table I. Fragment P-6 was found to have an N-terminal sequence of: H_2N-Ile-

[1] Since this abstract was published the ordering of amino acids in bovine albumin has been modified. Residues 306–307 of the current sequence were then numbered 329–330.

Leu-Asp-Arg- (Table III) which corresponds to residues 453–456 in the albumin sequence. The composition, molecular weight, and C-terminal data (Tables I and II) suggest that P-6 ends at residue 503. This termination point can also be inferred from the presence in the digests of large amounts of P-Phe which has been previously described by Peters and Hawn (1967) and shown to be composed of residues 504–581. Fragment P-6 can, therefore, be designated $P_{453-503}$.

Discussion

A series of studies by Foster and his associates (e.g., see review by Foster, 1960) has established that bovine albumin undergoes a partial acid expansion in the pH range 4.5 to 3.5 which has been termed the N–F transition. Below pH 3.5 the molecule undergoes additional changes into a fully expanded form. All such changes are completely reversible. Several models accounting for the nature of the acid expansion were based on the assumption that globular parts in the molecule were linked by flexible peptide chain segments such that electrostatic repulsion at low pH caused a separation of the globular parts. A consequence of such a structure, as noted by Weber and Young (1964a), was that an acid protease should preferentially cleave peptide bonds in the linking segments while liberating a small number of globular units. As early as 1939, Holiday digested horse albumin with pepsin at pH 2.1 for 5 min and 30 min in an attempt to determine how far one could degrade an antigen before its affinity for antibody was lost. It is of interest that he suggested from ultracentrifugal and electrophoretic studies that the 5-min digest probably contained quarter molecules and the 30-min digest eighth molecules. These may correspond to the small fragments we have isolated. Similar immunological studies of partially purified albumin fragments have been reported by Porter (1957), Smet et al. (1963), and Habeeb et al. (1974), but only Smet et al. employed pepsin as the proteolytic enzyme.

The biochemical characterization of the peptic fragments of bovine albumin was initiated by Weber and Young (1964a,b) and pursued by Peters and Hawn (1967). These investigators performed the digestion at pH 3.0 or below where the molecule exists in its fully expanded form. Recently, King (1973) employed milder conditions when he digested albumin for 20 min in the presence of the protecting ligand, octanoic acid, at pH 3.7 where the molecule is only partially expanded. From such hydrolysates, King purified two large fragments, P-B and P-A, which correspond to the amino and carboxyl halves of the molecule. Extending King's work at pH 3.7, we reported on the relative susceptibility of various peptide bonds to peptic cleavage both in the presence and absence of octanoic acid (Feldhoff and Peters, 1974).[1] In digests without octanoic acid, the principal product was found to be a large fragment with a mol wt of about 44,000. In all digests, two classes of small fragments with molecular weights of about 8000 or 15,000 were found containing either one or two disulfide loops. Our peptide purification procedures included an initial gel filtration step at pH 3.0 followed by a separation based on charge at pH 5.0. These low-pH procedures have tended to circumvent the problem of fragment aggregation which occurs near pH 7.

To date, three tryptic and nine peptic fragments have been prepared without disulfide bond reduction. These fragments result from the enzymatic cleavage of six of the eight proposed inter-loop links in Brown's model (Figure 6). Four of the fragments correspond to individual disulfide loops.

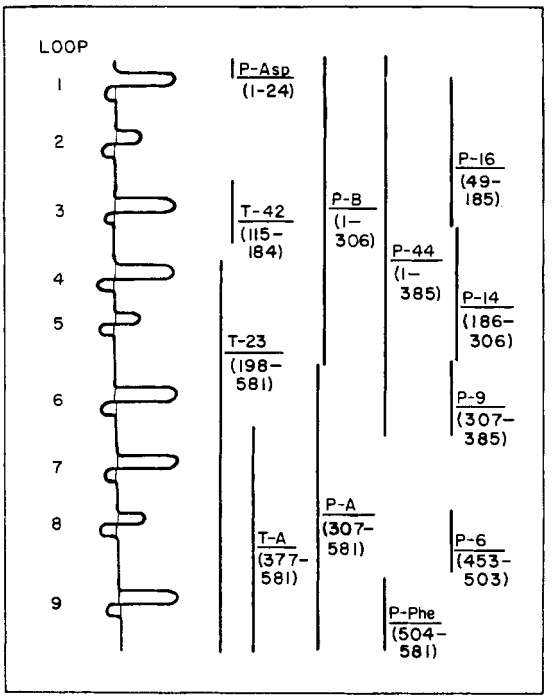

FIGURE 6: Diagram of tryptic and peptic fragments isolated from bovine albumin without breaking S–S bonds. The disulfide bridging pattern is according to the model proposed by Brown (1975). Fragments T-A, T-23, and T-42 were described by Peters and Feldhoff (1975); fragments P-Asp and P-Phe by Peters and Hawn (1967); fragments P-A and P-B by King (1973) and confirmed in this paper; fragments P-44, P-16, P-14, P-9, and P-6 in this paper. Peptides resulting from cleavages in six of the eight connecting links have been isolated.

These are the P-Phe fragment of Peters and Hawn (1967) (loop 9), the tryptic fragment $T_{115-184}$ (Peters and Feldhoff, 1975) (loop 3), and the two single-loop fragments described in this paper, P-9 (loop 6) and P-6 (loop 8). Additionally, the multiple-loop structures P-44, P-B, P-A, P-16, and P-14 have been isolated which correspond to loops 1–6, 1–5, 6–9, 1–3, and 4–5 in the model. Investigations of these 12 proteolytic fragments with respect to secondary structure, palmitate binding, bilirubin binding, and immunological reactivity will be presented in subsequent papers.

The P-B and P-A peptides (loops 1–5 and 6–9) are the only two fragments which together account for all of the residues in BSA. At pH 7.4 P-A and P-B associate to form an albumin-like complex (Feldhoff et al. 1975). The ligand-binding properties and immunological features of this complex are currently under study.

In recent publications, Braam and co-workers (1971a,b, 1974) have investigated the peptic digestion of bovine albumin in the pH range of 2.5 to 4.5 with particular emphasis on the fragments obtained after digestion between pH 3.6 and 3.9. They have purified five peptides observed on polyacrylamide gels and have termed these 4, 5, 8, 9, and 10. We have attempted to correlate their published analyses of these fragments with the nine peptic fragments shown in Figure 6. Although there are some discrepancies in amino acid composition and molecular weight, it seems likely that their fragments 4, 5, and 10 correspond to P-44, P-B, and P-Phe, respectively. Pearlman and Fong (1972) purified two peptic fragments by fractionation with trichloroacetic acid in an effort to obtain steroid-binding peptides. Their fragments, termed KL and VI, appear to correspond to P-9 and P-Asp, respectively.

Franglen and Swaniker (1968) have investigated five

peptic fragments of human albumin, but it is difficult to correlate their peptides with those obtained in this work since in our hands the peptic digestion pattern of human albumin is much different from that of bovine albumin. This difference is probably related to alterations in sequence which have changed the relative susceptibility of various peptide bonds to enzymatic hydrolysis.

Acknowledgments

We are indebted to Dr. James R. Brown for personal communications concerning the albumin sequence. We are grateful to Dr. Roberta G. Reed, Dr. Leonard A. Sauer, and Pamela A. Feldhoff for comments regarding this manuscript.

Supplementary Material Available

A full version of Table III showing results of Edman degradation of peptic fragments of bovine albumin will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Business Office, Books and Journals Division, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.00 for photocopy or $2.50 for microfiche, referring to code number BIO-75-0000.

References

Andrews, P. (1970), *Methods Biochem. Anal. 18,* 1.

Behrens, P. Q., Spiekerman, A. M., and Brown, J. R. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 591.

Braam, W. G. M., Harmsen, B. J. M., and Van Os, G. A. J. (1971a), *Biochim. Biophys. Acta 236,* 99.

Braam, W. G. M., Harmsen, B. J. M., Walters, J. A. L. I., and Van Os, G. A. J. (1971b), *Int. J. Protein Res. 3,* 277.

Braam, W. G. M., Hilak, M. C., Harmsen, B. J. M., and Van Os, G. A. J. (1974), *Int. J. Peptide Protein Res. 6,* 21.

Brown, J. R. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1389.

Brown, J. R. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 591.

Feldhoff, R. C. and Peters, T., Jr. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1235.

Feldhoff, R. C., Reed, R. G., and Peters, T., Jr. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 591.

Foster, J. F. (1960), in The Plasma Proteins, Vol. I, Putnam, F. W., ed., New York, N.Y., Academic Press, pp 179–233.

Franglen, G., and Swaniker, G. R. E. (1968), *Biochem. J. 109,* 107.

Habeeb, A. F. S. A., Atassi, M. Z., and Lee, C-L. (1974), *Biochim. Biophys. Acta 342,* 389.

Holiday, E. (1939), *Proc. R. Soc. London, Ser. B 127,* 40.

King. T. P. (1973), *Arch. Biochem. Biophys. 156,* 509.

King, T. P., and Spencer, M. (1970), *J. Biol. Chem. 245,* 6134.

Pearlman, W. H., and Fong, I. F. F. (1972), *J. Biol. Chem. 247,* 8078.

Peters, T., Jr., and Feldhoff, R. C. (1975), *Biochemistry* (in press).

Peters, T., Jr., and Hawn, C. (1967), *J. Biol. Chem. 242,* 1566.

Peters, T., Jr., Taniuchi, H., and Anfinsen, C. B., Jr. (1973), *J. Biol. Chem. 248,* 2447.

Porter, R. R. (1957), *Biochem. J. 66,* 677.

Smet, F., Lontie, R., and Preaux, G. (1963), *Protides Biol. Fluids Proc. Colloq. II,* 119.

Smith, I. (1953), *Nature (London) 171,* 43.

Weber, G., and Young, L. B. (1964a), *J. Biol. Chem. 239,* 1415.

Weber, G., and Young, L. B. (1964b), *J. Biol. Chem. 239,* 1424.

Weber, K., Pringle, P. R., and Osborn, M. (1972), *Methods Enzymol. 26C,* 3.