Fragments of Bovine Serum Albumin Produced by Limited Proteolysis: Complementary Behavior of Two Large Fragments†

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ABSTRACT: Two fragments of bovine serum albumin (BSA), prepared by limited peptic digestion, have been shown to form a 1:1 complex which demonstrates several properties of BSA not observed for either fragment alone. The two fragments are composed of residues 1–306 and 307–581 of BSA. T. P. King ((1973), *Arch. Biochem. Biophys. 156,* 509) showed that an equimolar mixture of these fragments had a higher capacity for binding octanoate than would be expected based on the sum of the individual binding capacities. We have found that the equimolar complex migrates as a BSA-like band during thin-layer gel filtration on Sephadex G-100 and upon electrophoresis on cellulose acetate. From the electrophoretic results the association constant for complex formation at 22 °C is estimated to be 1.5×10^5 M^{-1} at pH 8.6 and 7.4×10^3 M^{-1} at pH 7.4. The binding of tryptophan and 8-anilino-1-naphthalenesulfonate by the 1:1 complex is enhanced by a factor of two over that predicted from the sum of the binding affinities of the individual fragments. In contrast, binding of palmitate and bilirubin by the complex is comparable only to the simple sum of binding by the individual fragments. On double immunodiffusion with antiserum to BSA, the equimolar complex reacts as effectively as does BSA, whereas each fragment demonstrates only a partial identity with BSA. These results are consistent with the characterization of albumin as a protein composed of several domains, with each fragment retaining an intact domain and another domain being regenerated in the complex. Precipitin curves with anti-BSA suggest that the amino-terminal fragment, 1–306, is primarily in a configuration different from its configuration in the parent molecule, but that it tends to assume the native configuration when it is allowed to interact with the complementary fragment 307–581.

Fragments of bovine serum albumin (BSA)[1] obtained from limited proteolysis have been shown to retain many of the structural and functional characteristics of the parent molecule (Reed et al., 1975). Among the fragments which have been isolated and characterized, BSA-P-(1-306)[2] and BSA-P-(307-581) are of particular interest for complementation studies since together they constitute the entire amino acid sequence of BSA. Isolation of these fragments was first reported by King (1973), who noted that an equimolar mixture of the fragments had a higher capacity for binding octanoate than would be expected based on the binding capacity of either fragment alone. He suggested that the enhanced binding resulted from formation of a complex of the fragments. Additional evidence for complex formation has been cited by Taylor and Vatz (1973), who reported that the catalytic activity of BSA in the decomposition of the Meisenheimer complex is not observed for either fragment alone but is restored when the fragments are mixed in an equimolar ratio.

In the present work, electrophoretic, immunological, gel filtration, and ligand-binding properties of the fragments, alone and in combination, demonstrate the existence of a 1:1 complex, designated BSA-P'[2], which possesses some properties of albumin not present in either fragment alone. This represents a continuation of our studies on the purification and properties of proteolytic fragments of bovine serum albumin (Reed et al., 1975; Feldhoff and Peters, 1975; Peters and Feldhoff, 1975). There has been a preliminary report on some of this work (Feldhoff et al., 1975).

Experimental Section

Materials. Bovine serum albumin was crystalline bovine plasma albumin from Armour Pharmaceutical Co., Kankakee, Ill. For immunological studies, the sulfhydryl group of BSA was blocked with half-cystine by addition of a solution of L-cystine, 5 mol/mol of albumin, which had been dissolved in a minimum of 0.1 N NaOH, to a 2%, w/v, solution of BSA. The pH of the albumin solution was maintained between 7.0 and 7.5. After 5 h at 22 °C the solution was desalted in a mixed bed resin (Rexyn-I-300, Fisher Scientific Co.). Less than 0.01 mol/mol of free sulfhydryl was detectable by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) at pH 8.0 (Janatova et al., 1968). Fragments BSA-P-(1-306) and BSA-P-(307-581) were prepared from a 20-min peptic digest of half-cystinyl BSA in the presence of octanoic acid at pH 3.7 and 25 °C. The purification scheme included gel filtration at pH 3.7 on Sephadex G-150 followed by ion-exchange chromatography on CM-cellulose and DEAE-cellulose. Details of the preparation and purification have been described previously (Feldhoff and Peters, 1975).

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[1] Abbreviations used are: BSA, bovine serum albumin; Ans, 8-anilino-1-naphthalenesulfonate; SD, standard deviation; CM, carboxymethyl; DEAE, diethylaminoethyl.

[2] The peptide fragments of BSA have been designed by an adaptation of the rules of the Commission on Biochemical Nomenclature (IUPAC-IUB Commission on Biochemical Nomenclature (1967), *J. Biol. Chem., 242,* 555–557). The prototype is "trivial name (x-y)" where trivial name denotes the origin of the peptide and x and y the NH_2- and COOH-terminal amino acid residues, respectively. BSA-P-(1-306) and BSA-P-(307-581) represent fragments of BSA which are composed of 306 and 275 residues, respectively. These fragments were designated B and A by King (T. P. King, 1973) and P-B and P-A by Feldhoff and Peters (1975). The "P" between the trivial name and the residue numbers indicates that the fragments were isolated from a digestion of BSA with pepsin. The complex formed from the two fragments has been designated BSA-P'.

Rabbit antibovine albumin was prepared by injecting bovine albumin, precipitated with alum, into four adult female rabbits. In the first week 1.25 mg was injected subcutaneously, then at days 3, 4, and 5 the same dose was given intravenously; in the second week the injections were repeated using 2.5 mg per injection; the third week 5 mg was used per injection. The animals were bled by cardiac puncture at the end of the fourth week. The pooled antisera precipitated 1.0 mg of bovine albumin per ml of antiserum at equivalence.

Goat antibovine albumin was prepared by injecting 10 mg of bovine albumin in Freund's complete adjuvant into an adult goat. This preparation was conducted at the Animal Farm of the National Institutes of Health. After 3 and 6 weeks 10 mg of bovine albumin in Freund's complete adjuvant was injected. The animal was bled at week 7 and exsanguinated at week 11. The serum precipitated 0.5 mg of bovine albumin per ml of antiserum at equivalence.

Methods. Double immunodiffusion was carried out in 1% agar in 0.1 M NaCl–0.02 M sodium phosphate buffer at pH 7.4 containing 0.1 mg/ml sodium azide as a preservative. The center well contained 10 μl of antiserum and the outer wells contained 2 μl of a 1 mg/ml solution of protein. After 48 h at 22 °C, the agar slides were dried at 37 °C and stained with 0.5% Ponceau S in 5% trichloroacetic acid–5% sulfosalicylic acid.

Precipitin reactions were studied by adding 75 μl of clear antiserum to desired aliquots of antigen in 125 μl of the above phosphate-buffered saline in 1.5-ml polystyrene centrifuge tubes (Fisher No. 4-989-145). The tubes were mixed on a Vortex mixer and allowed to sit for 2 h at 37 °C and then for 16 h at 3 °C. The tubes were then centrifuged for 5 min at 600*g*, and the precipitates were washed three times with 200 μl of buffered saline with recentrifugation. The precipitates were dissolved in 1.0 ml of 0.05 N NaOH and protein assayed by an automated modification of the method of Lowry et al. (1951), which used a Technicon AutoAnalyzer. The standard was bovine serum albumin, the concentration of which was established by the factor, $A_{280nm}^{1mg/ml} = 0.661$.

Bilirubin binding was determined by kinetic assay using a peroxide–peroxidase system to oxidize the unbound bilirubin (Jacobsen and Wennberg, 1974). Palmitate binding was evaluated by partition of unbound palmitate between heptane and the aqueous phase as described by Spector et al. (1969). The binding of Ans was detected by the increase in its fluorescence in the presence of peptide or protein. The details of these procedures have been described (Reed et al., 1975). Tryptophan binding was determined by equilibrium dialysis. Protein (0.1 mM) and L-tryptophan (0.05–3 mM), each in 0.1 M NaCl–0.02 M sodium phosphate buffer at pH 7.4, were placed in opposing 1-ml compartments of an acrylic plastic dialysis cell. To each compartment containing tryptophan was added 0.1 μCi of L-[G-^{3}H]tryptophan (Amersham/Searle) of specific activity 9.7 Ci/mmol. After equilibration at 37 °C for 24 h, the [^{3}H]-tryptophan content of each compartment was determined by scintillation counting. Data were plotted according to the method of Scatchard (1949). The binding capacity was estimated from the *y*-axis intercept of a curve based on a series of successive equilibria (Spector et al., 1971).

Electrophoresis on cellulose acetate (Millipore Corp., Bedford, Mass., or Helena Laboratories, Beaumont, Texas) was performed at 22 °C either at pH 8.6 in sodium barbital buffer, μ = 0.075, at pH 7.4 in 0.04 M sodium phosphate buffer, or at pH 5 in 0.048 M pyridine–0.05 M acetic acid. Peptides were stained with 0.5% Ponceau S in 5% trichloroacetic acid–5% sulfosalicylic acid. Relative band densities were determined using a Helena AutoScanner.

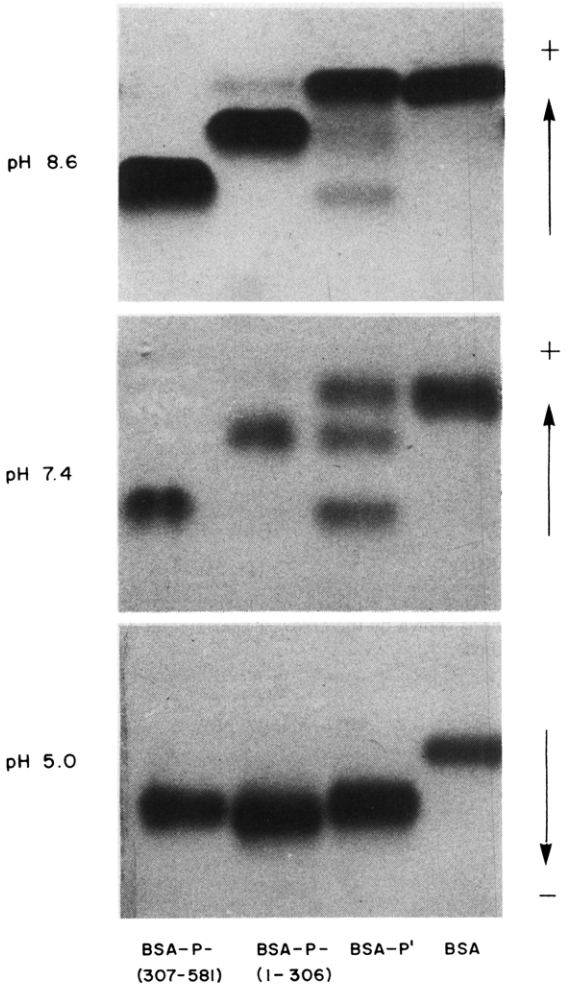

FIGURE 1: Electrophoresis on cellulose acetate demonstrating dissociation of BSA-P′ with decreasing pH. The direction of migration is shown by the arrow as being toward the anode (+) or cathode (−). Experimental details are described under Methods.

Thin-layer gel filtration followed the procedure of Andrews (1970), using Ultrafine Sephadex G-100 in 0.1 M NaCl–0.02 M sodium phosphate buffer of pH 7.4 at 22 °C. Running time was 4 h. Paper imprints were heated at 110 °C for 5 min and stained with 2% Light Green SF Yellowish (Matheson Coleman and Bell) in 8% sulfosalicylic acid.

Results

Electrophoresis. Electrophoresis of an equimolar mixture of BSA-P-(1-306) and BSA-P-(307-581) produced a major band at the position of BSA and minor bands at the positions of the two fragments (Figure 1). Both fragments migrated to positions less anodic than that of BSA or the complex BSA-P′. At pH 7.4, the electrophoretic pattern was similar to that at pH 8.6 except that the complex was appreciably more dissociated. At pH 5 the migration pattern was that of two noninteracting fragments. Dissociation could also be effected with increasing urea concentration at pH 8.6, with complete dissociation occurring in 4 M urea.

At pH 8.6, approximately 20% of each of the fragments migrated to the position of the free fragment and 80% to the position of BSA. At pH 7.4, 60% migrated as free fragment and 40% as BSA. The equilibrium concentrations of the three components were calculated from the concentration of the

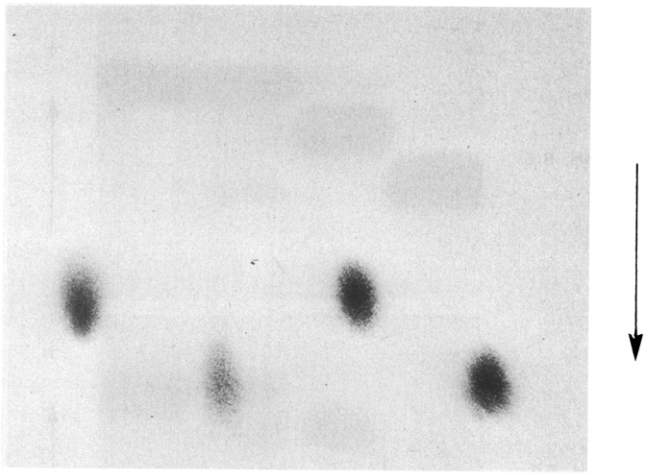

FIGURE 2: Thin-layer gel filtration on Sephadex G-100. The direction of migration is shown by the arrow. Experimental details are described under Methods.

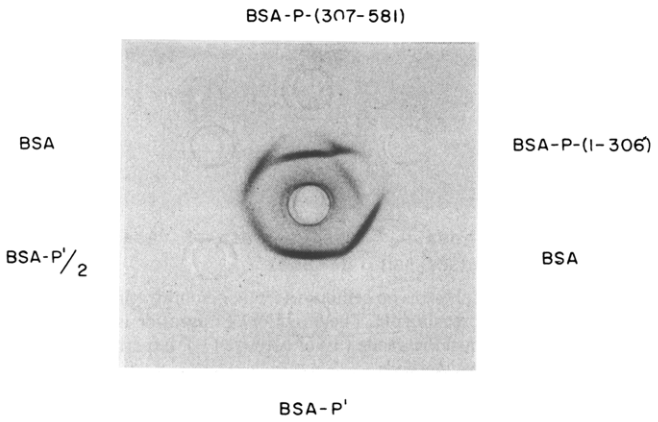

FIGURE 3: Double immunodiffusion demonstrating the relative antigenicities of BSA and fragments. The center well contained rabbit antiserum to BSA. The outer well designated BSA-P′/2 contained 1 μg of protein; all others contained 2 μg.

initial application (20 mg/ml) and the relative areas of the three bands upon integration. The magnitude of the association constant was estimated using the following equation:

$$K_a = \frac{[\text{BSA-P}']}{[\text{BSA-P-(1-306)}][\text{BSA-P-(307-581)}]}$$

At pH 8.6 (22 °C), K_a was determined to be 1.5×10^5 M^{-1}; at pH 7.4 (22 °C), it was 7.4×10^3 M^{-1}. These association constants are similar in magnitude to the value 6.3×10^5 M^{-1} reported for the association of two tryptic fragments of staphylococcal nuclease determined by fluorescence techniques (Light et al., 1974). They correspond to free energies of formation, ΔG, of −7.0 and −5.3 kcal/mol, respectively.

Gel Filtration. The size of the complex was estimated by comparison with BSA and each of the fragments on thin-layer gel filtration (Figure 2). BSA-P′ was found to migrate the same distance as BSA, reflecting a complex composed of one molecule of BSA-P-(1-306) and one of BSA-P-(307-581).

Immunoassay. The immunodiffusion experiment shown in Figure 3 was carried out by the Ouchterlony technique using rabbit antiserum to BSA. Single spurs between BSA and each

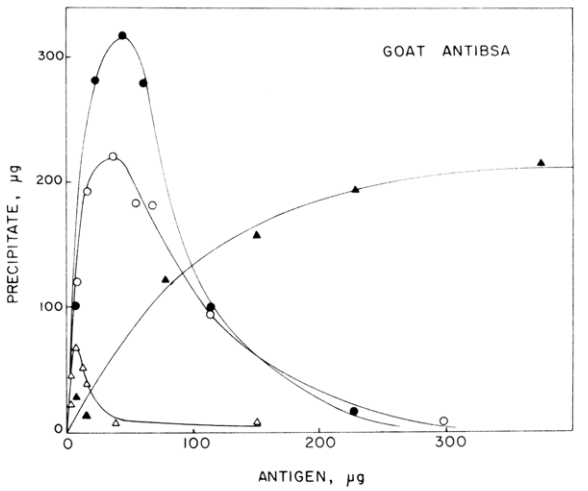

FIGURE 4: Precipitin curves of (●) BSA, (○) BSA-P′, (▲) BSA-P-(1-306) and (△) BSA-P-(307-581) with goat antiserum to BSA. The curve for BSA-P-(1-306) shows a decrease in precipitate formation when the antigen level exceeds 500 μg. Similar results were observed with rabbit antiBSA.

fragment indicate the absence of some antigenic sites of BSA in each fragment. The smooth fusion between BSA and BSA-P′ indicates that the complex contains all of the precipitin sites found in BSA. Similar results were observed with goat antiserum to BSA.

Precipitin behavior of each fragment with anti-BSA is markedly different from the behavior of BSA itself (Figure 4). BSA-P-(307-581) exhibits a classic precipitin curve which rises to a maximum at a relatively low antigen concentration and falls in the zone of antigen excess. This curve is lower and sharper than that for BSA, even when compared on a molar rather than a weight basis. On the other hand, BSA-P-(1-306) requires a relatively large antigen concentration to reach maximum precipitate formation and shows little decrease in the zone of antigen excess. The precipitin curve of BSA-P′ resembles that of BSA, showing the classic precipitin shape and maximum precipitate formation in the same range of antigen concentration.

Ligand Binding. Ligand binding properties of BSA-P′ and the individual fragments are compared with BSA in Table I. The binding capacity of BSA is defined as 100%; binding by the fragments and BSA-P′ is reported as a percentage of the capacity of BSA. Comparison of BSA-P′ (Table I, line 5) with the fragment total (Table I, line 4) indicates that the complex has approximately twice the affinity for L-tryptophan and Ans as the sum of its component fragments. The data of King (1973) for octanoate binding are listed for comparison and show a similar effect.

Titration of BSA-P-(1-306) with BSA-P-(307-581), monitored by Ans fluorescence, is shown in Figure 5. The observed fluorescence increased more rapidly than predicted by simple additivity until an equimolar amount of BSA-P-(307-581) had been added to BSA-P-(1-306). Addition of more than equimolar amounts of BSA-P-(307-581) resulted in a fluorescence increase directly proportional to the amount added, with no further enhanced fluorescence. The equivalence point for the titration occurred at a 1:1 molar ratio of the two fragments.

In contrast to the results with tryptophan, Ans and octanoate, little or no enhancement was seen for the more tightly bound ligands, palmitate and bilirubin (Table I, last two columns). Binding of palmitate by individual fragments totaled 71% of the capacity of BSA based on the sum of the *y*-axis

TABLE I: Relative Ligand Binding Properties of Albumin and Its Fragments.

	Relative Binding of				
	L-Tryptophan[a]	Ans[b]	Octanoate[c]	Palmitic Acid[d]	Bilirubin[e]
BSA	100	100	100	100	100
BSA-P-(1-306)	11 ± 5	26 ± 3	0.03	20 ± 4	275 ± 25
BSA-P-(307-581)	30 ± 8	20 ± 2	12.5	51 ± 6	0.6 ± 0.5
Fragment total	41 ± 13	46 ± 5	12.5	71 ± 10	276 ± 26
BSA-P′	83 ± 10	90 ± 7	23.8	74 ± 6	253 ± 25
Level of significance[f]	$p <$ 0.001	$p <$ 0.001		NS	NS

[a] Based on K_a for a single site ± SD. For BSA, K_a is 2.2×10^3 M^{-1}. [b] Relative fluorescence ± SD (Reed et al., 1975). [c] Based on association constants K_1 reported by King (1973). For BSA, K_1 is 1.6×10^5 M^{-1}. [d] Based on the range of y-axis intercepts of Scatchard plots. For BSA, the intercept is 4.5×10^7 M^{-1} (Reed et al., 1975). [e] Based on K_a for a single binding site ± SD. For BSA, K_a is 2.0×10^7 M^{-1} (Reed et al., 1975). [f] Significance of the difference between the sum of individual fragment binding and binding by BSA-P′ using Student's t-test. NS = not significant.

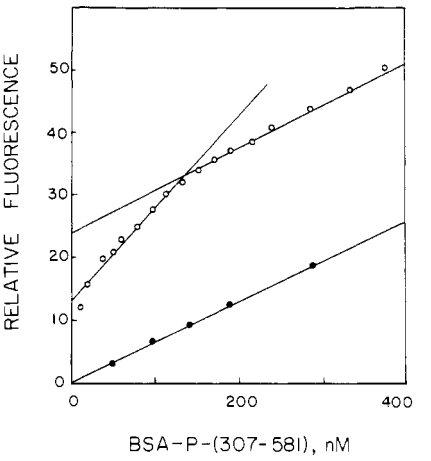

FIGURE 5: Relative fluorescence of Ans. (○) Observed fluorescence for Ans in the presence of 140 nM BSA-P-(1-306) and varying amounts of BSA-P-(307-581). (●) Observed fluorescence of Ans in the presence of varying amounts of BSA-P-(307-581) in the absence of its complementary fragment. The relative fluorescence of Ans in the presence of 140 nM BSA-P-(1-306) was 11. The slope of the initial portion of the upper curve (○) is 0.135; the latter portion, 0.070. The slope for the lower curve (●) is 0.066. The slopes were determined by linear regression and are reported in relative fluorescence units/nM BSA-P-(307-581).

intercepts of Scatchard plots. The complex showed a negligible increase to 74% of the capacity of BSA. A theoretical Scatchard plot (not shown) for BSA-P′ based on the sum of the two fragments diverges from the experimental binding curve for BSA-P′ as $\bar{\nu}$ increases, with BSA-P′ binding more effectively than predicted, although not as well as BSA itself. Treatment of the binding data for BSA-P′ by assuming a series of stepwise equilibria, as described by Spector et al. (1971), gave results compatible with an additive effect for the three strongest palmitate binding sites, plus a regeneration of additional, weaker binding sites.

The amino-terminal fragment BSA-P-(1-306) demonstrated a strong bilirubin-binding site (Table I) which was unaffected by the presence of the complementary carboxy-terminal fragment. The latter demonstrated only negligible binding affinity for bilirubin. The nature of the binding of bilirubin by BSA-P-(1-306), including comment on the stronger binding by the fragment than the parent molecule, has been discussed in an earlier work (Reed et al., 1975).

Discussion

The association of two fragments of BSA to form a complex has been characterized by a variety of experimental techniques. The complex is composed of one fragment of BSA-P-(1-306) and one fragment of BSA-P-(307-581) as demonstrated by gel filtration and by fluorimetric titration. The gel filtration pattern in Figure 2 shows the 1:1 complex to be about the same size as BSA. The maximum enhancement of Ans fluorescence occurred when the fragments were present in equal amounts—also indicative of a 1:1 complex of the two fragments.

The complex appears to be joined by many of the same noncovalent forces which determine the tertiary structure of BSA since the association of the fragments regenerates properties which are characteristic of BSA. The free energy for association of the two fragments, −7.0 kcal/mol, based on the electrophoretic separation at pH 8.6, is half of the 14 kcal/mol electrostatic free energy assigned to the initial unfolding of albumin with decreasing pH (Tanford, 1961). Although the electrophoretic patterns do not represent a true equilibrium situation, the relative concentrations of the complex and fragments may be taken as a lower limit of the degree of association. Kinetic studies on the association of the fragments of staphylococcal nuclease indicate that association is a relatively slow process, with a half time on the order of 16 s (Light et al., 1974). Their dissociation would be expected to be considerably slower. If the association of the fragments of albumin occurs on a similar time scale, little displacement from equilibrium should occur during the 20-min period required for electrophoretic separation.

It is noteworthy that each fragment has a lower net negative charge than BSA and migrates to a less anodic position than does BSA (Figure 1). Salaman and Williamson (1971) reported that the isoelectric point of BSA rose from 5 to 6 when 6 M urea was included in their isoelectric focusing medium. They interpreted this finding as consistent with the exposure of seven basic groups which were "buried" in native BSA but exposed in urea-denatured BSA. Exposure of some of these same basic groups in the fragments may be responsible for the electrophoretic migration of the free fragments to positions less anodic than BSA. Since BSA-P′ migrates to the same position as BSA, such positively charged groups would also be "buried" in the complex.

Immunologically, BSA-P′ behaves much like BSA. The Ouchterlony double diffusion pattern (Figure 3) shows a smooth transition between BSA and BSA-P′ indicating the presence of all precipitin sites of BSA in BSA-P′. The precipitin reaction of BSA-P′ with both goat and rabbit antisera to BSA demonstrated a dependence on antigen concentration resembling that of BSA (Figure 4). The fragments, on the other hand, reached maximum precipitate formation at antigen/antibody ratios quite different from that for BSA or BSA-P′. Precipitin curves typically exhibit a shape similar to those of BSA, BSA-P′, or BSA-P-(307-581) in Figure 4. The high antigen concentrations required for maximum precipitate formation and the failure of excess antigen to inhibit precipitation, as seen for BSA-P-(1-306), have also been observed with peptide fragments of sperm whale myoglobin (Atassi and Singhal, 1970) and of human serum albumin (Lapresle and

Doyle, 1975). Sachs (1974) has suggested that the late-forming plateau indicates that the antigenic fragment is not in its native conformation but may be induced into native form by the interaction of a single antigenic site with antibody. Other antigenic sites on that fragment would then be more reactive toward antibody than would sites on free fragments. Consequently, excess free fragment would not be very effective in inhibiting precipitate formation.

Extension of this interpretation to the data in Figure 4 would imply that the carboxy-terminal fragment, BSA-P-(307-581), is in a conformation similar to that of the carboxy-terminal half of native BSA, whereas BSA-P-(1-306) is not in the conformation of the amino-terminal half of native BSA. Although the circular dichroic spectrum of BSA-P-(1-306) was indicative of retention of some secondary structure (Reed et al., 1975), the unusually high affinity for bilirubin and the anomalous shift in the spectrum of bilirubin in the presence of this fragment lend support to the suggestion that this fragment is not in the native configuration. Another protein of comparable size, penicillinase (mol wt 28 880), has been reported to refold sequentially, regenerating the native circular dichroic spectrum at an intermediate stage in the process, before other properties of the native form have been regenerated (Robson and Pain, 1976). Similarly, BSA-P-(1-306) may be in an intermediate form which is neither a completely random peptide nor the native configuration.

The classic precipitin behavior of the complex BSA-P′ suggests that BSA-P-(1-306) assumes a conformation with antigenic sites characteristic of native BSA in the presence of its complementary fragment. Presumably the amino-terminal fragment is induced into native conformation by interaction of the carboxy-terminal fragment in much the same way as suggested by Sachs (1974) for induction of native structure by antibody interaction with a single antigenic site.

Many of the ligand-binding properties of the individual fragments have been characterized in an earlier work (Reed et al., 1975). The principal sites of palmitate binding to BSA were assigned to specific regions of the molecule, with one strong site occurring in BSA-P-(1-306) and two in BSA-P-(307-581). The single strong binding site for bilirubin has been assigned to a region completely within BSA-P-(1-306). Little enhancement of binding was observed for either of these ligands to BSA-P′. In contrast, binding of Ans and tryptophan was considerably enhanced in the complex, suggesting that tryptophan and Ans are bound at sites regenerated by the association of the fragments. Binding of tryptophan is thought to occur at the same principal site as medium-chain fatty acids—a site which is also believed to be a secondary binding site for long-chain fatty acids (King and Spencer, 1970; Cunningham et al., 1975). The enhancement of tryptophan binding, secondary palmitate binding, and octanoate binding in BSA-P′ supports the suggestion of regeneration of such a common site.

Albumin has been reported to be capable of binding as many as 15 Ans molecules, but only four of these contribute to the observed fluorescence (Kolb and Weber, 1975). These four Ans molecules are bound to four independent and equivalent binding sites on BSA. Kolb and Weber (1975) termed these four as "inner sites" and characterized them as being buried within hydrophobic regions of the protein, protected from quenching by isolation from solvent water molecules. The data in Table I would be consistent with one site residing entirely in BSA-P-(1-306), one site residing entirely in BSA-P-(307-581), and the other two sites being restored in BSA-P′.

The evidence presented above illustrates the concept that BSA behaves as a multidomain protein. Each fragment maintains a certain integrity as evidenced by the presence of some antigenic and ligand-binding site (domains) of albumin in each individual fragment. Other sites are regenerated only when the two fragments are mixed and an additional domain is allowed to form through the noncovalent interactions of residues from each fragment.

References

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

Atassi, M. Z., and Singhal, R. P. (1970), *Biochemistry 9,* 3854.

Cunningham, V. J., Hay, L., and Stoner, H. B. (1975), *Biochem. J. 146,* 653.

Feldhoff, R. C., and Peters, T., Jr. (1975), *Biochemistry 14,* 4508.

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

Jacobsen, J., and Wennberg, R. P. (1974), *Clin. Chem. 20,* 783.

Janatova, J., Fuller, J. K., and Hunter, M. J. (1968), *J. Biol. Chem. 243,* 3612.

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

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

Kolb, D. A., and Weber, G. (1975), *Biochemistry 14,* 4476.

Lapresle, C., and Doyle, N. (1975), *Biochem. J. 151,* 637.

Light, A., Taniuchi, H., and Chen, R. F. (1974), *J. Biol. Chem. 249,* 2285.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Peters, T., Jr., and Feldhoff, R. C. (1975), *Biochemistry 14,* 3384.

Reed, R. G., Feldhoff, R. C., Clute, O. L., and Peters, T., Jr. (1975), *Biochemistry 14,* 4578.

Robson, B., and Pain, R. H. (1976), *Biochem. J. 155,* 331.

Sachs, D. H. (1974), in Current Topics in Biochemistry—1973, New York, N.Y., Academic Press, p 73.

Salaman, M. R., and Williamson, A. R. (1971), *Biochem. J. 122,* 93.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.

Spector, A. A., Fletcher, J. E., and Ashbrook, J. D. (1971), *Biochemistry 10,* 3229.

Spector, A. A., John, K., and Fletcher, J. E. (1969), *J. Lipid Res. 10,* 56.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley, p 517.

Taylor, R. P., and Vatz, J. B. (1973), *J. Am. Chem. Soc. 95,* 5819.