Fragments of Bovine Serum Albumin Produced by Limited Proteolysis. Conformation and Ligand Binding†

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ABSTRACT: Twelve fragments of bovine serum albumin, isolated following limited tryptic or peptic hydrolysis, have been studied to define secondary structure and locate ligand-binding sites. Based on circular dichroism, the conformational pattern of albumin (68% α helix and 18% β structure) is substantially retained by individual fragments, indicating that secondary configuration is locally determined and is not destroyed during the cleavage process nor during fragment purification. The strong bilirubin-binding site of bovine serum albumin is present in 3 of the 12 fragments. Residues 186–238 are common to the three fragments and absent from those fragments which do not bind bilirubin; consequently the strong bilirubin-binding site is suggested to involve this region. By similar reasoning, the presence of palmitate-binding sites in some fragments and not in others indicates that the three strongest sites for the binding of palmitate are located in the carboxyl-terminal two-thirds of the molecule. The first site ($K_A \approx 2 \times 10^7\ M^{-1}$) is suggested as residues 377–503; the second site ($K_A \approx 8 \times 10^6\ M^{-1}$), residues 239–306; the third site ($K_A \approx 2 \times 10^6\ M^{-1}$), residues 307–377. Bromocresol Green, a reagent used in the assay of albumin, was bound by fragments roughly in proportion to their size but showed particular affinity for the region of the strong bilirubin-binding site. The fluorescent probe, 8-anilino-1-naphthalenesulfonate, was in general bound by large fragments, supporting the concept that this ligand is held principally in clefts between domains of the macromolecule.

Fragments of serum albumin obtained by proteolysis suffer minimal chemical damage, e.g., disulfide bonds remain intact and amino acid side chains are not altered. Such fragments are more nearly native than fragments prepared by harsher chemical cleavage and hence they would be expected to retain many of the structural features of albumin including ligand-binding sites. Fragments of BSA[1] prepared by tryptic hydrolysis (King and Spencer, 1970) or peptic hydrolysis (King, 1973) and representing the carboxyl-terminal portion of the molecule retained strong binding sites for both octanoate and L-tryptophan, whereas a similar fragment prepared by cyanogen bromide cleavage (King and Spencer, 1970) showed negligible binding.

Several fragments of BSA have been isolated following limited tryptic hydrolysis (Peters and Feldhoff, 1975) and limited peptic hydrolysis (Feldhoff and Peters, 1975) and their positions within the BSA molecule have been determined according to the sequence proposed by Brown (1975). Knowledge of the positions of these fragments allows correlation of the properties exhibited by the fragment with a particular region or site in BSA, thus identifying active areas of BSA and their positions relative to one another.

In this work the secondary structure and binding of palmitate, bilirubin, Bromocresol Green, and 8-anilino-1-naphthalenesulfonate have been determined and assigned to specific regions within the BSA molecule.

Materials and Methods

Preparation, purification, and identification of the three tryptic and nine peptic fragments of BSA are described elsewhere (Peters and Hawn, 1967; Peters and Feldhoff, 1975; Feldhoff and Peters, 1975). Crystalline bovine plasma albumin, Armour Pharmaceutical Co., was used without further purification; however, for palmitate binding studies, BSA was defatted with an anion exchange resin, AG1-X8 (Bio-Rad Laboratories), using the procedure of Scheider and Fuller (1970).

Unless otherwise indicated, proteins and peptides were dissolved in PBS,[1] pH 7.4, of the composition 0.1 M NaCl–0.0162 M Na_2HPO_4–0.00375 M NaH_2PO_4 containing 0.01% NaN_3 as a preservative, and their concentrations were determined from absorbance values at 280 or 215 nm using published values for $A_{280\ nm}^{1\ mg/ml}$ of the individual fragments (Peters and Feldhoff, 1975; Feldhoff and Peters, 1975) or a mean value of 17.5 for $A_{215\ nm}^{1\ mg/ml}$, a value derived from dry weight measurements, for fragments with little or no absorbance at 280 nm.

Circular Dichroism. Spectra were recorded on a Cary 60 recording spectropolarimeter with a Model 6002 attachment generously made available for this work by Professor T. E. King. Protein samples were dissolved in 0.005 M phosphate buffer (pH 7.4, containing 0.1 M NaCl) which had previously been passed through a 0.45-μ Millipore filter to remove any suspended material. Stock solutions of 2% protein were diluted to approximately 0.3 mg/ml immediately before CD[1] spectra were to be taken. A Cary cell of path length 0.1 cm was used. Spectra were recorded from 250 to 190 nm. Observed ellipticity, θ_{obsd}, was converted to mean residue ellipticity, $[\theta]$, by the following equation:

$$[\theta] = \frac{\theta_{obsd} \times (\text{av residue wt})}{10 \times (\text{path length in cm}) \times (\text{mg/ml of protein})}$$

Calculation of secondary structure from circular dichroism was based on the assumption that at each wave-

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[1] Abbreviations used are: BSA, bovine serum albumin; CD, circular dichroism; PBS, phosphate-buffered saline; Ans, 8-anilino-1-naphthalenesulfonate.

length the protein CD spectrum may be described as a linear combination of contributions from α-helix, β-structure, and random forms. The mean residue ellipticity would then be the sum of the contributions from each form expressed as the product of the fraction, f, of the molecule in that form and the ellipticity if the polypeptide were totally in that form (eq 1). The fractions of α helix, β structure, and random configuration were calculated from eq 1 using experimental mean residue ellipticities at two wavelengths and the relationship that $f_\alpha + f_\beta + f_R = 1$. The values for $[\theta]_\alpha$, $[\theta]_\beta$, and $[\theta]_R$ were taken from Table V in the work of Chen et al. (1972). Values of f_α, f_β, and f_R were calculated using all possible pairs of wavelengths at 3-nm intervals between 204 and 237 nm. Mathematical solutions which gave positive values for f_α, f_β, and f_R were averaged to give mean values for the fraction of each type of secondary structure. These averages are reported in Table I. No single wavelength or pair of wavelengths was deliberately weighted since the analysis of Barela and Darnall (1974) indicates that no choice of wavelength is predictably better than any other in this type of analysis.

$$[\theta] = f_\alpha[\theta]_\alpha + f_\beta[\theta]_\beta + f_R[\theta]_R \quad (1)$$

Bilirubin Binding. Concentration of free bilirubin in equilibrium with protein-bound bilirubin was determined from the rate of oxidation of bilirubin by peroxide-peroxidase using the procedure described by Jacobsen and Wennberg (1974). Solutions of bilirubin (Nutritional Biochemicals Corp.) were prepared fresh daily in 5 m*M* NaOH containing 1 m*M* ethylenediaminetetraacetic acid. Concentration was determined spectrophotometrically using a molar absorptivity of 47,500 M^{-1} cm^{-1} at 440 nm for aqueous bilirubin (Jacobsen and Wennberg, 1974). An aliquot of the bilirubin solution containing between 5 and 30 nmol was added to 1 ml of PBS containing approximately 30 nmol of protein in a 1-cm cuvette. A spectrum of the resultant solution was recorded between 500 and 350 nm using a Hitachi Perkin-Elmer Coleman 124 recording spectrophotometer with cell compartment maintained at 37°C. Aliquots of horseradish peroxidase (Sigma Chemical Co.), 0.05 mg/ml in PBS, and ethyl hydrogen peroxide (Ferrosan, Malmo, Sweden), 0.05%, were added and the absorbance at λ_{max} was recorded for a period of 3–5 min. The kinetics of this system are explained in detail in the work of Jacobsen and Wennberg (1974). The oxidation of bound bilirubin was assumed to occur at a negligible rate. The concentrations of free and bound bilirubin calculated from oxidation rate were used to construct Scatchard plots (Scatchard, 1949) from which association constants were determined for a single binding site.

Palmitate Binding. Palmitate binding was evaluated by partition of unbound palmitate between aqueous and heptane phases as described by Spector et al. (1969). Approximately 30 nmol of protein or peptide in 1 ml of PBS was equilibrated with 1 ml of heptane containing from 2 to 250 nmol of [1-^{14}C]palmitic acid of known specific activity. Samples were equilibrated in 8-ml test tubes with Teflon-lined screw caps in a water bath-shaker at 37°C for 12 to 16 hr. Each phase was analyzed for ligand content by scintillation counting. Aliquots of the heptane phase and the aqueous phase, solubilized in NCS tissue solubilizer (Amersham/Searle), were dissolved in a toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene cocktail. Samples were counted in a Nuclear-Chicago Model 6850 Unilux liquid scintillation system which uses a channels-ratio method of determining efficiency. A partition coefficient of 400 was used to relate palmitic acid concentration in the heptane phase to unbound palmitate concentration in the aqueous phase. From values of free and bound palmitate concentrations, Scatchard plots were constructed and individual site binding constants were determined using a graphic curve-fitting procedure described by Weder et al. (1974).

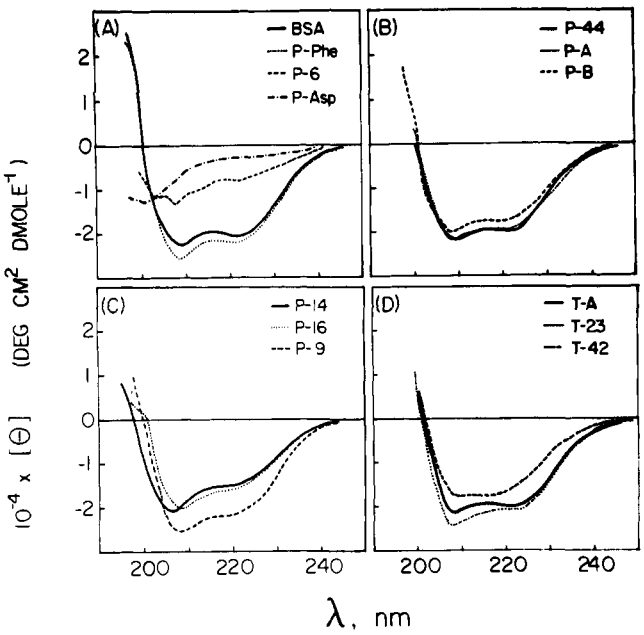

FIGURE 1: Circular dichroic spectra of BSA and its peptic and tryptic fragments. [θ] is reported as mean residue ellipticity.

Interaction with Bromocresol Green. Conditions of a commonly used clinical assay for serum albumin were employed. Three or four aliquots of peptide, 0.03–0.2 mg in 0.75 ml of water, were mixed with 0.25 ml of a fourfold concentrate of the reagent of Doumas and Biggs (1972). Peptide concentrations were determined by summation of amino acid analysis after complete hydrolysis. After 30 min at 22°C the absorbance was measured at 630 nm against a blank solution without peptide. Final concentrations were: 0.155 m*M* Bromocresol Green (Eastman Kodak 1782), 0.075 *M* sodium-succinate buffer (pH 4.2), 1.2 g/l. Brij-35 (Technicon Corp.), and 0.1 g/l. sodium azide. Results of replicates agreed within about ±2% and were averaged. There was no significant effect of doubling the Bromocresol Green:peptide ratio.

Interaction with Ans[1]. Conditions were modified after the report of Rees et al. (1954). Three or four aliquots of peptide, 0.08–0.2 mg, determined as above, were diluted to 10 ml with PBS containing 0.049 m*M* Ans (Eastman Kodak T484, recrystallized from water as the magnesium salt). After 30 min at 22°C, the relative fluorescence was measured in a Turner 110 Fluorometer, against a blank without peptide, using a 360-nm band-pass primary filter (no. 7-60) and secondary filters to exclude light below 460 nm (no. 48) and 520 nm (no. 3). The ×3 aperture setting was used. Results of replicates agreed within about ±3% and were averaged. There was no significant effect of varying the Ans:peptide ratio.

Results and Discussion

Secondary Structure. The CD spectrum of BSA (Figure 1A) shows two minima at 222 and 208 nm. This pattern is

Table I: Secondary Structure of Albumin and Albumin Fragments.

Protein or Peptide		Circular Dichroism[a]		Sequence[b]	
Name	Residues	% α	% β	% α	% β
BSA	1–581	68	18	52	13
P-44	1–385	66	16	54	13
T-23	198–581[c]	71	12	48	12
P-B	1–306	57	21	53	11
P-A	307–581	63	17	50	15
T-A	377–581	62	21	46	14
P-16	49–185	51	22	40	17
P-14	186–306	54	18	54	0
P-9	307–385	76	12	52	18
P-Phe	504–581	74	12	62	6
T-42	115–184	50	18	31	24
P-6	453–503	25	33	12	23
P-Asp	1–24	9	26	79	0

[a] Calculated from CD using the simultaneous equations derived by Chen et al. (1974). [b] Calculated from the amino acid sequence of BSA (Brown, 1975) using the rules put forth by Chou and Fasman (1974). [c] Some portions of T-23 are apparently missing peptide segments in the sequence 204–238 (Peters and Feldhoff, 1975).

Table II: Bilirubin Binding to Albumin and Albumin Fragments.

Protein or Peptide	λ_{max} (nm)[a]	$K_A(M^{-1})$[b]
BSA	470	2.0×10^7
P-44	429, 460	1.0×10^7
T-23	440	6.0×10^4
P-B	424	5.5×10^7
P-A	450	1.3×10^5
T-A	440	6.5×10^4
P-16	440	5.0×10^3
P-14	424	5.4×10^6
P-9	440	[c]
P-Phe	440	[c]
T-42	440	1.8×10^5
P-Asp	440	[c]

[a] Determined in PBS, pH 7.4, 37°C. Protein concentration was 20–30 μM; bilirubin concentration, 15 μM. [b] Determined using the peroxidase–peroxide oxidation as described by Jacobsen and Wennberg (1974). [c] No binding was detected.

substantially retained by 10 of the 12 fragments (Figure 1) indicating very little alteration in secondary structure upon proteolytic cleavage. Only the two smallest fragments, P-Asp and P-6, show relatively low ellipicities (Figure 1A). Calculations of α helix and β structure based on CD indicate that BSA and most of its fragments are 50–76% helical with less than 22% β structure (Table I).

The α helix and β structure may be predicted from amino acid sequence (Chou and Fasman, 1974) based on the concept that certain patterns of amino acid residues occur frequently in regions of α helix or β structure while others are rarely found in such regions. For BSA and its fragments, predictions based on sequence, in agreement with CD findings, indicate these peptides to be predominately helical (50%) with a small amount of β structure (14%); however, they consistently underestimate the amount of secondary structure as determined by CD. The only exception is P-Asp. This small fragment is predicted to have 72–79% helical content but by CD showed only 9%.

The most significant feature of the CD spectral data is the apparent retention of secondary structure by 11 of the 12 proteolytic fragments, supporting the premise that proteolytic fragments may be expected to exhibit properties similar to those of the parent molecule. It is noteworthy that P-Asp, the only fragment not exhibiting helical structure as predicted from its sequence, is also the only fragment which does not contain a pair of disulfide bonds and is not one of the double loops of BSA.

Bilirubin Binding. Most bilirubin present in serum is bound tightly to serum albumin, rendering the tetrapyrrole nontoxic while transporting it to the liver for subsequent conjugation and excretion. A number of other ligands, including drugs and fatty acids, have been reported to displace bilirubin from albumin leading to increased levels of free bilirubin in the plasma and increased probability of kernicterus and brain damage. Knowledge of location and chemical nature of the bilirubin-binding site would help to clarify the role of albumin in bilirubin metabolism.

Scatchard plots of bilirubin binding to BSA predict the presence of a single high-affinity binding site ($K_A = 2 \times 10^7\ M^{-1}$, Table II). This is consistent with spectral data of Wennberg and Cowger (1973) indicating that BSA binds bilirubin in a 1:1 complex.

Three peptic fragments of BSA have an affinity for bilirubin comparable to that of the parent compound (Table II). These fragments, designated P-44 (residues 1–385), P-B (residues 1–306), and P-14 (residues 186–306), have residues 186–306 in common. No other fragment investigated contains this entire region and no other fragment investigated binds bilirubin to an appreciable extent. However, the largest tryptic fragment, T-23, contains part of this region (residues 239–306) yet shows no evidence of a strong bilirubin site, suggesting that residues 186 to 238 (roughly comprising loop 4) constitute the essential region for bilirubin binding.

The visible spectrum of free bilirubin has an absorption maximum at 440 nm (Figure 2A). In the presence of fragments which do not contain the strong bilirubin-binding site, the spectrum of bilirubin resembles that of free bilirubin, e.g., bilirubin–P-A, Figure 2C. In the presence of BSA or fragments P-44, P-B, or P-14 the spectrum of bilirubin is altered in both position and shape. Bilirubin bound to BSA exhibits an absorption maximum at 470 nm, 1.3 times more intense than free bilirubin (Figure 2B). In the presence of fragments P-B and P-14 bilirubin exhibits an absorption maximum at 424 nm, 1.5 times more intense than free bilirubin (Figures 2C and 2D). When bilirubin is bound to fragment P-44 the spectrum resembles a composite of the bilirubin–BSA and bilirubin–P-B spectra (Figure 2D).

The shifts in λ_{max} of bilirubin from 440 to 470 nm upon binding to BSA and to 424 nm upon binding to fragment P-B probably represent conformational changes in bilirubin. Blauer and King (1970) have suggested that the dipyrrole units rotate around the methylene bridge bringing the two planar systems into or out of a parallel alignment. In an analysis of the conformation of bilirubin bound to human serum albumin, Blauer and Wagnière (1975) reported an inversion of the Cotton effect as the pH was changed from 4 to 6. Calculation of theoretical CD spectra for various conformations of bilirubin led them to conclude that at the higher pH the two dipyrrole units are rotated in a right-handed configuration with respect to one another whereas at low pH they assume a left-handed configuration. The inversion in chirality of bilirubin was suggested to result from pH-dependent conformational changes in the protein.

When the visible spectrum of bilirubin–BSA was studied as a function of pH (Wennberg and Cowger, 1973), a strik-

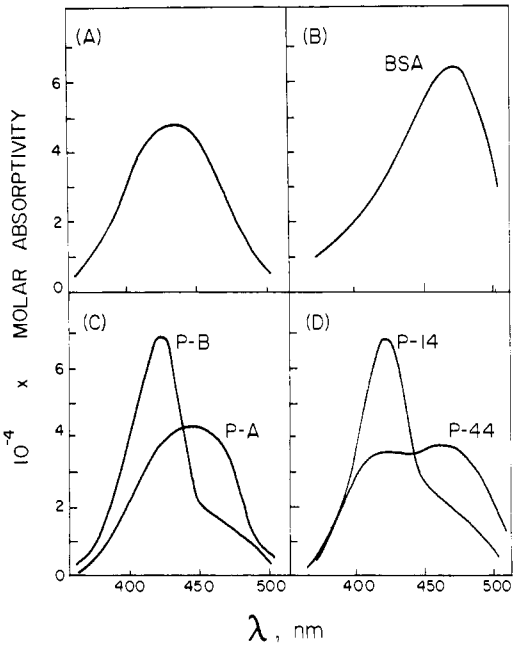

FIGURE 2: Bilirubin absorbance spectra in pH 7.4 PBS: (A) free bilirubin; (B-D) 15 nmol of bilirubin in the presence of 30 nmol of BSA or peptide.

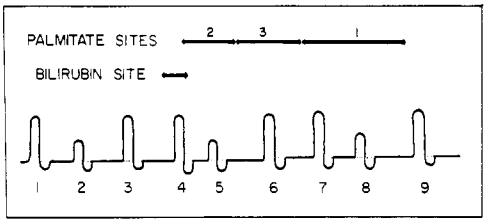

FIGURE 3: Schematic drawing of BSA showing the pattern of nine disulfide loops and the locations of palmitate and bilirubin binding sites.

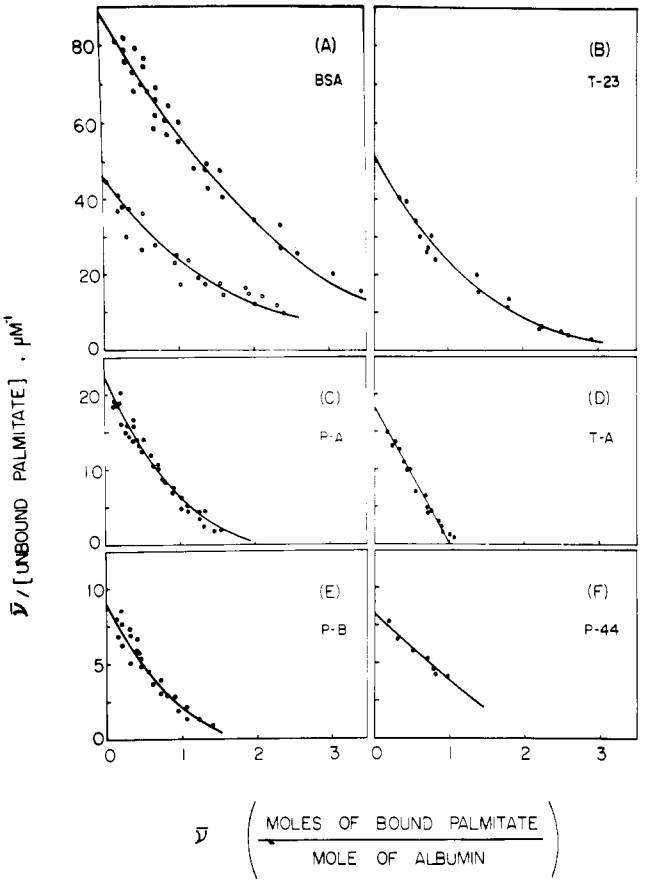

FIGURE 4: Scatchard plots of binding of palmitate to BSA and five large tryptic and peptic fragments. Solid lines represent the binding curve calculated from the association constants reported in Table III for the principal sites: (A) lower curve, BSA which had been defatted 6 months prior to binding studies; upper curve, BSA which was defatted immediately prior to binding studies.

ing change in λ_{max} from 470 nm near physiologic pH to 421 nm at pH 3.4 was observed. This was not a smooth shift from 470 to 421 nm, but instead a peak at 421 nm grew at the expense of the peak at higher wavelength as the pH was lowered from 4.5 to 3.4. This pH range is coincident with the N-F transition of albumin (Aoki and Foster, 1956). The slight expansion of albumin during N-F transition has been attributed to the separation of "domains" within the molecule. The recent publication of the sequence of BSA with its repeating loop-link structure (Brown, 1975) provides a basis for the division of albumin into domains by the spreading apart of the loops in various combinations (Figure 3). It is highly likely that the N-F transition involves the separation of loops 1-5 from loops 6-9 along the link extending from residues 288 to 313. Pepsin cleavage at pH 3.7 occurs readily between Asp-306 and Phe-307, supporting the premise that at pH 3.7 the link is extended and highly vulnerable (King, 1973; Feldhoff and Peters, 1975). The alteration in the bilirubin-BSA absorbance spectrum as the pH is lowered through the region of the N-F transition results in a spectrum similar to that of bilirubin bound to P-B or P-14, two fragments which have only loops 4 and 5 in common. Thus, these fragments simulate a domain terminating at loop 5 of BSA and containing the strong bilirubin-binding site in loop 4. The similarity of the bilirubin spectrum in the presence of BSA at pH 3.7 to that in the presence of fragments P-B or P-14 at pH 7.4 suggests that when BSA is in the N form loop 6 influences the spectral properties of bilirubin bound in the region of loop 4, but in the F form loop 6 is no longer close enough to exert such an influence. The composite nature of the bilirubin-P-44 spectrum might be interpreted in terms of two forms of P-44: the first being similar to the normal N form of BSA with loop 6 near the domain of loops 1-5 and the second being similar to the expanded F form with loop 6 away from the region of the bilirubin-binding site.

Palmitate Binding. An important function of serum albumin is to transport free fatty acids through the blood. Circulating albumin typically has only one or two fatty acid molecules bound although it is reported to be capable of binding as many as 69 fatty acid molecules (Spector et al., 1969). Studies with defatted albumin indicate that the configuration and stability of the protein depend upon the presence of a molecule of fatty acid (Sogami and Foster, 1968); when fatty acid is added to defatted albumin, the protein is believed to undergo conformational changes to form more stable hydrophobic regions.

Two Scatchard plots for BSA are shown in Figure 4A. One represents binding of palmitate to freshly defatted BSA. The other represents binding to BSA which had been defatted 6 months prior to the investigation and had been stored at 4°C in PBS containing sodium azide as a preservative. Storage of BSA in a defatted state apparently resulted in some permanent disruption of the structure as evidenced by diminished palmitate-binding ability. The bind-

Table III: Palmitate Binding to Albumin and Albumin Fragments.

Protein or Peptide	K_A (μM^{-1})		
	1st Site	2nd Site	3rd Site
BSA[a,b]	34	8.1	3.0
P-44	5.5	2.7	<0.1
T-23[b]	40	8.4	2.8
P-B	8.0	0.4	<0.1
P-A	20	2.0	<0.2
T-A	18	<0.2	

[a] BSA was stored 6 months at 4°C in the defatted state before use in binding studies, a condition comparable to the state of the fragments. When BSA was studied immediately after defatting, the corresponding association constants were 55, 20, and 10 μM^{-1}. [b] Scatchard plots suggest the presence of more than three sites, but quantitation of more than three association constants by graphical procedures was not feasible.

Table IV: Interaction of Bromocresol Green and 8-Anilino-1-naphthalenesulfonate with Albumin and Albumin Fragments.

Protein or Peptide			ΔA (630 nm) with Bromocresol Green, 1 g/l.	Fluorescence with Ans, relative 1 mg/l.
Name	No. of Residues	Mol Wt		
BSA	581	66,210	2.54	7.47
P-44	385	44,140	2.32	2.84
T-23[a]	349[a]	39,442[a]	1.36	3.26
P-B	306	34,953	2.76	5.25
P-A	275	31,275	1.32	4.10
T-A	205	23,138	1.16	3.97
P-16	137	15,766	1.23	1.52
P-14	121	13,554	3.52	1.39
P-9	79	9,205	0.65	0.11
P-Phe	78	8,623	0.82	0.38
T-42	70	8,313	0.92	3.50
P-Asp	24	2,808	0.44	0.12

[a] Some portions of T-23 (residues 198–581) are apparently missing peptide segments in the sequence 204–238 (Peters and Feldhoff, 1975).

ing of palmitate by freshly defatted BSA probably more closely reflects the binding capacity of native BSA. The stored BSA sample, however, should more closely approximate the condition of the fragments which did not have the benefit of stabilization by fatty acid during storage.

The Scatchard plots (Figure 4) of palmitate binding were analyzed graphically to determine the association constants for individual fatty acid binding sites. The association constants, 34, 8.1, and 3.0 μM^{-1}, for the three strongest sites of BSA are in agreement with the values 32.0, 7.78, and 3.48 μM^{-1} reported by Spector et al. (1971) who used a least-squares computer analysis to determine association constants for nine fatty acid binding sites of BSA. In the present investigation BSA and its fragments also showed weaker binding sites in addition to the three reported, but graphical analysis of association constants for these sites was not feasible.

Association constants for palmitate binding to the five largest fragments (Table III) may be grouped into four distinct classes: 18–34, 5.5–8.4, 2–3, and <1 μM^{-1}. A site with K_A between 18 and 34 μM^{-1} is found in fragments T-23, P-A, and T-A but not P-B or P-44. A site with K_A between 5.5 and 8.4 μM^{-1} is found in fragments P-44, T-23, and P-B but not P-A or T-A. A site with K_A between 2 and 3 μM^{-1} is found in fragments P-44, T-23, and P-A but not T-A or P-B. A site or several sites with K_A considerably less than 1 μM^{-1} may be present in all of the fragments but the data did not warrant extrapolation beyond two or three sites. BSA exhibited a single site with an association constant in each of the first three ranges and several sites with K_A less than 1 μM^{-1}.

Since the parent molecule exhibits only one site with an association constant greater than 8.4 μM^{-1}, the sites in T-A, P-A, and T-23 with association constants of 18, 20, and 40 μM^{-1} likely represent binding at the same location as the first site in BSA. The occurrence of this site in the three carboxyl-terminal fragments of BSA suggests that, in the parent molecule, the strongest fatty acid binding site occurs between residues 377 and 581. Since P-Phe (residues 504–581) showed little affinity for palmitate, the principal interaction with fatty acid probably involves only residues 377–503. In support of this suggestion, fatty acid is known to protect the Leu_{385}-Ile_{386} bond from peptic cleavage (Feldhoff and Peters, 1975) and fatty acid binding would be expected to occur near that region.

Since BSA exhibits only one site with an association constant in the range of 5–9 μM^{-1}, the sites in P-44, P-B, and T-23 with association constants of 5.5, 8.0, and 8.4 μM^{-1} likely represent binding at the same location as the second site in BSA. The region of the parent molecule common to these fragments is residues 198–306. The discontinuity in subfractions of T-23 resulting from the absence of residues between 204 and 238 (Peters and Feldhoff, 1975) suggests that the principal interaction for the second fatty acid binding sites involves residues 239–306.

BSA exhibits only one site with an association constant between 2 and 3 μM^{-1}. A site of similar strength is located in fragments P-A, T-23, and P-44 but not in T-A and as such would be expected to involve residues 307–377.

The above assignment of regions of binding is not intended to imply that residues outside the suggested regions do not contribute to binding. That binding at the proposed regions is comparable in strength to that by intact albumin is evidence, however, that the contribution of residues outside the designated regions must be small.

Bilirubin is known to be displaced from albumin by fatty acids and the site of bilirubin binding is believed to serve as a fatty acid binding site as well. The locations of binding sites as proposed in Figure 3 place bilirubin at a site which is independent of the three principal fatty acid sites, indicating that any competition would occur at a weaker fatty acid site(s). This is consistent with the observation reported by Woolley and Hunter (1970) that bilirubin is not displaced from albumin until oleate:albumin ratios reach about seven.

Interaction with Bromocresol Green and Ans. The interaction of fragments with Bromocresol Green and with Ans was tested in an attempt to outline the regions of the molecule responsible for the binding of these commonly used ligands. Bromocresol Green is a popular reagent for the assay of albumin in serum (Doumas and Biggs, 1972), and Ans is used both for clinical assays (Rice, 1966) and for the study of conformation by fluorescence (Anderson and Weber, 1969). Conditions employed were those of clinical assays; the ligand-to-albumin ratios were apparently at near-saturation levels since variation of the ratio caused no significant effect.

The color change with Bromocresol Green at pH 4.2 and the fluorescence with Ans at pH 7.4 are listed for BSA and

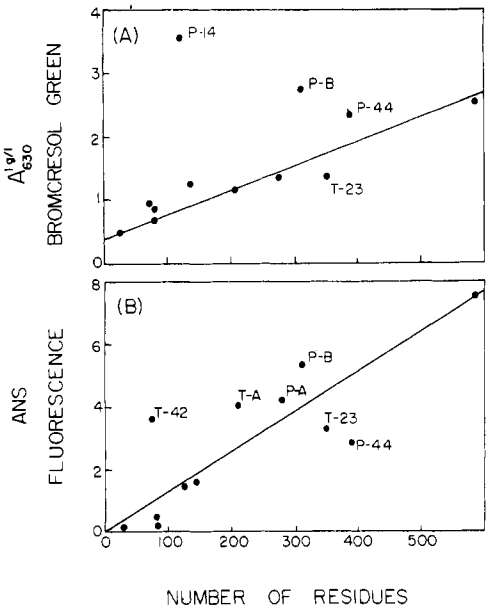

FIGURE 5: (A) Interaction of BSA and peptide fragments with Bromocresol Green; the solid line is the least-squares fit omitting the points for P-B and P-14; (b) Ans fluorescence in the presence of BSA and peptide fragments; the reference line was drawn between the origin and BSA.

11 fragments in Table IV. Results were calculated relative to the mass of peptide present. In Figure 5 the binding activities are compared to the size of the fragments, permitting detection of peptides which are particularly active or inactive for their size.

The interaction with Bromocresol Green, in general, reflects the size of the peptide (Figure 5A). The smallest peptide (24 residues) was about one-fifth as active on a weight basis as was intact albumin. Three peptides showed unexpectedly high activity—P-14, P-B, and P-44. These are all derived from the amino-terminal portion of the molecule, and have in common disulfide loops 4 and 5. Hence this region, which is also predicted to be active in bilirubin binding, appears to be a major site of interaction with Bromocresol Green. Loops 4 and 5 are also present in peptide T-23, but loop 4 is lacking a major peptide segment, which probably accounts for the abnormally low activity of this fragment.

The fluorescence with Ans (Figure 5B) is much more dependent upon peptide size than is Bromocresol Green binding. Cleavage of an average of only five bonds by pepsin is known to destroy 75–80% of the fluorescence with Ans (Weber and Young, 1964; Peters and Hawn, 1967). Peptides of less than 100 residues showed negligible binding, with the exception of fragment T-42. This fragment, which is disulfide loop 3, is notably hydrophobic, containing six tyrosines and one tryptophan in only 70 residues, and this may explain its strong interaction with Ans despite its small size. The retention of Ans-binding activity by the larger fragments may be interpreted in relation to the nature of the Ans-binding sites proposed by Anderson and Weber (1969). They suggested that five Ans molecules are bound in clefts between large domains of the molecule. Calculated on a molar basis, fragment P-B retained 40% of the Ans fluorescence of BSA (two sites?), and fragments P-A and T-A retained about 20% each (one site?). Hence, the cleavage of the BSA molecule at residue 306 and subsequent separation of the two pieces destroyed two of the binding sites for Ans. Two were preserved in the amino-terminal fragment, P-B, and one in the C-terminal fragment, which, since it appears in T-A, must be located in the region of loops 7–8–9.

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