Conformational Properties of Bovine Plasma Albumin with a Cleaved Internal Peptide Bond†

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ABSTRACT: As shown previously, proteinases frequently associated with plasma albumin samples catalyze a very limited and specific cleavage of the albumin molecule when it exists in the F conformational state near pH 3.7. The primary proteolytic product, BPA*, has a molecular weight similar to or identical with that of the parent protein but yields two large fragments of molecular weight approximately 46000 and 23000 on reduction. Evidence is presented here that cleavage occurs within the disulfide loop between Cys^{390} and Cys^{434} with no detectable loss of small peptides, the amino acid composition of BPA* being identical with that of the parent protein within experimental error. Cleavage exposes a new amino-terminal phenylalanine residue and may occur at the Glx^{392}-Phe^{393} bond although the possibility exists that it occurs at another X-Phe bond in the unsequenced region of residues 400–402. The damaged protein has a somewhat altered secondary structure as judged from optical rotatory dispersion and circular dichroism measurements, probably an approximate 15% loss in helicity. The hydrodynamic volume is increased by approximately 20%. However, various physical studies indicate the tertiary structure to be strikingly similar to that of the native protein. Of most significance is the fact that the protein still undergoes the N-F and N-B transitions, although in both cases they occur at somewhat more moderate pH than in the parent protein. Moreover a sensitivity of the N-B transition to Ca^{2+} is still seen and binding behavior toward the dye 8-anilino-1-naphthalenesulfonic acid is essentially unaltered. The results are best understood in terms of the concept of a multidomain structure which has been suggested frequently for plasma albumin. Bond cleavage damages one domain but leaves the overall structure essentially unaltered except for some weakening of the interaction between domains.

It has been observed repeatedly that limited proteolytic cleavage of either bovine or human plasma albumin yields large fragments. Such observations have been made with a variety of proteolytic enzymes including trypsin (Lapresle et al., 1959; Markus et al., 1967; King and Spencer, 1970), chymotrypsin (Porter, 1957; Richard and Kegeles, 1959), spleen cathepsins (Lapresle and Webb, 1960), pepsin (Weber and Young, 1964; Peters and Hawn, 1967; Franglen and Swaniker, 1968; Braam et al., 1971; King, 1973), and subtilisin (Adkins and Foster, 1966; Pederson and Foster, 1969). Such results, together with other extensive physical-chemical evidence, have led to the conclusion that the single peptide chain of which these proteins are formed must be folded into two or more compact subregions (Harrington et al., 1956; Foster, 1960; Bloomfield, 1966). Such elements of structure are now commonly referred to as domains (Edelman et al., 1969). The published information available on the disulfide pairings in bovine plasma albumin is completely consistent with such a model (Pederson and Foster, 1969; King and Spencer, 1970; Brown, 1975) although the precise number of domains cannot yet be specified.

In general, the aforementioned cleavage fragments separate without the necessity of reduction; hence, the peptide cleavages occur most readily between domains, as might be anticipated. An important exception to this rule is seen in the cleavage near pH 3.7 catalyzed by proteinase contaminants in the albumin (Wilson and Foster, 1971). The F form of the bovine plasma albumin molecule existing at this pH appears to be cleaved with great specificity in a single bond to yield predominantly two fragments of molecular weight approximately 46000 and 23000 which are, however, disulfide bonded. This product has been called BPA*[1] and it can be isolated in reasonably good yield since subsequent cleavages occur at a significantly slower rate. This protein may be regarded as having one domain nicked and it is a matter of some interest to ascertain how seriously this damage affects the overall three-dimensional conformation of the protein and to what extent it still undergoes the conformational transitions which are well known for the intact bovine plasma albumin molecule. Wilson and Foster (1971) concluded that BPA* does not undergo the N-F transition. It is shown here that this conclusion was incorrect and that in fact the solution behavior of BPA* is remarkably similar to that of the parent protein.

Experimental Section

Materials. Bovine fraction V, lots G36612 and F33106, and crystallized bovine plasma albumin, lot C78012 and F71601, were purchased from the Armour Pharmaceutical Company.

Sodium dodecyl sulfate, 95%, was purchased from Matheson Coleman and Bell, and recrystallized from 95% ethanol. Spectrophotometric grade guanidine hydrochloride was obtained from Heico, Inc. Acrylamide, *N,N*-methy-

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[1] Abbreviations used are BPA*, bovine plasma albumin with one internal peptide bond cleaved through action of the proteinases associated with the albumin at pH near 3.7–3.8; BPA**, a product of further action by the proteinases; ANS, 8-anilino-1-naphthalenesulfonic acid.

lenebisacrylamide, naphthol blue black, and iodoacetic acid were obtained from Eastman. The acrylamide and iodoacetic acid were twice recrystallized from chloroform. Coomassie brilliant blue R250 was obtained from Colab Laboratories, Inc., and 5,5′-dithiobis(2-nitrobenzoic acid) and iodoacetamide were purchased from the Aldrich Chemical Company. The iodoacetamide was recrystallized from water.

8-Anilino-1-naphthalenesulfonic acid was purchased from Nutritional Biochemicals Corp. as the magnesium salt and recrystallized from water. 3-Bromo-1,1,1-trifluoropropanone was obtained from Penninsular Chem. Research, Inc., dansylated amino acids from Nutritional Biochemicals Corp., and dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) from the Pierce Chemical Co. Polyamide layer sheets manufactured by the Cheng Chin Trading Company, Ltd., were obtained from Gallard-Schlesinger. All other chemicals were of the highest purity commercially available.

All water was distilled and deionized with a specific resistance greater than 10^6 ohms, and filtered through sintered glass before use.

Albumin Preparations. Defatted albumin (Chen, 1967; Sogami and Foster, 1968), monomeric albumin, and mercaptalbumin (Hagenmaier and Foster, 1971) were prepared and stored as described previously (Zurawski and Foster, 1974).

Blocking of the free sulfhydryl group of various albumin preparations with iodoacetamide was accomplished using the method described by Nikkel and Foster (1971). Occasionally the reaction was run at 25°C for 15–20 min instead of at 2°C. No noticeable differences in the properties of the blocked albumin were found. For most careful work the low temperature approach is recommended, however. Free sulfhydryl content was determined by the method of Ellman (1959) using techniques recently reported (Zurawski and Foster, 1974).

Albumin Cleavage Product. The initial cleavage product (BPA*) of Wilson and Foster (1971) was prepared from bovine fraction V purchased from Armour Pharmaceutical Co. Recently obtained samples of this fraction V contained much less of the proteinase activity; hence, another grade of albumin, leptalb-7, lot K580138, provided by the same vendor, was used in some later experiments. The nature of the BPA* produced was evidently the same.

Protein samples were placed in sealed, sterilized vessels, dissolved in a 0.01 *M* sodium acetate–0.15 *M* sodium chloride buffer at pH 3.75, and allowed to incubate at 25°C until about 50–60% of the albumin monomer was cleaved. Progress of the reaction was monitored via disc gel electrophoresis and/or sodium dodecyl sulfate gel electrophoresis.

Crude digests were purified using gradient elution chromatography on SP-Sephadex at pH 4.70. A linear ionic strength gradient from 0.1 to 0.4 was used. Also a stepwise gradient consisting of 0.1, 0.2, and 0.4 steps in ionic strength was useful in some cases. Best results were obtained using a 0.1 followed by a 0.2 step, then a 0.2–0.4 linear ionic strength gradient. The order of elution from the column was uncleaved albumin followed by BPA** and then by BPA*.

BPA* monomer was prepared by passing the purified material from this ion-exchange chromatography through a Sephadex G-150 column equilibrated with a 0.02 *M* phosphate–0.10 *M* sodium chloride buffer at pH 6.80. The presence of a small amount of dimeric protein in samples not so treated appeared not to affect the properties herein examined.

Preparation of Fragments. Pooled samples of BPA* were reduced and carboxymethylated essentially by the procedure of Crestfield et al. (1963). After 15 min the reaction was stopped by dialysis against 20% acetic acid. After several hours, the precipitated, reduced, and carboxymethylated protein was redissolved by dialysis against 0.1% dodecyl sulfate in pH 7.0, 0.01 *M* phosphate buffer and the fragments were separated by chromatography on a Sephadex G-200 column equilibrated with this same dodecyl sulfate containing buffer. It was found later that precipitation could be avoided by conducting the reduction and blocking in 6 *M* guanidine hydrochloride and then dialyzing against 0.1% dodecyl sulfate in 0.05 *M* Tris-acetate buffer (pH 8.4) followed by chromatography in this solvent.

Determination of Amino-Terminal Residues. The amino-terminal residues of BPA* and its fragments were determined by the dansylation method of Gray and Hartley (1963). The conditions for dansylation were those suggested by Gros and Labouesse (1969). Chromatography was performed by the method of Woods and Wang (1967) as modified by Hartley (1970). The hydrolysate was dissolved with either acetone–acetic acid (3:2 v/v), pyridine, or ethyl acetate saturated with water. The dansyl amino acids were identified by comparison with a standard mixture of the dansyl amino acids chromatographed on the reverse side of the polyamide sheet.

The above procedure was also used for manual sequence determinations. The amino-terminal residue was removed by the Edman degradation method as described by Weiner et al. (1972). However, we did not go beyond the second residue due to problems in redissolving the protein in the coupling buffer (0.5 *M* $NaHCO_3$, pH 9.8).

Amino Acid Analyses. Amino acid compositions of bovine plasma albumin, BPA*, and the fragments were determined on a single-column Durrum D-500 amino acid analyzer. Protein samples were hydrolyzed in 6 *M* HCl in vacuum sealed tubes at 110° for 24, 48, and 72 hr. At least three analyses were made at each time of hydrolysis and averaged. Values for threonine, serine, and cystine were obtained by extrapolation to zero time. Values for valine, isoleucine, and leucine were obtained by extrapolation to 96 hr. Values for all other amino acids were taken by averaging all data.

Optical Rotation and Circular Dichroism Measurements. Optical rotatory dispersion and optical rotation at 300 and 233 nm were carried out with a Cary 60 recording spectropolarimeter using methods outlined by Zurawski and Foster (1974). Circular dichroism measurements were performed in a similar manner on the Cary 60 equipped with a Model 6002 circular dichroism accessory.

Hydrodynamic Measurements. Investigation of the elution volume of albumin and BPA* was performed on a Sephadex G-150 column equilibrated with 0.01 *M* sodium acetate–0.15 *M* sodium chloride at pH 5.50. Bovine mercaptalbumin, commercial fraction V samples, and BPA* solutions were prepared in 0.16 *M* sodium chloride and applied to the column. Stokes radii of the hydrodynamic particles were calculated using the procedure of Ackers (1967).

Sedimentation velocities for BPA* and uncleaved albumin were determined using a Beckman Model E analytical ultracentrifuge equipped with a schlieren optical system. Protein solutions were prepared at pH 5 in 0.16 *M* sodium chloride at concentrations of 3 mg/ml. BPA* and un-

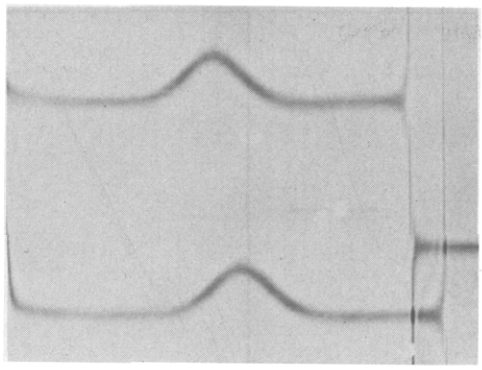

FIGURE 1: Sedimentation patterns of BPA* (upper, prism cell) and bovine mercaptalbumin monomer (lower, standard cell). Sedimentation is from right to left, 64 min at 59780 rpm and 26°, 0.3% isoionic protein in 0.16 *M* NaCl.

cleaved albumin samples were run at 59780 rpm simultaneously in a double sector cell to assure identical conditions for comparison. Photographs of schlieren peaks were taken at 16-min intervals over a period of 2 hr.

Anionic Dye Binding. The binding of the organic dye, 8-anilino-1-naphthalenesulfonic acid, was determined using the fluorometric method described by Jonas and Weber (1971). A Perkin-Elmer MPR-2A fluorescence spectrophotometer was used in the direct mode of operation with frequent referencing to a sample of known fluorescence. Concentrations of the dye were determined by optical density using a molar extinction coefficient of 6.15×10^3 M^{-1} cm^{-1} at 350 nm. Protein solutions were in 0.15 *M* sodium chloride buffered to pH 5.5 with 0.01 *M* sodium acetate.

Miscellaneous. Protein concentrations were determined with either a Cary 15 or Cary 118 recording spectrophotometer assuming an extinction coefficient E_{279nm} (1%) 6.67 for both uncleaved albumin and BPA*. The validity of this number for BPA* was confirmed by determination of the optical density of a number of aqueous solutions prepared from carefully weighed dry protein.

Intrinsic fluorescence of protein dissolved in 0.16 *M* sodium chloride at pH 5.75 was measured using the Perkin-Elmer MPF 2A fluorescence spectrophotometer. Emission spectra (260–380 nm) were obtained with a 3.0-nm excitation slit and a 4.5-nm emission slit using excitation at both 275 and 285 nm. The solutions were matched in optical density using the Cary 118 just prior to obtaining emission data.

Disc gel and sodium dodecyl sulfate gel electrophoresis were performed essentially by the method outlined by Wilson and Foster (1971). In the case of the former, gels were usually run at 2 mA/tube to avoid overheating which might distort bands. With the latter, samples were applied and gels stained and destained using the methods described by Weber and Osborn (1969).

Preparation of trifluoroacetonylated derivatives and measurement of ^{19}F nuclear magnetic resonance (NMR) spectra were accomplished as previously described (Zurawski and Foster, 1974).

Results

Figure 1 shows a typical sedimentation schlieren pattern for the purified product of the enzymatic cleavage, BPA*, together with that of unmodified bovine mercaptalbumin. By this criterion and by disc gel electrophoresis (not shown) BPA* appeared to be as homogeneous as the native protein.

Table I: Amino Acid Composition of BPA* and the Two Fragments Obtained on Reduction and Carboxymethylation, and Comparison with the Composition of the Parent Protein and Calculated Compositions Based on Sequence.[a]

Amino Acid	BPA* Large Fragment		BPA* Small Fragment		Sum of Two Fragments	BPA*	Bovine Plasma Albumin	
	Anal	Seq[b]	Anal	Seq[b]		Anal	Anal	Seq
Cys						32.0	31.8	35
CMCys	24.0	24	11.4	11	35.4			
Asp	41.7	42	12.3	11	54.0	53.6	53.0	53
Thr	15.8	15	17.8	19	33.6	32.9	33.2	34
Ser	18.7	19	9.7	9	28.4	27.9	28.5	28
Glu	56.6	55	22.1	23	78.7	79.3	78.3	78
Pro	16.5	17	11.3	11	27.8	27.9	30.6	28
Gly	12.5	11	3.8	4	16.3	15.8	16.1	15
Ala	33.0	33	12.3	13	45.3	45.6	45.7	46
Val	18.0	18	17.7	18	35.7	34.7	35.3	36
Met	1.8	2	1.9	2	3.7	4.0	4.1	4
Ile	9.2	10	3.9	4	13.1	12.8	13.8	14
Leu	41.8	42	18.0	19	59.8	59.5	59.5	61
Tyr	15.9	15	3.1	4	19.0	19.6	19.5	19
Phe	18.6	18	6.8	8	25.4	26.3	26.4	26
His	13.2	14	3.2	3	16.4	16.9	17.0	17
Lys	40.3	40	19.1	19	59.4	59.2	58.7	59
Arg	14.9	15	8.1	8	23.0	22.9	22.3	23
Trp	(2)	2	(0)	0	(2)	(2)	(2)	2
Total		392		186				578

[a] Based on 578 residues, excluding the gap of three unknown residues shown at positions 400–402 in the sequence of Brown (1975). [b] Assuming cleavage to occur at the Glx^{392}–Phe^{393} bond.

Table I summarizes amino acid analyses on BPA* and unmodified bovine plasma albumin, together with the values predicted on the basis of the primary sequence for bovine plasma albumin proposed recently (Brown, 1975). Within experimental error, the composition of BPA* is the same as that of the starting protein, consistent with the conclusion of Wilson and Foster (1971) that the cleavage occurs without loss of any peptide by-products. Similarly, the sums of the contents of the various amino acids in the large and small fragments agree well in each case with the values for BPA* and for the parent protein.

Wilson and Foster (1971) found that reduction of BPA* yields two fragments, one of approximately 46000 daltons representing the amino-terminal portion of bovine plasma albumin and the other, approximately 23000, having amino-terminal phenylalanine and representing the remainder of the albumin molecule. In accord with this, and lending additional support that our BPA* preparation was essentially homogeneous, the dansylation method indicated Phe as the only amino-terminal residue other than Asp, which is the amino-terminal residue of bovine plasma albumin. Inspection of the sequence presented by Brown (1975) shows that cleavage must occur within the large disulfide loop lying between residues Cys^{390} and Cys^{434} in order that fragments of the correct size would be obtained on reduction. The sequence of Brown (1975) shows only one Phe in this disulfide loop, namely that at position 393. Table I compares the analytical figures for both the large and small fragments with the values predicted on the basis of the Brown sequence assuming cleavage to occur at the Glx^{392}–Phe^{393} bond. The agreement of all values appears to be well within experimental error. However, results of preliminary Edman degradations of both BPA* and the small fragment are in disagreement with the conclusion that the cleavage

Table II: Values of $-\alpha]_{233}$ for Bovine Plasma Albumin and BPA* at Various pH Values.

	pH	$-\alpha]_{233} \times 10^{-3}$
Bovine plasma albumin	2.50	6.9[a]
	3.75	8.1[a]
	5.50	9.1
	9.50	8.5
BPA*	3.34	8.0
	5.20	8.7
	9.82	8.2

[a] Data from Sogami and Foster, 1968.

Table III: Values for the Moffit–Yang Parameters a_0 and b_0 for Bovine Plasma Albumin and for BPA* in 0.16 M NaCl at Various pH Values.

	pH	a_0	b_0
Bovine plasma albumin	5.65	329	256
	7.64	333	251
	8.89	312	246
BPA*	3.59	342	201
	5.25	380	210
	8.80	350	210

occurs at this bond. BPA* yielded Thr and Val on dansylation following one stage of Edman degradation. Since Thr is the second residue in the parent albumin molecule, this result suggests Val to be the penultimate residue in the small fragment. Dansylation following one stage of Edman degradation on the isolated small fragment also indicated Val to be the second residue rather than Glu as would be predicted by the Brown sequence. This discrepancy will be considered further in the Discussion.

Table II gives values of the magnitude of the first Cotton trough in the optical rotatory dispersion spectrum, $-\alpha]_{233}$, for BPA* and a few values on the parent protein for comparison. There is surprisingly little difference, the value for BPA* being about 95% as great in the isoionic pH range. Table III gives comparable values for the computed Moffitt–Yang parameters a_0 and b_0. Again the differences between BPA* and the native protein are relatively minor. Since b_0 is generally thought to be approximately proportional to helix content (Moffitt and Yang, 1956) it can be concluded that cleavage of the one disulfide loop has resulted in approximately a 15% loss of helix content. A similar conclusion can be reached on the basis of the diminished circular dichroism in the 210–225-nm region (Figure 2). The circular dichroism spectra of the cleaved and uncleaved albumin in the near-ultraviolet range (250–310 nm) are identical at isoionic pH, except for a very small decrease in the magnitude of the ellipticity of the BPA* spectrum from about 250 to 265 nm. No loss or gain of circular dichroism bands is associated with the peptide bond hydrolysis, indicating little or no change in chromophore asymmetry of residues which absorb in this region. This is in contrast to spectral alterations noted for the N–F and N–B transitions.

Inspection of Tables II and III indicates that BPA* shows a decrease in both of the parameters $-\alpha]_{233}$ and b_0 on lowering the pH. It is well known that in bovine plasma albumin corresponding changes occur in two steps, the N–F transition near pH 4 and the so-called expansion reaction at lower pH (Foster, 1960). Wilson and Foster (1971) observed the decrease in $-\alpha]_{233}$ for BPA* at low pH but concluded it resulted only from expansion and suggested that BPA* existed at neutral pH in essentially the F conformation. However, it is known that measurements of the optical rotation at a wavelength near 300 nm are much more sensitive to changes in tertiary structure and hence a better criterion of the presence or absence of the N–F transition (Leonard and Foster, 1961; Sogami and Foster, 1968). Figure 3 presents data on $-\alpha]_{300}$ vs. pH in the acid range for both BPA* and the parent albumin. There can be little doubt that the modified protein does in fact show the N–F transition but at a pH approximately 0.3–0.4 unit above that of the intact albumin. The results also suggest that expansion occurs in the same pH range in both cases so that the plateau corresponding to existence of the unexpanded F form is broader for BPA* than for the intact protein. Figure 4 shows similar results in the range of the neutral transition. BPA* shows a transition which is very similar in magnitude to that shown by albumin. Again the curve is displaced somewhat toward neutrality but in this case the cooperativity appears to be slightly greater than for native protein (Hill coefficient approximately 1.7 as compared to 1.0). Even more interestingly, the neutral transition is affected by Ca^{2+} in much the same manner as is that of albumin. The transition is shifted downward in pH and the high-pH plateau value of $-\alpha]_{300}$ is elevated. However, the increase in cooperativity due to Ca^{2+} is less than is observed with albumin itself.

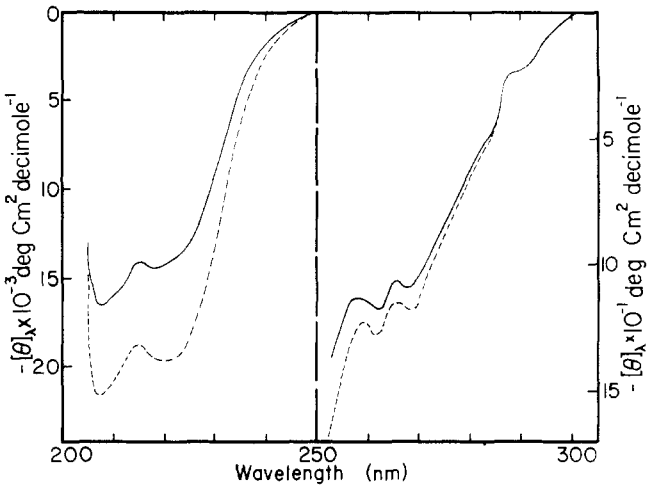

FIGURE 2: Circular dichroism spectra for BPA* (—) and bovine plasma albumin (- - -). Note 100-fold difference in scales for near-ultraviolet (right) and far-ultraviolet (left) ranges.

Fluorescence emission spectra of BPA* and bovine plasma albumin were obtained near isoionic pH with excitation at both 275 and 285 nm. Table IV summarizes the spectral parameters. Examination of these data clearly indicates an increased quenching of the fluorescence in BPA* as compared with the intact molecule, but no blue shifting of the spectrum is noticeable. This latter fact is clearly demonstrated by comparison of the F_{max}/F_{310} ratios. This result is in striking contrast to that obtained on conversion of the native protein to the A isomer where enhanced quenching and blue shifting were demonstrated (Stroupe and Foster, 1973). In that case the ratio F_{max}/F_{310} is only 1.6 (Zurawski, 1973). Lack of any blue shifting indicates no substantial change in the environment of tyrosyl chromophores to be associated with the bond cleavage. Enhanced quenching near the emission maximum probably results from an increased exposure to the aqueous solvent of one or both of

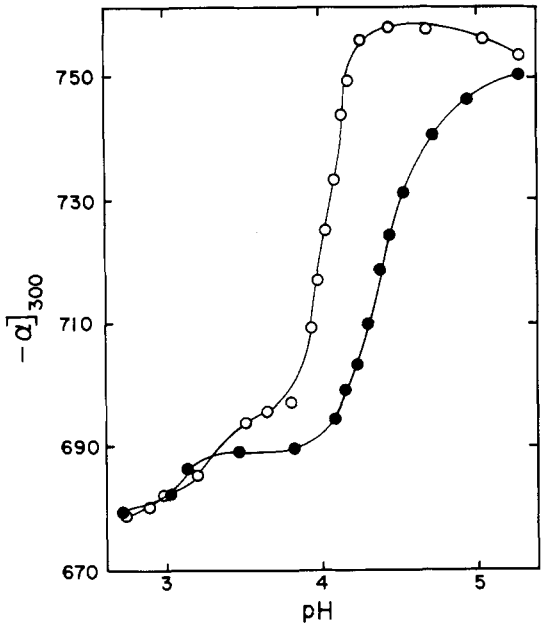

FIGURE 3: Specific rotation at 300 nm vs. pH in the acid range for BPA* (●) and bovine plasma albumin (○). In both cases experiments employed 0.13% protein in 0.16 *M* NaCl at 25°.

Table IV: Summary of Fluorescence Data on BPA* and Bovine Plasma Albumin.

Sample	Excitation Wavelength (nm)	Emission Maximum (nm)	Relative Fluorescence at Maximum Wavelength	F_{max}/F_{310}
Native	285	340	94.8	2.83
BPA*	285	339	77.5	2.55
Native	275	340	56.3	2.72
BPA*	275	340	45.6	2.42

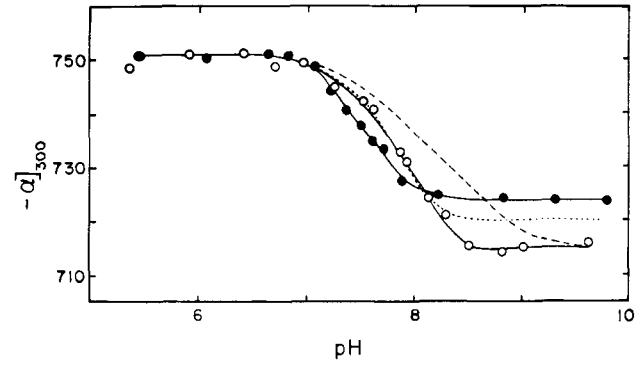

FIGURE 4: Specific rotation at 300 nm vs. pH for BPA* in the neutral range. Experiments employed 0.13% protein at 25° in 0.16 *M* NaCl (○; pH midpoint 7.89, Hill coefficient 1.66) or in 0.154 *M* NaCl plus 0.002 *M* $CaCl_2$ (●; pH midpoint 7.54, Hill coefficient 2.03). Shown for comparison are results on intact bovine plasma albumin under similar conditions without calcium (- - -; pH midpoint 8.36, Hill coefficient 1.00) and with calcium (· · ·; pH midpoint 7.68 and Hill coefficient 2.69).

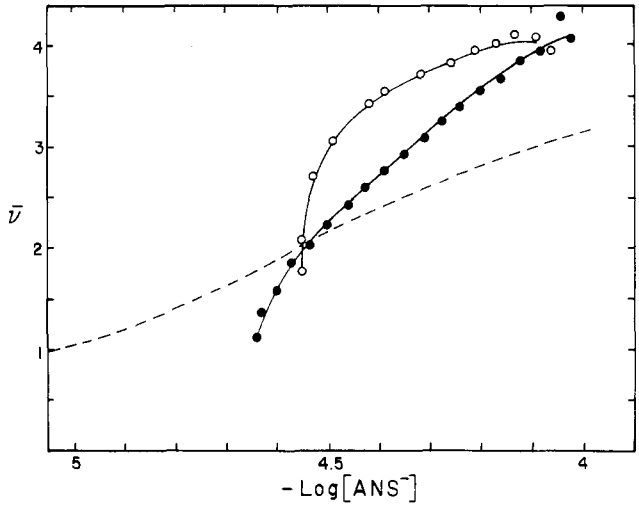

FIGURE 5: Binding isotherms for 8-anilino-1-naphthalenesulfonic acid to BPA* (●) and bovine mercaptalbumin (○) at 25° in 0.16 *M* NaCl (pH 5.2). Dashed line is theoretical for four equivalent and noninteracting sites with $K_{Asn} = 3.6 \times 10^4$.

the two tryptophan residues which dominate the spectrum (Teale, 1960; Cowgill, 1967). Alternatively it may suggest a change in the proximity of some specific quenching groups on the folded peptide chain. In any event, the differences caused by cleaving the peptide bond are smaller than those associated with the structural isomerizations of intact albumin (Steiner and Edelhoch, 1961; Chen, 1966; Halfman and Nishida, 1971), in agreement with the circular dichroism results suggesting very little change in the aromatic chromophores.

Figure 5 presents data on the binding of the anionic dye 8-anilino-2-naphthalenesulfonic acid (Daniel and Weber, 1966; Jonas and Weber, 1971) to both BPA* and the parent protein. There appear to be four binding sites in both cases; furthermore, the apparent binding affinity is virtually unaffected by the bond cleavage. The curve for native mercaptalbumin is somewhat sharper than that for BPA* suggesting a slight loss of binding cooperativity to result from cleavage of the peptide bond.

We made use of the ^{19}F nuclear magnetic resonance (NMR) technique to obtain evidence on the environment of the sulfhydryl residue. Zurawski and Foster (1974) examined the effect of the N-F and N-B transitions on the chemical shift of the signal obtained on the protein labeled with the trifluoroacetonyl group at the single sulfhydryl residue. Chemical shifts relative to a trifluoroacetic acid reference were respectively 375, 342, and 334 Hz upfield for the N, F, and B forms. In this case near the isoionic pH in 0.16 *M* NaCl we obtained 358 Hz, a value intermediate between those for N and the isomerized forms. This can be taken as an indication that cleavage of the peptide bond does not alter greatly the orientation of groups in the environment of the sulfhydryl residue.

Wilson and Foster (1971) noted that BPA* elutes slightly ahead of uncleaved albumin on Sephadex G-150, suggesting that some increase in the effective molecular volume results from cleavage of the peptide bond. A more detailed examination of the elution of BPA* and bovine mercaptalbumin on Sephadex G-150 was undertaken. Based on assumed Stokes radii of 33 Å for albumin monomer and 43.5 Å for the dimer (Laurent and Kellander, 1964) and employing the method of Ackers (1967) we calculate the Stokes radius of BPA* to be 35 Å. This corresponds to a 6% increase in radius or nearly 20% increase in effective molecular volume resulting from bond cleavage. A similar conclusion is reached on the basis of sedimentation studies. Sedimentation coefficients for BPA* and bovine mercaptalbumin were determined under identical conditions by employing

two cells (Figure 1 shows one frame from such an experiment). The resulting values corrected to water at 20° were 4.47 mercaptalbumin and 4.21 for BPA*. Since the molecular weights must be identical or nearly so, as indicated by dodecyl sulfate gel electrophoresis and amino acid compositions, this corresponds to a 6% increase in Stokes radius.

Discussion

The results presented here, together with those already published (Wilson and Foster, 1971), demonstrate beyond reasonable doubt that the initial cleavage of bovine plasma albumin in the F state by the associated proteinases occurs at an X-Phe bond within the peptide loop between Cys^{390} and Cys^{434}. The molecular weight and the amino acid composition are unaltered in so far as can be ascertained. Hence the resulting protein, BPA*, has one broken internal bond. Although the amino acid analyses on the two fragments resulting from reduction of BPA* are consistent with cleavage at the Glx^{392}-Phe^{393} bond, the position of cleavage cannot be taken as established. The main reason for this is that the sequence of Brown (1975) has a gap of at least three unknown amino acid residues at positions 400–402. In fact, by homology to the sequence of the human protein this gap might contain four residues[2] including a Phe since the sequence of the human protein in the region of this gap is Lys-Phe-Gln-Asn- (Behrens et al., 1975). There is the additional fact that our preliminary results, on both BPA* and on the small fragment, by Edman degradation followed by dansylation indicate the newly exposed sequence to be Phe-Val-, a sequence that does not occur in this region of the Brown sequence. While there remains some possibility this result is artifactual, we suggest that there may be a Phe-Val- sequence in the region of residues 400–402 or 400–403 and that this is the principal locus of cleavage. Correction of the calculated amino acid compositions for the large and small fragments in Table I to correspond to this alteration in the point of cleavage does not seriously affect the quality of the agreement with the experimental values and in fact improves agreement in several cases, for example, Phe and Tyr. It is ironic that the very region which has given special trouble in sequencing because of difficulty in obtaining suitable overlapping peptides by enzymatic cleavage[2] is exactly the locus which is most vulnerable to cleavage, by the proteinases under consideration here, when the protein is in the F conformation. We are taking advantage of this fact in an attempt to isolate peptides which will facilitate completion of the sequence in this region of the molecule as well as providing a precise location of the point of cleavage.

The optical rotatory properties clearly indicate some loss of secondary structure to accompany the bond cleavage. In the pH range 5–9 the parameter b_0, which is presumed proportional to the content of α helix (Moffitt and Yang, 1956), decreases on bond cleavage from approximately 250 to 210 (Table III) suggesting a loss of approximately 15% in the number of residues existing in this conformation. The decrease in $-\alpha]_{233}$ (Table II) and in molar ellipticity in the far-ultraviolet (Figure 2) are in qualitative accord with this conclusion, the first result suggesting a somewhat smaller, the second a larger loss in helix content.

Sogami and Foster (1968) have calculated bovine plasma albumin to contain approximately 52% α helix on the basis of various optical rotatory parameters including b_0. To a first approximation, then, we estimate the native protein to contain approximately 300 and BPA* approximately 255 amino acid residues in the helical state. Thus approximately 40–50 residues must lose their helical conformation as a consequence of the bond cleavage. A similar loss of helix content occurs in the N-F transition (Sogami and Foster, 1968). That the helical region(s) destroyed in the bond cleavage and in the N-F transition are substantially the same is indicated by the fact that the N-F transition in BPA* results in very little additional destruction of α helix as shown by the similar b_0 values at pH 5.25 and 3.59 (Table III). This suggests a plausible model to account for the hypersensitivity of the Cys^{390}-Cys^{434} loop toward enzymatic cleavage in the F conformation. Application of the helix-forming potential and rules of Chou and Fasman (1974) indicates that a substantial fraction of the residues in this loop, perhaps at least 30, might exist in the helical state. It seems entirely possible that destruction of the native conformation of this loop could occur in the N-F transition and place some of the peptide bonds in a position of exposure and even unusual strain so that their enzymatic cleavage would be favored.

Other than the slight apparent loss of helicity, the conformational properties of the protein must be altered very little by the bond cleavage. Most striking in this regard is the persistence of the two conformational transitions, the N-F transition at low pH and the N-B transition in the neutral range. The N-F transition has been pictured as primarily an alteration of tertiary structure, probably essentially a separation of folded domains (Foster, 1960) with a modest accompanying loss of helicity as described in the preceding paragraph. Similarly, the N-B transition is a change in tertiary structure with even less alteration of secondary structure (Leonard et al., 1963; Zurawski and Foster, 1974), although a very small decrease in the b_0 parameter is seen as shown by comparison of the values at pH 5.65 and 8.89 in Table III. It is of some interest that BPA* shows absolutely no evidence of any alteration in secondary structure associated with the N-B transition, the b_0 values being identical at pH 5.25 and 8.80 (Table III). Possibly for this reason the N-B transition of BPA* is a more truly two-state transition than is that of the intact protein, accounting for the enhanced cooperativity. It seems most significant that the Hill coefficient for the transition is increased from 1.0 to 1.7 as a result of the bond cleavage (Figure 4). It is noteworthy, however, that both the N-F and N-B transitions take place at pH values somewhat closer to isoionic in BPA* as compared to the native protein. It is also of significance that the effect of Ca^{2+} on the N-B transition persists, although to a somewhat less dramatic degree in the cleaved protein. The pH midpoint is shifted somewhat downward and the cooperativity is enhanced slightly (Figure 4). Evidently the binding sites for calcium ions as well as for the 8-anilino-1-naphthalenesulfonic acid anion are essentially unaltered. In a general way we believe the cleaved protein, BPA*, can be regarded as essentially identical with the native protein but having one damaged domain. The loosened structure of this domain weakens the overall interaction between domains, resulting in an increased hydrodynamic volume and reducing the breadth of the pH span over which the native tertiary conformation is stable.

The excellent agreement between these results and results obtained in the laboratory of Van Os and Harmsen in Nijmegen should be pointed out. In that group careful studies have been made of the degradation of bovine plasma albumin by pepsin in the region of existence of the F form.

[2] Dr. James R. Brown, private communication.

Large fragments of molecular weight 40000 and 47000 representing the N-terminal portion of the albumin molecule have been isolated (Braam et al., 1974). In their case, in contrast to ours, the fragments separate without reduction, indicating that cleavages occur outside disulfide loops. However, the cleavages clearly occur in the same general region as ours indicating again an unusual vulnerability of the protein to cleavage in this domain when in the F conformation state. More recent results not known to us at the time of the work reported here (Hilak et al., 1974) have shown these large N-terminal fragments to possess conformational properties very similar to the native protein and to undergo transitions near pH 4–5 which are similar in character to the N–F transition. Those authors conclude that the tertiary changes characteristic of the N–F transition involve the N-terminal portion of the protein. Of particular interest is the fact that, on the basis of their results and contrary to earlier conclusions (Wilson and Foster, 1971), they predict that BPA* would be found to yield an N–F transition if examined by the criterion of the pH dependence of $-\alpha]_{313}$. The results reported here strikingly confirm their prediction. Moreover, they have presented evidence that the loss of helix content accompanying the N–F transition occurs in the carboxy-terminal half of the protein, in complete agreement with our conclusion that this structural breakdown occurs mainly in the Cys^{390}–Cys^{434} loop.

In conclusion, we believe these results provide further evidence for a domain structure for bovine plasma albumin. The separation of domains which occurs in the N–F transition is accompanied by a disruption of the secondary structure of one of the domains, rendering it hypersensitive to enzymatic cleavage. Proteinase contaminants in many or most bovine albumin preparations cleave very selectively in one disulfide loop in this domain. The resultant damaged protein, BPA*, is still capable of reforming essentially the native tertiary structure at neutral pH. However, the cleaved domain does not reassume the native secondary conformation so that the resultant protein has a somewhat weakened stability and enhanced hydrodynamic volume.

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The Mechanism of Action of Vinblastine. Binding of [*acetyl*-^{3}H]Vinblastine to Embryonic Chick Brain Tubulin and Tubulin from Sea Urchin Sperm Tail Outer Doublet Microtubules†

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ABSTRACT: Tritium-labeled vinblastine, specific activity 107 Ci/mol, was prepared by acetylation of desacetylvinblastine with [^{3}H]acetic anhydride, and has been employed in a study of vinblastine binding to tubulin. There are two high affinity vinblastine-binding sites per mole of embryonic chick brain tubulin (K_A = 3–5 × 10^5 l./mol). Binding to these sites was rapid, and relatively independent of temperature between 37 and 0°C. Vincristine sulfate and desacetylvinblastine sulfate, two other active vinca alkaloid derivatives, competitively inhibited the binding of vinblastine. The inhibition constant for vincristine was 1.7 × 10^{-5} *M*; and for desacetylvinblastine, 2 × 10^{-5} *M*. The vinblastine binding activity of tubulin decayed upon aging, but this property was not studied in detail. Vinblastine did not depolymerize stable sea urchin sperm tail outer doublet microtubules, nor did it bind to these microtubules. However, tubulin solubilized from the B subfiber of the outer doublet microtubules possessed the two high affinity binding sites (K_A = 1–3 × 10^5 l./mol). These data suggest that vinblastine destroys microtubules in cells primarily by inhibition of microtubule polymerization, and does not directly destroy preformed microtubules.

The antimitotic drug, vinblastine, belongs to a class of chemically related dimeric alkaloids known commonly as the vinca alkaloids. Along with vinblastine, other active vinca alkaloids such as vincristine and desacetylvinblastine inhibit mitosis by destroying the microtubules of the mitotic apparatus. This results in accumulation (due to blockage) of cells at the metaphase stage (reviewed in Olmsted and Borisy, 1973; Margulis, 1973; Wilson and Bryan, 1974). In addition to its antimitotic effects, vinblastine and other active vinca alkaloids can destroy microtubules in a wide variety of cells and tissues, thereby disrupting the many biological functions which depend upon this class of subcellular organelles (Olmsted and Borisy, 1973; Margulis, 1973; Wilson and Bryan, 1974). In many cases, dissolution of microtubules within cells and tissues by vinblastine is associated with the formation of highly regular, birefringent crystals (Schochet et al., 1968; Bensch and Malawista, 1969; Nagayama and Dales, 1970; Bryan, 1971). Bryan (1971, 1972a,b) isolated vinblastine-induced crystals from unfertilized sea urchin eggs, and found them to be composed of tubulin complexed to vinblastine, in a molar ratio of 1 mol of vinblastine/mol of tubulin. The finding that vinblastine caused crystal formation in cells in vivo was shortly followed by the demonstration that high concentrations of vinblastine precipitate tubulin in vitro. It has been thought that these two actions of vinblastine are mechanistically related (Bensch et al., 1969; Marantz et al., 1969). However, the ability of high vinblastine concentrations to precipitate tubulin in vitro seems to be a nonspecific effect, since vinblastine can precipitate a large number of other acidic proteins, including muscle actin, as well as nucleic acids (e.g., double-stranded DNA) (Wilson et al., 1970). These data suggested there might be two classes of binding sites for vinblastine on tubulin: a high affinity class (biologically important) and a low affinity class (biologically unimportant). Owellen et al. (1972, 1974) have studied the high affinity binding of vinblastine to purified pig and rat brain tubulin utilizing a DE81 filter paper assay, and obtained a binding constant of 5–6 × 10^6 l./mol at 37°C, and one vinblastine binding site per 2 mol of tubulin.

In this report, we have prepared tritium-labeled vinblastine, and have studied its binding to purified embryonic chick brain tubulin, and tubulin solubilized from sea urchin sperm tail outer doublet microtubules, utilizing a number of different assay procedures. The high affinity binding of vinblastine to brain tubulin has been shown to be biologically specific, and the properties of the binding reaction have been partially characterized. We have also investigated the binding of vinblastine to intact sea urchin sperm tail outer doublet microtubules. Our results suggest that vinca alkaloids disrupt microtubules in cells primarily by preventing

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