wavelength region is not easily amenable to interpretation by this procedure since it is composed of a mixture of the amide $\pi-\pi^*$ bands and the strong tyrosyl absorption in the 192-nm region.

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Presence of Arginine Residues at the Strong, Hydrophobic Anion Binding Sites of Bovine Serum Albumin*

Ana Jonas and Gregorio Weber

ABSTRACT: Derivatives of bovine serum albumin were prepared by partial chemical modification of the native protein with acetic anhydride, formaldehyde, and glyoxal. Rotational relaxation time, sedimentation velocity coefficient, and pH dependence of fluorescence polarization measurements indicate that the overall tertiary structure of the derivatives is the same as that of bovine serum albumin in the pH range from 4.5 to 10. However, the formaldehyde- and glyoxal-treated albumins with 30 and 80% modified arginine residues, respectively, have a binding affinity for 1-anilinonapthalene-8-sulfonate almost two orders of magnitude lower than the acetylated and native albumins. The results suggest that there are arginine residues at or close to the strong hydrophobic anion binding sites of bovine serum albumin.

For a long time serum albumin has been known to have a remarkable binding capacity for all kinds of cationic, anionic, and neutral ligands. The vast literature on this subject has been reviewed several times in the last three decades (Edsall, 1947; Klotz, 1953; Foster, 1960; Putnam, 1965).

Much of the work on the binding properties of serum albumin was carried out with ionic ligands having hydrophobic side chains. The affinity of the protein for such ligands was shown to depend on the hydrophobic character of the molecule as well as on the charged group. For example, increasing binding affinity was correlated with the length of the aliphatic chains of fatty acids and detergents. Also, serum albumin was shown to bind anions more tightly than cations or neutral molecules with similar hydrophobic groups, even at pH values where the protein itself was anionic. Numerous studies indicated that many ligands, in particular anionic ligands, had at least two classes of binding sites on serum albumin differing in binding affinity by one or two orders of magnitude. The strongest binding sites were, in most cases, less than ten and overlapped for a large number of similar ligands.

Beyond these general properties of the strong anion binding sites, very little is known about the structural characteristics of serum albumin which determine its unusual binding capacity. Except for the fact that the strong anion binding sites on serum albumin include cationic and hydrophobic amino acid residues, and indications that tryptophan residues are near the binding sites (Herskovits and Laskowski, 1962; Polet and Steinhardt, 1968; Swaney and Klotz, 1970), there is no direct information about the structure of these sites.

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In a previous paper (Jonas and Weber, 1970b) we described the fragmentation of BSA[1] by α-chymotrypsin and showed that BSA has regions of positive and negative charge with respect to its net charge. Corresponding to one of the positively charged regions, we isolated, after chymotryptic digestion, a heterogeneous small peptide fraction representing nearly 20% of the total protein weight. This fraction had a high content of hydrophobic and basic residues (mainly arginines), included at least one of the tryptophan residues, and had no disulfide bridges. In addition, the small peptide fraction was shown to enhance the binding affinity of a BSA digest for ANS. We concluded that the positively charged region of BSA, which gives rise to the small peptide fraction, is associated with the strong anion binding sites.

In the present work, we studied the binding of ANS by derivatives of BSA having chemically modified arginine residues, in an attempt to show that arginine residues are involved in the strong binding of hydrophobic anions.

Materials and Methods

Crystalline BSA,[2] lot no. F71703, was obtained from Armour Pharmaceutical Co. BSA-DNS[3] was prepared by the method of Weber (1953). The magnesium salt of ANS was prepared according to Weber and Young (1964a). Analytical reagent grade 30% formaldehyde solution and acetic anhydride were purchased from Mallinckrodt Chemical Works, and the 37% aqueous solution of glyoxal was obtained from Matheson Coleman & Bell. The reagents used in the various assays and the common acids, bases, and salts were commercially available, reagent grade chemicals. Sucrose, an Allied Chemical product, was recrystallized once from ethanol. Deionized and glass-distilled water was used during the course of these experiments.

Fluorescence polarization values, fluorescence spectra, and fluorescence intensities were obtained on the instruments described in a previous paper (Jonas and Weber, 1970a). Absorbance values were measured on a Zeiss spectrophotometer. A Beckman analytical ultracentrifuge, Model E-359, was employed in determining sedimentation velocity coefficients.

Preparation of BSA Derivatives. Reaction with formaldehyde. A three to five times molar excess of formaldehyde over the total number of lysine and arginine residues in the protein was added gradually to 1% solutions of BSA and BSA-DNS in water. The reaction was carried out at 22–25°, with continuous stirring and manual adjusting of pH to 7.5–8.5 with 1 M NaOH. Dialysis against water, at 5°, was started after allowing the reaction solution to stand at room temperature for 1 hr. All the dialyzed solutions were subsequently lyophilized, and the dry material was stored at −5°.

Reaction with glyoxal. The conditions for the reaction of BSA with glyoxal were essentially the same as those used by Nakaya *et al.* (1967) for the reaction of lysozyme and the B chain of insulin with glyoxal. Approximately 1% protein solutions in water were reacted with a 29-fold excess of glyoxal per each arginine and lysine residue. The pH was maintained near pH 9, and the reaction solution was left at room temperature for 3 hr before dialysis against water.

Reaction with acetic anhydride. For the preparation of BSA and BSA-DNS with 20% acetylated lysine residues, 40 ml of 1.5% protein solutions in water was treated with 0.127 mmole of acetic anhydride. The reaction was carried out at room temperature (22–25°), maintaining the pH at 6.5–7.5. After the pH became stationary, the solution was allowed to stand for 0.5 hr at room temperature before desalting on a Sephadex G-25 column.

Assays. Free lysine residues were determined in acetylated BSA using the ninhydrin assay (Harding and MacLean, 1916), and in formaldehyde and glyoxal derivatives of BSA using the trinitrobenzenesulfonic acid procedure of Habeeb (1966).

Arginines were determined by the colorimetric method of Sakaguchi (1925), and tryptophans by the Spies and Chambers (1949) procedure.

Protein concentrations in BSA and BSA-DNS derivative solutions were obtained by the Folin method (Folin and Ciocalteau, 1927). BSA and BSA-DNS concentrations were determined spectrophotometrically using a percent extinction coefficient at 280 mμ of 6.6×10^2 g^{-1} cm^2 for BSA (mol wt 67,000) and a molar extinction coefficient of 4.3×10^3 M^{-1} cm^{-1} for the DNS label at 340 mμ (Weber, 1952).

Concentrations of ANS solutions were obtained by using a molar extinction coefficient of 6.15×10^3 M^{-1} cm^{-1} at 350 mμ (J. Stewart, 1970, personal communication).

Physical Measurements. Rotational relaxation times and pH dependences of the fluorescence polarization of formaldehyde, acetic anhydride, and glyoxal derivatives of BSA were determined following the same procedure as in a previous work (Jonas and Weber, 1970a). Changes in polarization were indicative of structural changes since the limiting polarization (polarization value in the absence of Brownian rotations) and the fluorescent lifetime of the DNS label, as judged from fluorescence spectra, were identical for BSA-DNS and for its derivatives.

Quantum yields of tyrosine ($q_{T_{Yr}}$) and tryptophan ($q_{T_{rp}}$), defined as the ratio of quanta emitted by tyrosine to quanta absorbed by the protein, and the ratio of quanta emitted by tryptophan to quanta absorbed by the protein, respectively, were determined according to Weber and Young (1964b).

Binding of ANS by BSA and by its derivatives was followed by two methods: fluorescence titration and equilibrium dialysis. The fluorescence method (Weber and Young, 1964a; Daniel and Weber, 1966) was used with native BSA and the formaldehyde and acetic anhydride treated protein. Experiments were carried out in 0.05 M sodium phosphate buffer (pH 7.0) by adding small amounts of a 2% protein solution to ANS solutions. After each protein addition, the solutions were mixed and the fluorescence intensities were measured at 470 mμ, using 345-mμ exciting light and 3.33- and 6.67-mμ excitation and emission bandwidths, respectively. Front face optics were used in the binding experiments. Since the fluorescence intensity corresponding to complete binding of ANS by the derivatives of BSA could not be determined experimentally, it was obtained from plots of $1/F$ *vs.* $1/P_0$, extrapolated to $1/P_0 = 0$ (F is fluorescence intensity, and P_0 is total protein concentration). Data were treated as in a previous paper (Jonas and Weber, 1970b).

The glyoxal derivative of BSA has excitation and emission

[1] The following abbreviations were used: BSA, bovine serum albumin; DNS, 1-dimethylaminonaphthalene-5-sulfonyl fluorescent label; BSA-DNS, BSA labeled with DNS; ANS, 1-anilinonaphthalene-8-sulfonate.

[2] Crystalline serum albumin is always associated with a small amount of fatty acid (Kendall, 1941; Goodman, 1958); Armour BSA is known to contain less than 1 mole of fatty acid/mole of protein (Chen, 1967). In this study the untreated crystalline BSA, which binds 5 moles of ANS/mole of protein with an intrinsic dissociation constant of 2×10^{-6} M^{-1} in 0.1 M sodium phosphate buffer, pH 7.0 (Daniel and Weber, 1966), is the standard material, and all the properties of the derivatives are compared to it.

[3] The physical properties of BSA and BSA-DNS are indistinguishable (Harrington *et al.*, 1956; Weber, 1952; Weber and Young, 1964a).

TABLE I: Percentage of Reacted Lysine and Arginine Residues in the Derivatives of BSA.

Derivatives of BSA	Lysines	Arginines
Formaldehyde	20	30
Glyoxal	28[a]	83
Acetic anhydride	20	0

[a] Value obtained after 2 days of dialysis against water and 1 day *vs.* acid at pH 2.5–3.0, in order to hydrolyze additional lysine-glyoxal derivatives.

spectra with wavelength maxima at 340 and 415 mμ, respectively, which overlap the spectral regions where ANS absorbs and emits light; therefore, the equilibrium dialysis technique, rather than the fluorescence titration method, was used in the binding experiments of this BSA derivative. Nearly 1% protein solutions in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.05 M NaCl were equilibrated with ANS solutions of varying concentrations prepared in the same buffer. After 20-hr dialysis (sufficient to attain equilibrium), ANS concentrations were determined in the buffer compartments of the cells.

Results

BSA Derivatives. The reaction products of BSA with acetic anhydride, formaldehyde, and glyoxal were assayed for the presence of unreacted lysines and arginines. The results, expressed in terms of percent reacted residues, with native BSA taken as 0%, are shown in Table I.

It has been reported that under reaction conditions similar to those used here to prepare the formaldehyde derivative of BSA, the only amino acids reacted in proteins are: lysines, arginines, and to some extent cysteines (Olcott and Fraenkel-Conrat, 1947). Glyoxal, according to Nakaya *et al.* (1967), gives a stable reaction product with arginine residues of proteins, but it does not change permanently the rest of the amino acids as determined by amino acid analysis. Those authors point out, however, that acid hydrolysis destroys some reaction products which are stable under less drastic conditions. We found from 40 to 20% modified lysines in our glyoxal-BSA preparations. Acetic anhydride reacts specifically with lysines, amino acids containing hydroxyl groups, and cysteines (Vallee and Riordan, 1969).

Tryptophan, determined by the Spies and Chambers (1949) method, was found intact in the derivatives of BSA. In addition, absorption and fluorescence spectra did not reveal any drastic changes in the spectral properties, on chemical modification of BSA, which could indicate destruction of this amino acid.

Structure of BSA Derivatives. Sedimentation velocity coefficients ($s_{20,w}$) and rotational relaxation times (ρ_h) were obtained for the derivatives of BSA in order to determine whether or not chemical modification of the protein resulted in major structural changes.

Sedimentation velocity coefficients were determined on 0.5% protein solutions in 0.1 M sodium phosphate buffer (pH 7.0) using Schlieren optics. Rotational relaxation times were obtained on dilute solutions of the DNS-labeled proteins, in the same buffer, at 25.0°. Plots of the inverse of the fluorescence polarization ($1/p$) *vs.* the temperature over viscosity ratio (T/η) (Figure 1) were used in calculating ρ_h values. A lifetime of 12 nsec (Steiner and McAlister, 1957) was used in all cases because the fluorescence spectra of DNS for the derivatives of BSA-DNS and for BSA-DNS itself were identical. Also, we showed in a previous paper (Jonas and Weber, 1970a) that extensive modification of BSA-DNS with citraconic anhydride, which results in marked changes in the structure of the protein, has no effect on the lifetime of the DNS label. Therefore, the assumption that the lifetime is unchanged by the chemical reactions carried out in this work seems justified. The blue fluorescence of the glyoxal-BSA derivatives did not interfere with the fluorescence polarization measurements of the DNS labels on glyoxal-BSA-DNS. Although both DNS and the glyoxal-BSA complex absorb light at 366 mμ (exciting wavelength), the emission filters pass fluorescent light corresponding to DNS alone (wavelengths longer than 490 mμ). Radiationless energy transfer from the glyoxal to the DNS chromophore does not seem to be important as the DNS emission spectrum is not altered by the presence of the glyoxal groups on BSA.

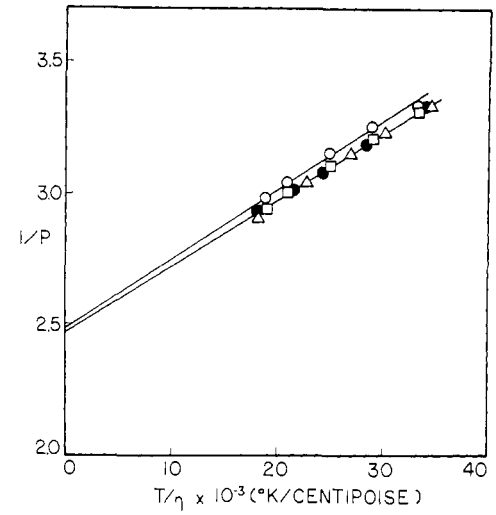

FIGURE 1: Rotational relaxation times of BSA derivatives. Plots of $1/p$ *vs.* T/η, at 25.0°, in 0.1 M sodium phosphate buffer, pH 7.0, for: (△,△) BSA, (□,□) acetylated BSA, (○,○) formaldehyde-treated BSA, and (●,●) glyoxal-treated BSA.

The results given in Table II indicate that, at pH 7.0 and ionic strength 0.1–0.2, the structure of the derivatives of BSA is very similar, in terms of $s_{20,w}$ and ρ_h, to that of BSA.

The pH dependence of the fluorescence polarization for BSA and the chemically modified proteins is shown in Figure 2. In the pH region from pH 4.5 to 10 all species have almost constant and identical polarization values, indicating a common, stable structure. At low pH, however, the decrease in

TABLE II: Sedimentation Velocity Coefficients and Relative Rotational Relaxation Times of BSA Derivatives.

Derivatives of BSA and BSA	$s_{20,w}$ (S)	$\rho_{h,25}$(Derivative)/$\rho_{h,25}$(BSA)
Formaldehyde	4.10	0.98
Glyoxal	4.23	1.02
Acetic anhydride	4.17	0.99
BSA	4.17	1.00

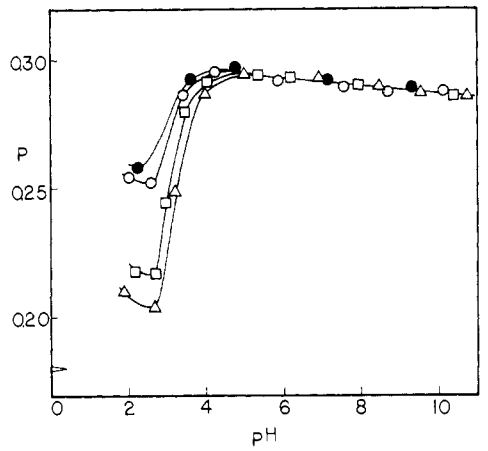

FIGURE 2: Dependence of fluorescence polarization on pH for BSA derivatives at 25°. (△,△) BSA, (□,□) acetylated BSA, (○,○) formaldehyde-treated BSA, and (●,●) glyoxal-treated BSA.

polarization reflects a different degree of expansion due, perhaps, to the different number of positive charges on each of the protein species.

Figure 3 shows the uncorrected intrinsic fluorescence spectra of native, acetylated, and formaldehyde-treated BSA in 0.05 M sodium phosphate buffer (pH 7.0). The results are summarized in Table III, which gives the wavelength of maximum fluorescence (excited at 275 mμ) and the quantum yields of tyrosine and tryptophan residues. Native and acetylated BSA have identical fluorescence spectra, while the formaldehyde treated protein has a 10-mμ shift in the wavelength maximum toward the blue, accompanied by a decrease in the quantum yield of tryptophan. The spectral changes suggest a change in the local environment of one or both tryptophan residues. The intrinsic fluorescence spectra of BSA treated with glyoxal are not included because the marked energy transfer from the tryptophan residues to the glyoxal-BSA chromophores obscures any spectral changes due to changes in the local environment of the tryptophan groups.

TABLE III: Intrinsic Fluorescence of BSA Derivatives.

Derivatives of BSA and BSA	Wavelength Max (mμ)	q_{Tyr}[a]	q_{Trp}
Formaldehyde	335	0.022	0.135
Acetic anhydride	345	0.021	0.170
BSA	345	0.020	0.176

[a] Absolute quantum yields of 0.20 and 0.21 for tyrosine and tryptophan in water, respectively (Weber and Teale, 1957), were used in the calculations.

Binding of ANS. Figure 4 gives the plots of the logarithm of the free ANS concentration against moles of bound ligand per mole of total protein (r) for BSA and its three derivatives. The binding curve for BSA was taken from the paper by Daniel and Weber (1966); data for glyoxal treated BSA were obtained by equilibrium dialysis, while the rest of the curves were calculated from fluorescence titration data.

Acetylated BSA has essentially the same binding affinity for ANS as the native protein; glyoxal and formaldehyde derivatives of BSA, however, show a marked decrease in affinity for the ligand when compared to BSA.

Discussion

Early investigations on the binding of hydrophobic anions by chemically modified serum albumin did not include studies on the effects of chemical modification on the structure of the protein (Klotz and Urquhart, 1949; Teresi, 1950). The decrease in binding affinity of acetylated and formaldehyde treated albumins for anionic ligands was attributed, in those

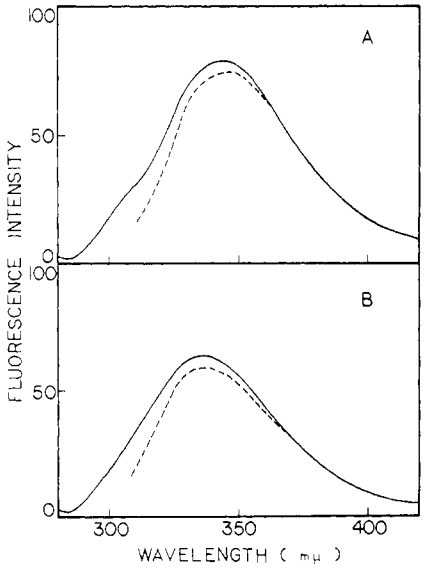

FIGURE 3: Intrinsic fluorescence spectra of BSA derivatives. (A) Spectra obtained for BSA and acetylated BSA, (B) spectra corresponding to the formaldehyde derivative of BSA. All the solutions had the same absorbance of 0.19 at 275 mμ, and were excited at 275 mμ (——) and 295 mμ (-----). Intensities are relative to the BSA spectra.

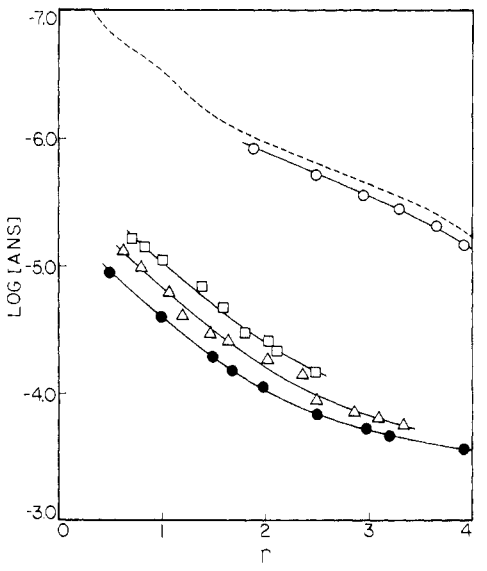

FIGURE 4: Binding curves of ANS by BSA derivatives. Plots of log [ANS] *vs.* r for: (-----) BSA, data of Daniel and Weber (1966); (○,○) acetylated BSA; (□,□) formaldehyde-treated BSA, 15% reacted arginines; (△,△) formaldehyde-treated BSA, 30% reacted arginines; and (●,●) glyoxal-treated BSA, 83% reacted arginines. Solutions in 0.05 M sodium phosphate buffer (pH 7.0) at 23°.

studies, to the destruction of nonspecific cationic sites alone. However, chemical modifications of serum albumin, especially those resulting in significant changes in the net charge, at neutral pH and low ionic strength, have been shown to produce unfolding of the protein. Reactions of BSA with dicarboxylic anhydrides (Habeeb *et al.*, 1958; Jonas and Weber, 1970a) and methylation (Sun, 1969) cause major changes in the tertiary structure of the protein. Since both hydrophobic and electrostatic interactions are responsible for the strong binding of anions with nonpolar side chains by serum albumin, at least two amino acids, one basic and one nonpolar, not necessarily adjacent in the linear amino acid sequence, are involved in the binding. As in the case of enzymes, where major alterations in the tertiary structure may drastically change the binding properties for substrates and effectors, BSA expanded by acid loses the strong ANS binding sites, while numerous sites of lower binding affinity become available for the same anion (Pasby, 1969). Similarly, peptic (Weber and Young, 1964a) and chymotryptic (Jonas and Weber, 1970b) digests of BSA are not capable of binding ANS with high affinity.

In the present work, under the conditions specified in the experimental section, acetic anhydride, formaldehyde, and glyoxal derivatives of BSA were prepared having the same gross structure as the native protein, in the pH range from 4.5 to 10, and ionic strengths higher than 0.05. Changes in the intrinsic fluorescence spectrum of formaldehyde treated BSA as compared to BSA itself and with the acetylated species suggest, however, changes in the environment of the tryptophan residues. The blue shift of the wavelength maximum may indicate a net change to a less polar environment, while the decrease in q_{Trp} may be caused by a quenching of tryptophan fluorescence by the new groups introduced in the protein. Thus, gross tertiary structure changes were eliminated as a possible factor in the binding experiments; local conformational changes in the formaldehyde treated protein, however, cannot be excluded and may influence the binding of ANS.

Modification of BSA with formaldehyde and glyoxal, which affects arginines as well as 20–30% of the lysine residues, markedly decreases the affinity of the protein for ANS, at binding ratios (r) below 4. Reaction of 20% lysines alone, in acetylated BSA, has no effect on the binding of ANS as compared to native BSA. It appears, therefore, that in the formaldehyde and glyoxal derivatives it is the modification of the arginines rather than of lysines that causes the change in the binding properties of the protein. The extrapolation of the results from the acetylated BSA to the formaldehyde and glyoxal treated proteins is valid under the assumption that the reaction of the lysines with acetic anhydride as well as with both aldehydes is random.

The binding affinity varies somewhat with the extent of reaction of the arginine residues; samples with 15, 30, and 80% reacted arginines have decreasing binding affinities for ANS. But the small differences in the effects caused by 3, 7, and 18 modified arginines suggest that either the arginines involved in the strong anion binding sites are more reactive than the rest or that reaction of a few arginines at the binding sites changes the configuration of the protein enough to affect the remaining sites.

The report by Pande and McMenamy (1970) that the stronger binding of SCN^- by BSA takes place at arginine residues, and the isolation of an arginine rich, small peptide fraction from the chymotryptic digest of BSA, which enhances the binding affinity of the total digest for ANS (Jonas and Weber, 1970b), support the evidence presented here that arginine residues are closely associated with the strong hydrophobic anion binding sites of serum albumin.

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