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Molecular Aspects of the Interaction of Bromosulfophthalein with High-Affinity Binding Sites of Bovine Serum Albumin

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SUMMARY

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Binding of the amphipathic dye bromosulfophthalein (BSP) to bovine serum albumin has been studied, especially at small molar ratios. Observations with regard to binding at the first two or three sites revealed that the absorption spectrum of BSP is shifted to longer wavelengths and that the color associated with bound BSP disappears when more of the dye is added. A circular dichroism effect is produced on attachment of the first 2 or 3 molecules but is abolished on further binding. The ellipticity is independent of pH in the range 5-8.3. Finally, binding of BSP is accompanied by absorption of protons to the binding site. These findings indicate cooperative behavior for binding at the different sites, and we have proposed a model for the attachment of BSP to albumin at a binding site that may contain a tryptophan and a histidine residue. The methods used should be suitable for the characterization of other BSP-binding proteins in the liver cell membrane.

INTRODUCTION

It is well known that bromosulfophthalein passes through the liver cell membrane rapidly in spite of its high affinity for serum albumin (1, 2). Arias *et al.* (3, 4) demonstrated that BSP[1] is also strongly bound to cytoplasmic proteins, and assumed that the distribution between plasma and liver cell is governed by the binding capacity of the proteins in the intracellular fluid and plasma.

Since the pK values of the sulfonic groups of BSP are below 2, it is unlikely that it passes through the membrane in the non-ionic form. Therefore a carrier mechanism probably is involved in the passage of BSP (5).

In order to elucidate the molecular mechanism of passage through the membrane, we chose bovine serum albumin as a model, since this protein has a very high affinity for amphipathic compounds and may have some typical binding properties in common with the hypothetical carrier protein in the membrane.

Several investigators have studied the number and affinity of BSP binding sites on albumin (6, 7) and their dependence on protein concentration (8) and on content of fatty acids and other compounds (9, 10).

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[1] The abbreviations used are: BSP, bromosulfophthalein; ANS, 1-anilino-8-naphthalenesulfonate.

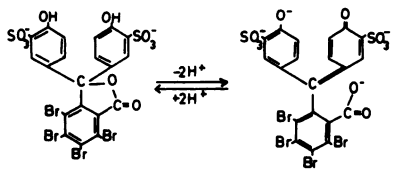
However the details of the molecular mechanism of binding have not yet been explained. Scholtan and Gloxhuber (6) investigated the interaction of BSP with albumin using optical rotatory dispersion measurements, but they did not determine ORD changes at small BSP concentrations, which are present in serum when BSP is employed for testing hepatobiliary function. Therefore we decided to reinvestigate the binding of BSP to serum albumin at smaller molar ratios.

Our studies reveal peculiar behavior at the first two or three binding sites, which is described in detail below. In addition, cooperative interaction between the different binding sites is discussed.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin was purchased from Behringwerke, Marburg, Germany (purest quality, 100% electrophoretically pure, batches 754 and 755; fatty acid content, 0.40 and 0.38 mole/mole of albumin, respectively). It was used without further purification. The albumin concentration was prepared by weight and determined according to a modified biuret method (11). Usually the albumin was brought to a final concentration of 65 μM (corresponding to one-tenth the serum concentration) by dilution in 10 mM sodium potassium phosphate buffer. [^{131}I]-Albumin was kindly supplied by Dr. Kallee, Tübingen.

BSP was a generous gift from Merck, Darmstadt, West Germany. It is a triphenylmethane dye which is colored only in the dissociated form after opening of the lactone group. The dissociation occurs in alkaline solution with a pK of about 8.5. The concentration of BSP was determined spectrophotometrically, using an absorption coefficient ϵ = 68,000 M^{-1} cm^{-1} at 578 nm and pH 12. [^{35}S]BSP was supplied by Amersham/Buchler, Braunschweig, West Germany. ANS was used as the ammonium salt.



Structure of BSP

In acidic solution BSP is colorless (left). On dissociation in alkaline solution (pK = 8.5; right) it absorbs with a maximum at 578 nm.

Optical measurements. Absorption measurements at constant wavelengths were performed in an Eppendorf photometer. Absorption spectra were determined with a Unicam photometer. ANS fluorescence was measured using the fluorescence equipment supplied with the Eppendorf photometer, with excitation at 405 and 430 nm and emission at 470–3000 nm. All absorption measurements were made in cuvettes of 1-cm pathlength and, unless indicated otherwise, at room temperature.

Circular dichroism was measured with a Jouan 185 apparatus, using cuvettes of 1-cm pathlength in the visible range and 1–0.1-cm pathlength in the ultraviolet region.

Equilibrium dialysis. Dialysis was performed in cells composed of two 1-ml Plexiglas chambers separated by a membrane of Visking cellulose. A number of cells were joined and simultaneously rotated for 16 hr at constant temperature. [^{35}S]BSP was added to either the protein solution or the other side of the membrane. In both cases the final equilibrium was the same.

We established that the absorption of both [^{131}I]albumin and [^{35}S]BSP to the Visking cellulose and the dilution of the albumin solution caused by the Donnan effect were negligible.

Ultrafiltration. Ultrafiltration centrifugation was used as an alternative method for the determination of binding affinities. Cellophane dialysis tubes were suspended as loops in centrifuge tubes and held in place with stoppers. Each dialysis tube contained 4.4 ml of a [^{35}S]BSP-albumin solution and was centrifuged three times at 600 $\times$ *g* for 20 min to yield three filtrate samples. In order to minimize errors from dilution with minute amounts of water trapped in the cellophane, the first sample was discarded; the other two samples were used to determine the concentration of unbound [^{35}S]BSP. The results did not de-

viate significantly from those obtained by dialysis. The specific activity of [^{35}S]BSP used in equilibrium and ultrafiltration measurements was 5.6 μCi/μmole.

Viscosity. Viscosity was measured with an Ostwald viscosimeter.

Measurements of proton binding. Proton uptake associated with the binding of BSP to albumin was measured with a pH electrode connected to a Knick pH meter and recorder. High sensitivity was achieved by suppression of the zero point with an external voltage source. Thus full-scale expansion of the recorder could be attained with a 5-mV signal, corresponding to a change in pH of 0.1, independent of the absolute pH.

The measurements were performed in unbuffered, carbon dioxide-free albumin solutions with stirring under an atmosphere of N_2. The number of protons bound by albumin after BSP addition was calculated from calibration spikes of known amounts of 5 mM HCl. After BSP addition the pH was readjusted with HCl to allow measurements at constant pH over a wide range of BSP to albumin ratios.

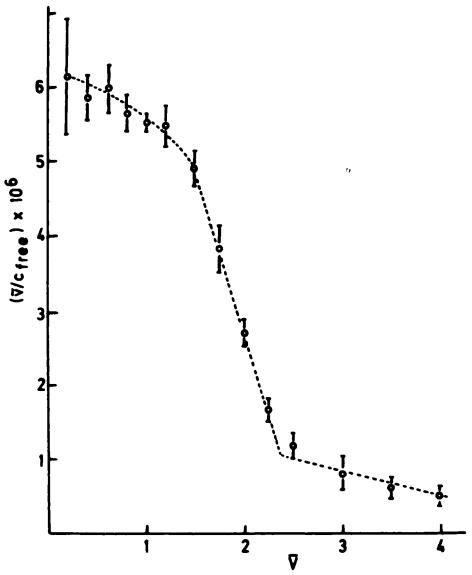


FIG. 1. *Scatchard plot (12) for binding of BSP to albumin*

$\bar{\nu}$ = molar ratio of bound BSP to albumin; c_{free} = free molar concentration of BSP. Data were obtained by ultrafiltration at 22° at an albumin concentration of 70 μM, in 10 mM phosphate buffer, pH 7.0. Points represent the mean values of three independent measurements.

RESULTS

Characteristics of binding of BSP to albumin. The Scatchard plot (12) in Fig. 1 represents data for the binding of BSP to albumin at pH 7 in 10 mM phosphate buffer. The points are the mean values of three independent measurements obtained by ultrafiltration. They are in full agreement with results obtained by equilibrium dialysis (not shown). The same curve was obtained with BSP in 150 mM NaCl plus 10 mM phosphate buffer.

At a BSP to albumin molar ratio between 1 and 2 the slope becomes steeper, suggestive of cooperative binding (13, 14). The binding curve is derived from characteristics of sites with different affinities. Therefore the number of binding sites and their affinities can be estimated only approximately from the slope and extrapolation to the intercept on the x axis. The slope is steepest up to a molar ratio of about 2.5, corresponding to an association constant of about $K_1 = 3.8 \times 10^6\ M^{-1}$ for two or three binding sites. At higher BSP to albumin ratios the slope is much smaller, and the resulting association constant is about $K_2 = 0.28 \times 10^6\ M^{-1}$.

Spectrophotometric measurements. At low BSP to albumin molar ratios the absorption of BSP at 578 shows anomalous behavior (Fig. 2). The effect is most pronounced at low ionic strength. In 10 mM phosphate buffer the absorption rises with increasing concentrations of BSP up to a BSP to albumin ratio of almost 2, then decreases, and finally approaches the same increase seen with BSP alone (curve *C*).

From the binding characteristics shown in Fig. 1 we can calculate that at molar ratios below 2, more than 99% of the BSP is bound to albumin. This indicates that the contribution of unbound BSP to the absorption in curves *A* and *B* (Fig. 2) is negligible. At higher ionic strength (curve *B*) the abnormal peak in the curve disappears. The absorption at the first two sites is much smaller. At higher BSP to albumin

ratios both curves become identical. Extrapolating curves *A* and *B* from high values of $\bar{\nu}$ to zero absorption shows that under each condition 2 molecules of BSP bound to albumin have lost their color.

Figure 3 shows the absorption of increasing amounts of BSP bound to albumin at different pH values. A comparison of the initial and final slopes of each curve reveals that the absorption at the first two binding sites is less pH-dependent than at the higher binding sites. Below pH 7.9 the absorption at the first two binding sites is greater than at further binding sites, whereas above pH 7.9 it is smaller. This suggests that the troughs of the curves at low ratios of BSP bound to albumin are due to diminished absorption at the first two or three binding sites, whereas further bound BSP itself causes an absorption increase. The higher the pH, the stronger is the absorption as the binding ratio increases. The maxima of the curves at smaller BSP to albumin ratios change to shoulders. Again, extrapolation to zero absorption shows that 2 or 3 Eq of BSP have lost their color.

Figure 4 shows the absorption curves at two different temperatures. The first 2 or 3 bound molecules of BSP exhibit an absorption which is independent of temperature, whereas the absorption of BSP at additional binding sites shows a temperature dependence similar to that of BSP alone.

The absorption spectrum of BSP changes upon addition of increasing amounts of albumin at pH 8.0 (Fig. 5). The maximum is decreased and undergoes a red shift from about 580 nm to 592 nm. The BSP spectrum is changed most drastically when it is bound to the two or three high-affinity binding sites but seems to be identical at all these sites. At higher binding ratios BSP maintains the same spec-

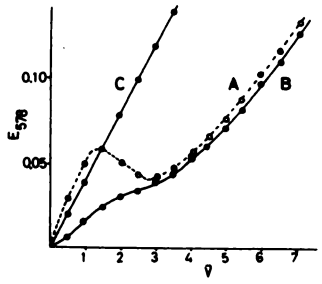


FIG. 2. *Absorption of BSP and of BSP-albumin complex*

Albumin (70 μM) was titrated with BSP at 22°, and the absorption at 578 nm was plotted against the molar ratio of BSP to albumin. Since more than 99% of the BSP is bound at the ratios indicated, the symbol $\bar{\nu}$, defined in Fig. 1, is also used for the molar ratio of added BSP to albumin. The absorption data were corrected for dilution by additions of the titrating agent. Curve *A*, 10 mM phosphate buffer, pH 7.5; *B*, 10 mM phosphate buffer, pH 7.5, plus 150 mM NaCl; *C*, BSP without albumin in 10 mM phosphate buffer, pH 7.5.

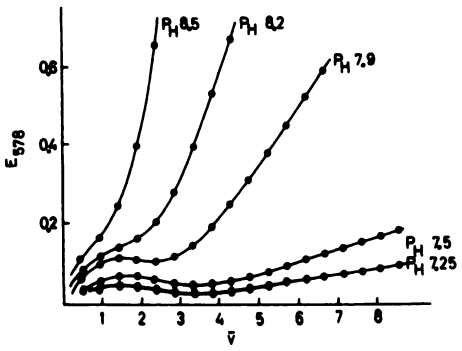


FIG. 3. *Absorption of BSP-albumin complex at different pH values*

Albumin (70 μM) in 10 mM phosphate buffer at various pH values was titrated with BSP at 22°. Details of the procedure are given in Fig. 2; $\bar{\nu}$ is the molar ratio of bound BSP to albumin. The trough of the absorption curves is most pronounced at low pH values.

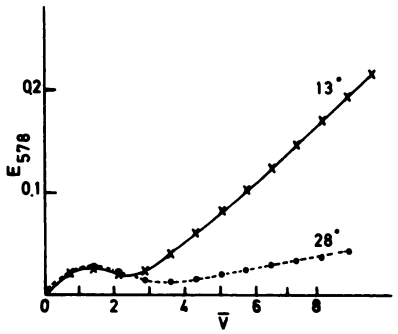


FIG. 4. *Absorption of BSP-albumin complex at different temperatures*

Albumin (70 μM) in 10 mM phosphate buffer, pH 7.25, was titrated with BSP at 13° and 28°. Details of the procedure are given in Fig. 2; $\bar{\nu}$ is the molar ratio of bound BSP to albumin.

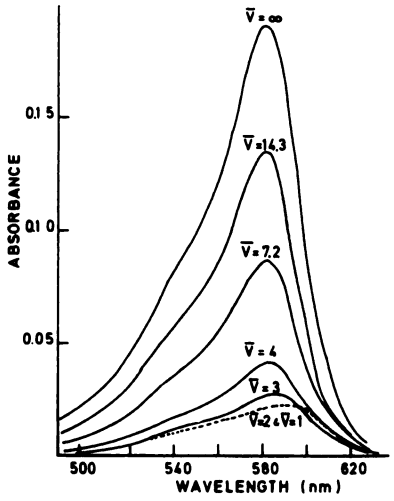


FIG. 5. *Absorption spectra of BSP-albumin complexes at different molar ratios*

Increasing amounts of albumin were added to a constant amount of BSP (50 μM) in 10 mM phosphate buffer, pH 8. Details of the procedure are given in Fig. 2; $\bar{V}$ is the molar ratio of bound BSP to albumin. Upon binding, the extinction coefficient of BSP decreases and its absorption maximum exhibits a red shift.

trum as in the absence of albumin. The gradual shift of the absorption maximum in Fig. 5 seems to result from the overlapping of these two absorption characteristics. The A_{580}:A_{592} extinction ratios plotted against increasing BSP to albumin concentration ratios ($\bar{V}$) (not shown) remain constant up to $\bar{V}$ = 2, but then increase and asymptotically approach the value found for free BSP.

Induced optical activity of BSP molecule. In aqueous solutions BSP is optically inactive. On binding to albumin, however, optical activity appears and can be measured in both the ultraviolet and the visible range. We measured the CD spectra of the BSP-albumin complex between 535 and 615 nm (not shown). With increasing BSP to albumin ratios the ellipticity rises to a maximum at $\bar{V}$ = 2. Further addition of BSP causes a decrease in ellipticity. In Fig. 6 the relative ellipticity at 580 nm and 295 nm is plotted against the molar ratio of BSP to albumin. In the visible range the ellipticity is positive, and in the ultraviolet range it is negative. The absolute ellipticity at $\bar{V}$ = 2 and 580 nm amounts to $\theta = 7 \times 10^3$ deg·cm^2 dmole^{-1} at 0.36 mM albumin.

Since BSP has a large absorption in the ultraviolet range, optical density becomes too high for accurate measurements above BSP to albumin molar ratios of 3 under the conditions indicated in Fig. 6. To obtain the curve at 295 nm, the contribution to the optical activity of the albumin molecule was subtracted. At 295 nm the ellipticity reaches a maximum near $\bar{V}$ = 3, followed by a slow decrease. In contrast, in the visible range ellipticity is maximal at $\bar{V}$ = 2 and then decreases faster and becomes very small at a BSP to albumin ratio of 8.

A comparison of the ellipticity curve in Fig. 6 with that of Fig. 2 shows that the decrease in ellipticity is paralleled by the diminution of absorption. The induced ellipticity was independent of pH variations in the range 5–8.3.

Measurements of ellipticity at the peptide absorption band indicate that the α-helix content of the albumin did not vary with complex formation up to a BSP to albumin ratio of 8.

Fluorescence measurements. The fluorescent dye ANS has been widely used as a probe to investigate conformational changes of macromolecules (15–17). Changes in quantum yield are assumed to be caused by alterations in viscosity and polarity in the microenvironment of its binding site (18). When bound to albumin, ANS shows a 200-fold increase in quantum yield (16). This high fluorescence is influenced by the binding of some ligands to albumin (19–21).

In the experiment of Fig. 7 ANS was added in equimolar concentration to a 1.7 μM albumin solution at pH 7.0. On addition of BSP the ANS fluorescence was gradually quenched (Fig. 7). This effect was sigmoidal, with a maximal slope at a BSP to albumin ratio between 2 and 3. The quenching of ANS fluorescence was not due to displacement of ANS from albumin, as shown by ultrafiltration measurements. The addition of BSP did not cause any significantly release of ANS from albumin unless the molar ratio of BSP to albumin exceeded 8.[2]

Proton absorption accompanying BSP binding to albumin. When BSP is added to

[2] Unpublished observations.

an albumin solution, protons are absorbed from the medium. The degree of H^+ binding depends on the pH, as shown in Fig. 8. At pH 4 about 1.5 protons are bound per BSP molecule added. This ratio holds over the entire range of BSP to albumin ratios up to a value of 17, where denaturation of the albumin molecule occurs (not shown).

With increasing pH values the ratio of protons bound per BSP molecule added decreases, as demonstrated by the slopes of the curves in Fig. 8. In addition, with increasing pH a tendency toward saturation of protonation can be observed. At pH 7 saturation is reached at a molar ratio of BSP to albumin of about 4 (measured

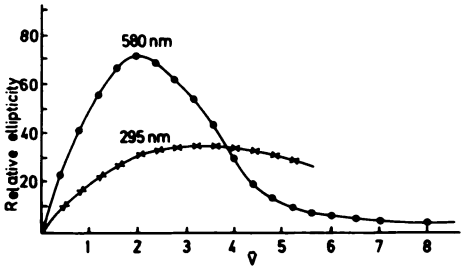


FIG. 6. *Ellipticity of BSP-albumin complex at different molar ratios*

The relative increases in ellipticity at 580 nm (positive) and 295 nm (negative) were plotted as functions of the molar ratio $\bar{\nu}$ of BSP to albumin. Measurements were made in 10 mM phosphate buffer, pH 8.3, at 22°. Because of the low molar ellipticity (θ/mM albumin = 7 at $\bar{\nu}$ = 2; 580 nm) the albumin concentration used in this case was 361 μM.

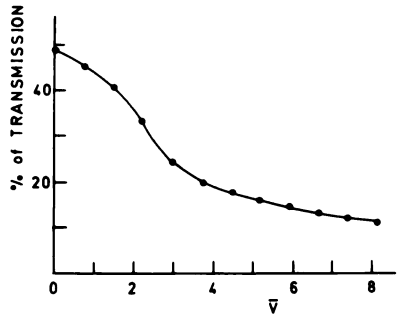


FIG. 7. *Effect of BSP on fluorescence of ANS bound to albumin*

Albumin (1.7 μM) and ANS (1.7 μM) in 10 mM phosphate buffer, pH 7.0, were titrated with BSP. Excitation (mercury filter) was at 405 and 430 nm; emission was measured between 470 and 3000 nm. $\bar{\nu}$ is the molar ratio of BSP to albumin.

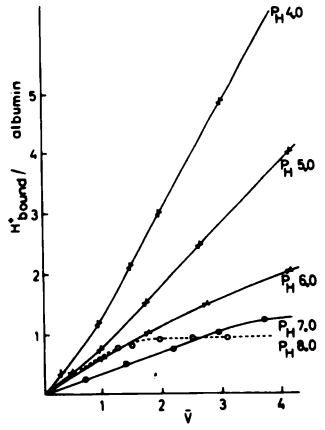


FIG. 8. *Absorption of protons accompanying binding of BSP to albumin*

Albumin (70 μM), free of buffer, was titrated with BSP at constant pH under an atmosphere of N_2. After each addition the pH was readjusted with HCl. $\bar{\nu}$ is the molar ratio of BSP to albumin. See MATERIALS AND METHODS for details on the measurement of pH.

points at $\bar{\nu} > 4$ are not shown). At pH 8 saturation is reached at $\bar{\nu} = 2$.

Proton binding is diminished with increasing ionic strength, however, and can no longer be detected at physiological salt concentration. To determine whether the protons were bound to the albumin or to the anionic groups of BSP, analogous measurements were performed with fluorescein. This dye is structurally similar to BSP, but has no sulfonic acid groups. Its extent of proton binding resembles that of BSP. Thus it can be concluded that protonation does not depend on sulfonic acid groups. Below pH 7 both dyes are more than 95% in their lactone form, which requires protonation. At least in acidic solution, therefore, the protons must be associated with an amino acid of the albumin.

Viscosity measurements. Up to a molar ratio of BSP to albumin of 6 there was no change in viscosity of the albumin solution.

DISCUSSION

The approaches which have been used to study the molecular nature of the binding of amphipathic compounds to albumin include competition experiments (22–27), studies on the conformational changes of the albumin molecule (28–30), and partial

digestion of the albumin molecule in order to isolate a fragment containing the specific binding sites (31). In general, two or three binding sites with high affinity, for which organic anions compete, seem to be available.

Figure 1 presents evidence that two or three binding sites with high affinity exist. This result agrees with studies of other authors, who assumed three high affinity binding sites (2, 4). The same number of sites with these particular properties was observed by means of CD and absorption measurements. The ellipticity at 580 nm shows an enhancement up to a molar ratio of BSP to albumin of 2, whereas at 295 nm it increases until a ratio of 3 is achieved (Fig. 6). This discrepancy may be due to changes of the intrinsic ellipticity of the protein at 295 nm.

The presence of two or three binding sites with high affinity is in agreement with the findings that the extinction of about 2 molecules is independent of temperature (Fig. 4), and that their color is lost if increasing amounts of BSP are added to the albumin (Fig. 2). We found that the exact number of high-affinity binding sites depends on the batch of albumin, which differs slightly with respect to the content of fatty acids and other ligands.

A cooperative interaction between anionic compounds and albumin has been discussed (6, 16). The Scatchard plot (Fig. 1) shows an increase of the negative slope at a molar ratio of BSP to albumin between 1 and 2. Such a convex curvature has been interpreted to indicate cooperative binding (13, 14). Baker and Bradley (7) presented a Scatchard plot with the usual linear course at small $\bar{V}$ values. Their plotted points, however, also permit one to draw a curve which would indicate a discontinuity at $\bar{V}$ values between 1 and 2, consistent with our findings. Because of the great standard deviation at low BSP concentrations, the described affinity measurements alone may not present sufficient evidence for cooperative binding behavior.

There is other evidence, however, for a cooperative interaction between the primary binding sites and the third and subsequent BSP molecules bound to albumin. The strongest evidence for cooperativity is provided by CD measurements. The ellipticity induced in BSP molecules on binding to albumin increases up to a molar ratio of BSP to albumin of 2 but decreases if additional binding sites are occupied. This behavior parallels the light absorption at 580 nm. Since ellipticity can only be observed in the absorbance region of an optically active compound, we assume that the diminished ellipticity is caused by a decreased absorption of 2 sterically fixed BSP molecules. This interpretation is further confirmed by the observation that at 295 nm the ellipticity decreases much more slowly, in accordance with the fact that the absorption at 295 nm, in contrast to that at 580 nm, is nearly the same in the lactone form as in the carboxylic form of BSP.

A cooperative effect is also indicated by fluorescence measurements. The well-known increase of ANS fluorescence, after addition to albumin in equimolar amounts, is quenched by BSP in a sigmoidal fashion, with a turning point at a BSP to albumin ratio near 2.5 (Fig. 7).

If BSP is dissolved in 60% ethanol, its absorbance maximum is shifted from 578 nm to 587 nm. An even stronger red shift can be observed when BSP is bound to the high-affinity binding sites of albumin (Fig. 5). Furthermore, the absorption of BSP bound to these binding sites is less pH-dependent than that of free BSP or BSP bound to sites with lower affinities (Fig. 3). In addition, the ellipticity at 295 nm is pH-independent between pH 5 and pH 8.3. The red shift and pH independence suggest that BSP may be bound to apolar regions of the protein if it interacts with the high-affinity binding sites. However, the BSP molecules seem to be incompletely immersed in a hydrophobic area, since the dissociated form is still visible in alkaline solution (Fig. 3), and binding of further equivalents of BSP leads to even greater disappearance of absorption.

It should be emphasized, however, that we cannot definitely distinguish between an effect on the dissociation of the lactone

group and other influences which may alter the apparent absorption coefficient. Our observations concerning spectra shifts show interesting parallels with those described by Lang and Lasser (32) for the binding of trypan blue to bovine serum albumin. They found that on addition of a third equivalent of trypan blue the dye molecules bound to the two high-affinity sites underwent a spectral shift, and a set of three new and equal binding sites was formed.

Any loss of color due to a diminished dissociation of the BSP molecule should be accompanied by binding of protons from the solution. Figure 8 shows proton binding, which, above pH 7, can indeed be ascribed to BSP protonation. Below pH 7, however, where according to its pK, BSP is almost completely in its lactone form, protonation seems to occur with the albumin, as we confirmed by comparative studies with fluorescein binding to albumin. The pH dependence of the initial proton attachment has a turning point around pH 6.5, pointing to histidine as the amino acid involved, since this is the pK of the imidazole nitrogen.

Our findings suggest that the first 2 or 3 BSP molecules are attached near an apolar region. Their binding induces a conformational alteration. A histidine residue located close to the binding site is thereby exposed and can be protonated. This protonation may compensate for the charge of a dissociated sulfonic acid group. Thus the BSP molecule is fixed sterically, and the optical activity would be induced.

Another amino acid should also be involved in the binding of BSP. BSP strongly quenches the tryptophan fluorescence of albumin. Addition of 2 Eq of BSP reduces the tryptophan fluorescence by 85%.[2] Bovine serum albumin contains 2 tryptophanyl residues, which seem to be located in different environments (33). Nevertheless they might contribute to two similar binding sites for BSP.

The model in Fig. 9 could explain the different cooperative behavior of ANS and BSP. On binding of further BSP molecules a conformational change may occur at the

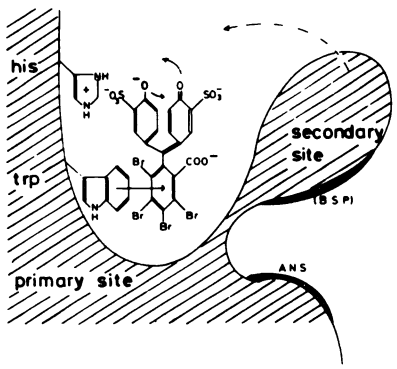


FIG. 9. *Schematic model of high-affinity binding site of albumin for BSP*

albumin molecule, moving the high-affinity site for BSP deeper into apolar regions of the protein. This results in the loss of color, because in an apolar environment the undissociated form of BSP is favored. The ANS binding site, on the other hand, is moved to more polar regions, and its fluorescence decreases. The conformational change of the albumin molecule cannot be large, however, since the viscosity and the α-helix content of the macromolecule are unchanged.

Alternatively, there could be a cluster of binding sites, which might explain our observations. The third and additional BSP molecules are bound near the first two binding sites. This accumulation of anionic groups in the microenvironment of the primary binding site diminishes the dissociation of the carboxylic and phenolic groups of the BSP molecules, as indicated by a loss of color. Furthermore, the ANS binding site should be located in the vicinity of the BSP binding region, and an energy transfer from ANS to BSP would result in quenching of the ANS fluorescence.

Our results suggest that albumin may have three characteristic features in common with a carrier protein for BSP in the liver cell membrane: BSP is bound with high affinity to the protein; the binding site is subjected to conformational changes; on binding, the charge of the ligand is partly compensated.

Further investigations should reveal

whether the attachment of amphipathic compounds to other proteins, such as carrier molecules, follows similar rules. The unsolved problem of permeation of amphipathic compounds through cell membranes might be explained if our assumption that albumin and carrier proteins have comparable properties proves correct. The results may also provide a model for reactions of receptor sites in the cell with drug molecules having properties similar to BSP.

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