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Binding Aspects of 2-(4-Hydroxyphenylazo)benzoic Acid to Bovine Serum Albumin in the Limited Mixing Ratio

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The binding of 2-(4-hydroxyphenylazo)benzoic acid (HABA) to bovine serum albumin (BSA) was studied in a dilute phosphate buffer of pH 7.0 and ionic strength 0.014 at 25 °C. Binding measurements were made for both undefatted BSA and defatted BSA (the protein naturally contains 2.4—2.5 mol/mol fatty acid). A metachromasy band appeared at 480 nm at the expense of the original absorption band at 350 nm in the presence of BSA. An isosbestic point in the absorption spectra was observed in the vicinity of 380 nm only in the limited range of the molar mixing ratios of HABA to BSA (D_0/P_0), where the ratio was 1—10 in the above buffer, regardless of the absence and presence of fatty acids. However, the isosbestic point was not observed by any feasible change of the D_0/P_0 ratio in a buffer containing 0.1 M NaCl. This fact seemed to indicate that the binding nature depended on the ionic strength as well as the D_0/P_0 ratio. Then, binding measurements were carried out in the limited range of D_0/P_0 ratios in a dilute phosphate buffer. The amount of HABA bound to BSA was determined by curve-fitting for changes of the apparent molar extinction coefficient, ε, between 300 and 550 nm, assuming one class of binding site: that is, only two species (free and bound dyes) contributing to the absorption changes. The simulated curves of ε were in good agreement with those obtained experimentally. By the use of the Scatchard plot, the binding parameters were determined. The binding constant was larger for defatted BSA than for undefatted BSA, the values being of the order of 10^5 M^{-1}. The number of binding sites of HABA was 1.4—1.5, not being affected very much by the absence and presence of fatty acids.

The azo dye, 2-(4-hydroxyphenylazo)benzoic acid (HABA) is well-known to exhibit a new absorption band characteristically, when it binds to serum albumin, which generally serves as a carrier protein for a wide variety of substances.[1] Consequently, the mode of interaction between HABA and serum albumin has been attracting renewed interest. During the last two decades, the system of HABA and bovine serum albumin (BSA) has often been studied.[2-7] The exhibition of a new band has been attributed to the azo-hydrazone tautomeric transition of the dye induced upon its binding to the protein.[2-9] However, most of these studies have been carried out by changing the HABA concentration over wide ranges, from 10^{-6} or 10^{-5} to 10^{-3} M (1 M=1 mol dm^{-3}), at a constant BSA concentration in rather concentrated buffers. In the present work, the interaction depended on both the ionic strength of the buffer and the molar mixing ratio of HABA to BSA, D_0/P_0. An isosbestic point in the absorption spectra of HABA was observed only over a limited range of the D_0/P_0 ratios and only in the buffer of low ionic strength. Some important facts seem to have been overlooked, probably because most of the preceding studies regarding the interaction were made under the above-stated experimental conditions. The present results suggest that a certain chemical equilibrium in the binding system exists only within a particular range of D_0/P_0 ratios.

Experimental

The crystalline BSA was purchased from Miles Laboratories Inc.[10] The preceding studies concerning this system emphasized the importance of the purity of the protein.[6] Therefore, the BSA sample was purified by passing it through Pharmacia MONOQ HR5/5 (ionic-exchange media) and Superose 12 (gel filtration media) using a Pharmacia high-performance liquid chromatography FPLC. HABA was purchased from Tokyo Kasei Co. and was purified by recrystallization from methanol before use. The protein concentration was determined spectrophotometrically using $E^{1\%}_{1\,cm}$=6.8 at 280 nm.[11] The molar extinction coefficient of HABA was assumed to be 18800 at 350 nm. A sodium phosphate buffer of pH 7.0 was extensively used. The final concentrations of NaH_2PO_4 and Na_2HPO_4 in the buffer were 3.33 and 3.56 mM, respectively; the ionic strength of the buffer was 0.014.[10]

The content of fatty acids in the present BSA was determined to be 2.4—2.5 mol/mol by the Dole method.[12] The removal of fatty acids was carried out by an acid-charcoal treatment.[13] The BSA fractionated as described above was mixed with charcoal, the amount of which was approximately half of that of the protein in weight. The pH of the BSA-charcoal solution was lowered to 3 by the addition of 0.2 M HCl. After the solution was stirred at 0—2 °C for 4 days, the charcoal was then removed by centrifugation. The period of 4 days was determined on the basis of a relation obtained between the stirring time and the quantity of fatty acids remaining in the BSA treated by charcoal. The clarified solution was brought to pH 7.0 by the addition of 0.2 M NaOH, and then dialyzed against the phosphate buffer of pH 7.0.

Absorbance measurements were made at 25 °C with a Hitachi double-beam spectrophotometer U-3200.

Results and Discussion

The binding properties of HABA to serum albumin have been studied so far by equilibrium dialysis,[2,5,9] rotating-disk polarography,[14] ultrafiltration,[14] spectrophotometric analysis,[3,5,6] and gel chromatography[7]

at rather high ionic strengths using mostly 0.05—0.1 M phosphate buffers.[2-9] The HABA concentration was in the range 10^{-6} or 10^{-5} to 10^{-3} M and the BSA concentration in the range 10^{-5} to 10^{-4} M in these preceding studies. When HABA binds to serum albumin, it exhibits a metachromasy band at 480 nm at the expense of the original absorption band at 350 nm, as has been observed by Baxter[2] and Moriguchi et al.[3,4] They concluded that the absorption band at 480 nm is due to the formation of a hydrazone form of HABA;[2-4] Terada et al. have supported this conclusion.[6] There are two kinds of binding sites for HABA in the protein. Sakurai et al. have shown that the absorption bands at 350 and 480 nm originate in HABA bound to a non-metachromasy site and a metachromasy site of BSA, respectively.[5] They have concluded that there are, at least, 5 binding sites consisting of 1.5—2 metachromasy sites and 3.5—3 non-metachromasy sites (Fig. 1 in Ref. 5).

Figure 1 shows the absorbances of 1×10^{-5} M HABA at various BSA concentrations in the present dilute buffer. The absorbance at the metachromatic band clearly increased with an increase in the BSA concentration up to a protein concentration of 1×10^{-5} M,

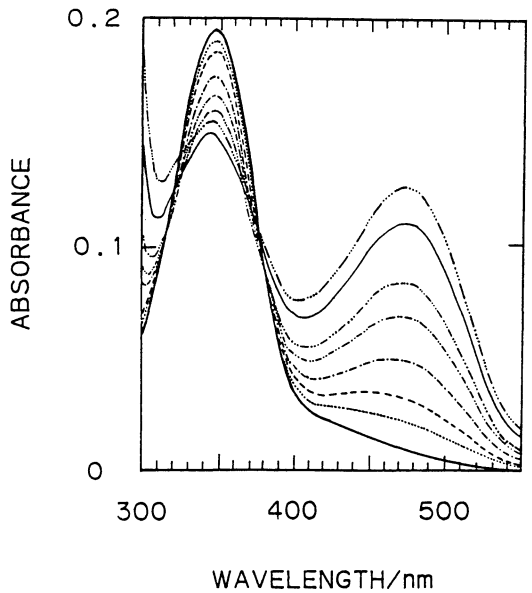

Fig. 1. Absorbance of 1.0×10^{-5} M HABA in the presence of defatted BSA of 0 (——), 1.0×10^{-6} (·····), 2.0×10^{-6} (----), 4.0×10^{-6} (-·-·-), 7.0×10^{-6} (-···-···-), 1.0×10^{-5} (-·····-·····-), 2.0×10^{-5} (——), and 3.0×10^{-5} M (-·····-·····-) at 25 °C.

showing an isosbestic point in the vicinity of 380 nm. However, when the BSA concentration was beyond 1.0×10^{-5} M against 1.0×10^{-5} M HABA, the spectrum did not pass through the isosbestic point. The absorption spectrum of HABA in 2×10^{-5} M BSA crossed the spectrum of HABA, itself, at a different wavelength from that of the isosbestic point obtained below 1×10^{-5} M BSA. The spectrum of HABA in 3×10^{-5} M BSA crossed the spectrum of HABA, itself, at another different wavelength. These results suggest that the HABA-BSA interaction, itself, differs with each other above and below this particular BSA concentration (1×10^{-5} M) at the constant HABA concentration: that is, the binding mechanism depends on the D_0/P_0 ratio.

Although absorbance measurements were also performed in a phosphate buffer of ionic strength 0.1 and in the present dilute buffer containing 0.1 M NaCl, the isosbestic point was not observed at any feasible concentration range of BSA at a constant concentration of HABA. In the present dilute buffer containing 0.1 M NaCl, the decreasing magnitude of the absorbance around 350 nm upon the addition of BSA was rather small (not shown) compared with that in a buffer without NaCl (Fig. 1). Figure 2 shows the effect of the added NaCl on the apparent molar extinction coefficients at 350 and 480 nm, ε_{350} and ε_{480}, in the presence of 1×10^{-5} M BSA. The value of ε_{350} increased and that of ε_{480} decreased with increasing NaCl concentration, indicating qualitatively that the increment of ionic strength inhibits the HABA binding to the protein.

The amount of HABA bound to BSA was determined spectrophotometrically by applying a curve-fitting method to the apparent molar extinction coefficient changes in the wavelength region 300—550 nm. This is the same method that has often been used to estimate the fractions of secondary structures, such as a helix in a polypeptide, using a reference circular dichroism spectra of the corresponding structures.[15-17] Assuming that only two species, the free HABA and the HABA bound to the protein, contribute to the absorption, the following relation is obtained:

$$\varepsilon_{obs}=f_{free}\,\varepsilon_{free}+f_{bound}\,\varepsilon_{bound},$$

where ε_{obs} is the experimentally observed apparent molar extinction coefficient of HABA in the presence of BSA; ε_{free} and ε_{bound} are the molar extinction coeffi-

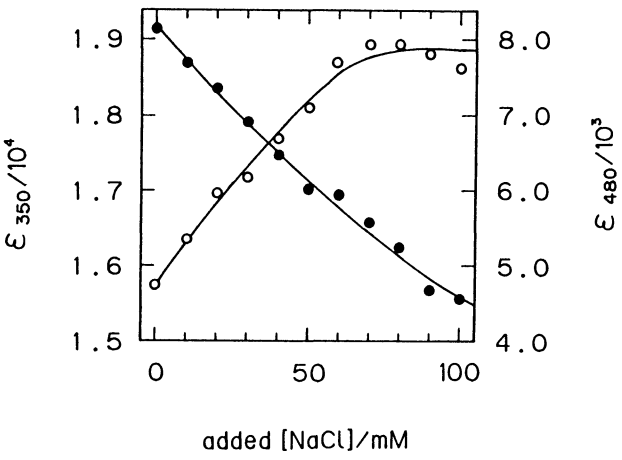

Fig. 2. Changes of extinction coefficients of HABA, ε_{350} (○) and ε_{480} (●) as a function of NaCl concentration added to the buffer of ionic strength 0.014 at 25 °C. BSA concentration was kept at 1.0×10^{-5} M.

cients of the free and bound species, respectively. The notations of f_{free} and f_{bound} are fractions of the free and bound species, respectively, and $\sum f_i=1$. Since the solution of HABA obeyed Beer's law in the concentration range used here (in the order of 10^{-5} M), the value of ε_{free} was easily determined at each wavelength. However, it was actually impossible to obtain the value of ε_{bound} only by the spectrophotometrical method, since the absorbance of 1×10^{-5} M HABA continued to increase with increasing BSA concentration up to 1×10^{-3} M. Then, the values of ε_{bound} were determined by the combined use of the equilibrium dialysis at the particular ratios of D_0/P_0 which enable the absorption spectra of HABA to pass through the isosbestic point in Fig. 1. The binding quantity estimated by the equilibrium dialysis method corresponds to the total amount of HABA bound to both the metachromasy and non-metachromasy sites. However, the dye is considered to bind predominantly to the metachromasy site under the present experimental conditions, because the binding numbers obtained in the present work are similar to those already obtained spectrophotometrically so far (as stated below). When the total HABA concentration (free HABA+bound HABA) was 1×10^{-5} M in the presence of 1×10^{-5} M BSA, the concentration of bound HABA was determined to be 8.8×10^{-6} M by an interpolation of the binding data obtained by equilibrium dialysis. Thus, the value of ε_{bound} was obtained at an arbitrary wavelength. The simulated wavelength region covers the two characteristic absorption bands. This method seems to be more reliable than an estimation at a particular wavelength. Figure 3 shows an example of the fitting of the simulated molar extinction coefficient to the experimentally obtained value in the above-mentioned wavelength region. The calculated curve agrees excellently with the experimentally obtained curve. This good agreement and the existence of the isosbestic point might suggest that the dye binds to one class of binding site in BSA. The binding profile was also examined in a dilute buffer containing 0.1 M NaCl. In this case, the binding number of the dye to the defatted BSA was determined by changes of ε_{obs} between 400 and 550 nm, since the absorbance below 400 nm did not change systematically with a change in the BSA concentration in the presence of 0.1 M NaCl.

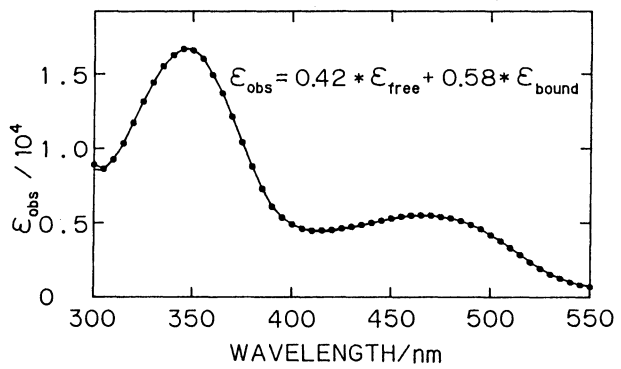

Fig. 3. Computed (——) and experimentally determined (-⊖-) molar extinction coefficient, ε_{obs}, of HABA in 5.3×10^{-6} M BSA. The scheme in the figure indicates that the computed spectrum consists of 42% of extinction coefficient of free HABA and 58% of extinction coefficient of bound HABA (see text). The binding quantity of HABA was calculated by the multiplication of the constant HABA concentration (1.0×10^{-5} M) by 0.58 and the subsequent division of it by the BSA concentration.

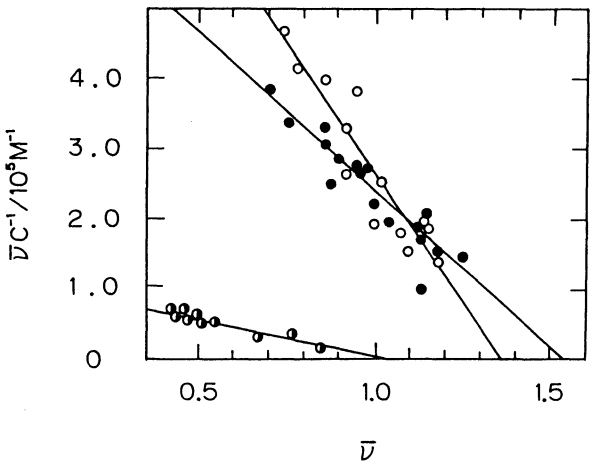

Fig. 4. Scatchard plot of binding of HABA to BSA. (○); defatted BSA, (●); undefatted BSA, (○); defatted BSA in 0.1 M NaCl. The binding data were taken in the range of D_0/P_0 between 1 and 10 at 25 °C.

Scatchard plots for these three kinds of binding profiles are shown in Fig. 4. The intercept to the ordinate equals the product of nK (n: number of binding site, K: binding constant) according to the Scatchard equation. Each product was of the order of 10^5 M^{-1}. Accordingly, the values of K are of the order of 10^5 M^{-1}, being larger by one order than those so far reported.[2,3,5–7] The values of K and n are listed in Table 1.

The magnitude of K was influenced by the presence of 0.1 M NaCl. At a low ionic strength, the BSA molecule more or less expands. Thus, the protein is in a more expanded state at an ionic strength of 0.014 than at a higher ionic strength. The metachromasy of HABA has been considered to be caused by its binding to the hydrophobic interior of a BSA molecule.[2,4,6]

Table 1. Binding Parameters of HABA to BSA

	Native BSA	Defatted BSA	Defatted BSA in 0.1 M NaCl
K_{int}[a]/10^5 M^{-1}	4.55	7.44	1.05
n[b]	1.5	1.4	1.0

a) Intrinsic binding constant obtained from the Scatchard plot. b) Number of binding site obtained in the same plot.

The environmental change of the binding site due to the expansion might be the reason for the increase in K with a lowering of the ionic strength. As can be seen in Table 1, the value of K is appreciably larger for defatted BSA than for undefatted BSA. Spector and Imig have studied an effect of the palmitate concentration on HABA binding to human serum albumin by means of equilibrium dialysis.[9] In their Scatchard plot (Fig. 1 in Ref. 9), the intercept on the ordinate (corresponding to the value of nK) is clearly larger in the absence of palmitate than in its presence, indicating a similar effect of the fatty acid on K to that observed in the present work.

The values of n are substantially compatible with those obtained spectrophotometrically at higher ionic strengths by Sakurai et al. (Fig. 1 in Ref. 5) and by Terada et al.[6] As can be seen in Table 1, however, the addition of 0.1 M NaCl decreases the number of binding sites in defatted BSA. This might also be related to an expansion of the BSA molecule at a low ionic strength. On the other hand, the removal of the fatty acids causes no appreciable difference in the number of binding sites (Table 1). Thus, it is believed that HABA binds to the same metachromasy sites independently of the fatty acids, although the removal of the fatty acids increases the magnitude of K.

Using a medium of such low ionic strength, we encountered the fact that the isosbestic point was observed in the restricted range of D_0/P_0 ratios in a dilute buffer. Assuming only two species, a good agreement was observed between the curve of experimentally observed apparent extinction coefficients and that of simulated ones. These suggest that only one kind of binding site exists under the present restricted conditions. Therefore, the reason why such an isosbestic point is not observed at higher ionic strengths might be due to a simultaneous appearance of plural kinds of binding sites. The difference in the numbers of binding site classes seems to be caused by the expansion nature of BSA due to a decrease in the ionic strength. The binding nature of BSA appeared to be easily affected by the coexistence of fatty acids or electrolytes. We can state that this is one of the aspects of serum albumin which serves as a carrier protein for various substances.

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