ASSIGNMENT OF CIRCULAR DICHROISM SPECTRA OF 2-(4'-HYDROXYPHENYLAZO)BENZOIC ACID INDUCED BY BOVINE SERUM ALBUMIN TO THE AZO FORM

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In the displacement study, the effects of tolbutamide on the absorption spectra and induced CD spectra of 2-(4'-hydroxyphenylazo)benzoic acid(HABA)-bovine serum albumin(BSA) complex were investigated using the samples obtained from equilibrium dialysis at pH 7.40. The binding of tolbutamide to BSA enhanced the binding of the azo form in spite of the displacement of the hydrazone form of bound HABA at the molar ratio of HABA to BSA of 0.65, causing the increase in the negative ellipticity at 445 nm, which was termed the C band. The C band could be assigned to the azo form of bound HABA.

Keywords—2-(4'-hydroxyphenylazo)benzoic acid; bovine serum albumin; tolbutamide; induced circular dichroism; equilibrium dialysis; absorption spectra; azo form; hydrazone form

Induced Cotton effects have been observed in optically inactive molecules, drugs, physiological substances, *etc.* by serum albumin.¹⁾ In the previous paper, the 2-(4'-hydroxyphenylazo)benzoic acid(HABA) bound to bovine serum albumin (BSA) shows the Cotton effects in the wavelength range of the absorption spectra of bound HABA in pH 7.40 phosphate buffer.²⁾ The circular dichroism(CD) spectra of HABA induced by BSA change in the molar ratio of HABA(D) to BSA(P) and can be resolved into four Gaussian bands. These are re-illustrated in Fig. 1 as A, B, C, and D bands. Kamisaka *et al.*³⁾ and Anderson⁴⁾ attributed the changes in the induced CD spectra to be due to the ligand-albumin ratio to the heterogeneity of the binding sites of the protein. As HABA molecules bound to BSA show the azo and hydrazone forms which have $\lambda_{\max}$ at 348 nm and 474 nm on the absorption spectra,⁵⁾ respectively, four Gaussian bands must correspond to either form of bound HABA. It was clarified that the A and B bands originate from one azo form and the D bands from the hydrazone form by displacement studies.²⁾ However, it is uncertain to which forms the C band corresponds.

In this paper, we are reporting that the C band originates from the azo form of bound HABA based on the result of investigation of the effects of tolbutamide on the absorption spectra and induced CD spectra of bound HABA.

All solutions were prepared with 1/15 M phosphate buffer(pH 7.40, ionic strength 0.16). 5 ml of the solution containing 0.5×10^{-4} M HABA and 0.6% BSA (D/P=0.65), and of the solution containing 2.0×10^{-4} M HABA and 0.3% BSA (D/P=5.20) were dialyzed against 15 ml of buffer solution for 20 hr at 20°. In displacement study, tolbutamide(0.1×10^{-4} M— 3.0×10^{-4} M) was added to the HABA solution. After equilibration, the concentration of total bound HABA was determined by analyzing the free HABA concentration, and the concentration of the hydrazone form was obtained from the absorption spectra of bound HABA(Hitachi 557 spectrophotometer), then that of the azo form was obtained by subtracting the hydrazone form from the total bound HABA.²⁾ CD measurements(JASCO 500C spectropolarimeter) were carried out using BSA-side solution at 20°.

The effects of salicylic acid, 6-*n*-propyl-2-

FIG. 1. *Four Gaussian Bands*²⁾ (dotted lines) obtained from Resolution of Observed CD Spectrum (solid line) at the Molar Ratio of HABA to BSA of 2.14

thiouracil, sulfadimethoxine and tolbutamide on the absorption spectra of bound HABA was investigated. The drugs except for tolbutamide (TB) displaced two forms of bound HABA, though the degree of the displacement of two forms was different between these three drugs. TB preferentially displaced the hydrazone form. The absorption spectra and the concentration of bound HABA with and without TB were shown in Fig. 2 and Fig. 3, respectively. At the D/P ratio of 0.65 (Fig. 3a), the decrease in the hydrazone form is clearly observed, though the total bound HABA did not change. The absorption spectra of the azo form having λ_{max} at 348 nm showed red shift and increase in the absorbance after the addition of TB (Fig. 2a). It is considered that the decrease in the hydrazone form was compen-

FIG. 2 *Effects of Tolbutamide on the Absorption Spectra of Bound HABA at the D/P Ratio of (a) 0.65 and (b) 5.20*

Added mol TB/mol BSA:

(a); (1) 0 (2) 0.13 (3) 0.66 (4) 1.32 (5) 2.65 (6) 3.97.
(b); (1) 0 (2) 0.27 (3) 1.32 (4) 2.65 (5) 5.29 (6) 7.94.

D/P is the molar ratio of HABA to BSA at the beginning of equilibrium dialysis.

FIG. 3 Effects of Tolbutamide on Total Bound HABA, Azo and Hydrazone Forms at the D/P Ratio of (a) 0.65 and (b) 5.20

— Δ —: total bound HABA, — $\circ$ —: azo form, — $\square$ —: hydrazone form.

sated with the increase in the azo form of bound HABA. The changes of the absorbance of the azo form caused by TB correspond to the changes of the concentration of the azo form of bound HABA obtained by subtraction (Fig. 2a, 3a). These facts clearly show that the binding of TB to BSA enhances the binding of the azo form in spite of the displacement of the hydrazone form. This suggests that the binding of TB to BSA caused the conformational change of the binding sites of the azo form on BSA.

The induced CD spectra of HABA with and without TB are shown in Fig. 4. The CD spectrum of HABA-BSA complex with the D/P ratio of 0.65 (Fig. 4a), which is characterized by two negative peaks at 360 nm and 430 nm, turned to the spectra with the positive peak at 360 nm and the negative peak at 445 nm after the addition of TB. These two ellipticity bands increase linking together upon addition of various concentrations of TB. The increase in the negative ellipticity at 445 nm, which corresponds to the C band, corresponds to the increase in the concentration of the azo form (Fig. 3a). Therefore, the C band would be assigned to the azo form. The

FIG. 4 Effects of Tolbutamide on the Induced CD spectrum of HABA-BSA Complex at the D/P Ratio of (a) 0.65 and (b) 5.20

added mol TB/mol BSA:

- (a); (1) 0 (2) 0.13 (3) 0.66 (4) 1.32 (5) 2.65 (6) 3.97.
(b); (1) 0 (2) 0.27 (3) 1.32 (4) 2.65 (5) 5.29 (6) 7.94.

presence of CD-spectrally distinguishable two azo forms of bound HABA, which show the A and C bands respectively, was clarified.

On the other hand, at the D/P ratio of 5.20 (Fig. 2b, 3b), only the addition of 0.27 mol TB/mol BSA caused the increase in the azo form of bound HABA. However, the CD spectrum of HABA-BSA complex, which is characterized by two negative peaks at 360 nm and 510 nm and one positive peak at 440 nm, turned to the same ellipticity pattern as in the case of D/P ratio of 0.65 after the addition of TB, showing the progressive increase in the negative ellipticity at 445 nm and positive one at 360 nm (Fig. 4b). It is considered that TB-induced conformational change of the binding sites of the azo form on BSA may cause the change of the CD spectra from the A band to the C band.

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REFERENCES

1) J.H.Perrin and D.A.Nelson: Induced optical activity

following the binding of warfarin, indomethacin, 4-hydroxycoumarin and salicylic acid to human serum albumin, *Life Science*, **11**, 277–283 (1972); J.C.Brand and T.Y.Toribara: Equilibrium dialysis and circular dichroic analysis of the novobiocin-human serum albumin complex, *Arch. Biochem. Biophys.*, **167**, 91–98 (1975); Y.Y.Thomas Su and B. Jirgensons: Optical activity studies of drug-protein complexes. The interaction of acetylsalicylic acid with human serum albumin and myeloma immunoglobulin, *Biochem. Pharmacol.*, **27**, 1043–1047 (1978).

- 2) T.Sakurai, S.Tsuchiya and H.Matsumaru: Effects of phenylbutazone and mefenamic acid on two classes of binding sites of 2-(4'-hydroxyphenylazo)benzoic acid on bovine serum albumin characterized by absorption spectra and circular dichroism spectra, *J. Pharm. Dyn.*, **4**, 345–355 (1981).
- 3) K.Kamisaka, I.Lisotwsky, J.J.Betheil and I.M.Arias: Competitive binding of bilirubin, sulfobromophthalein, indocyanine green and other organic anions to human and bovine serum albumin, *Biochem. Biophys. Acta.*, **365**, 169–180 (1974).
- 4) S.R.Anderson: The circular dichroism spectra of the complexes of 1-anilino-8-naphthalenesulfonate with bovine serum albumin. Evidence for heterogeneity of binding, *Biochemistry*, **8**, 4838–4842 (1969).
- 5) T.Sakurai, S.Tsuchiya and H.Matsumaru: Binding of 2-(4'-hydroxyphenylazo)benzoic acid to bovine serum albumin characterized by optical spectroscopy and equilibrium dialysis, *J. Pharm. Dyn.*, **4**, 65–68 (1981).