Drug Binding Properties of Glycosylated Human Serum Albumin as Measured by Fluorescence and Circular Dichroism

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Binding properties of Sudlow's site-specific drugs to glycosylated human serum albumin (G-HSA) were investigated using fluorescence and circular dichroism (CD). Dansylamide, phenylbutazone and warfarin were used as site I-specific drugs, and dansylproline, ibuprofen and flufenamic acid were used as site II-specific ones. Similar changes in the fluorescence intensity of dansylamide occurred in the presence of both G-HSA and intact human serum albumin (HSA), while the fluorescence enhancement of dansylproline caused by G-HSA was extremely weakened in comparison with that by HSA. These results suggest that the glycosylation of HSA inhibits the binding of the site II-specific drug, dansylproline, to HSA, while it does not influence the binding of the site I specific drug, dansylamide. The induced ellipticities of the complexes of ibuprofen, flufenamic acid and phenyl butazone with G-HSA were diminished in comparison with those with HSA. With the complexes of warfarin, the induced ellipticity was enhanced. These CD results suggest that the glycosylation of HSA induces microenvironmental changes in the binding sites for the above site-specific drugs which influence the drug binding ability of HSA.

Keywords glycosylated human serum albumin; drug binding; fluorescence; circular dichroism

Human serum albumin (HSA) is subject to modification by glucose *in vivo*, and diabetic patients have high amounts of the glycosylated albumin (G-HSA) in the serum.^{1–3)} To date, Lys 525⁴⁾ and Lys 199⁵⁾ have been identified as possible primary and secondary sites of non-enzymatic glycosylation of HSA *in vivo*. Further, Shaklai *et al.*⁶⁾ reported that the affinity of bilirubin or *cis*-parinaric acid for G-HSA was largely reduced relative to HSA. These studies suggest that the glycosylation of HSA may influence the binding ability of drugs which bind to the specific sites on HSA designated as Sudlow's site I and II. In this study, we investigated the drug binding properties of G-HSA with some of Sudlow's site-specific drugs using fluorescence and circular dichroism (CD) methods.

MATERIALS AND METHODS

HSA (lot No. 41) and fatty acid-free HSA (lot No. 22) were obtained from Miles, Inc., Kankakee, and G-HSA (lot No. 61H8090) was from Sigma Chem. Co., St. Louis. G-HSA contained 1.94 mol of glucose per mol of protein. Dansylamide (lot No. FCU01) was from Tokyo Kasei Kogyo Co., Tokyo. Dansylamide (lot No. 111H3341) and warfarin (lot No. 118F0111) were from Sigma Chem. Co. Phenylbutazone (lot No. M2A1280) was from Nakalai Tesque Co., Kyoto. Ibuprofen (lot No. ecg4380), flufenamic acid (lot No. TWM7307) and other reagents of the highest quality were from Wako Pure Chem. Ind., Osaka.

Fluorescence measurements were performed with a Hitachi 850 spectrofluorometer. The temperature of the sample was controlled by using a hollow cell holder through which water from a constant temperature bath regulated within 0.1 °C was circulated.

CD measurements were carried out on a Jasco J-720 spectropolarimeter. The spectrum was expressed as molar ellipticity based on the albumin concentration. For the

calculation of the albumin concentration, $E_{280\text{ nm}}^{1\text{ cm}, 1\%} = 5.3$ was used, and the validity of the value was confirmed by the Lowry method.⁷⁾ The molecular weights of HSA and G-HSA were assumed to be 66400 and 66800 respectively, according to the amino acid composition.⁸⁾ All fluorescence and CD measurements were carried out in 0.1 M sodium phosphate buffer, pH 7.0.

In this study, commercially available crystalline HSA and G-HSA were used. Neither albumin was defatted, and, therefore, they bound to a few residual fatty acids. For this reason the binding of the site I-specific drug, dansylamide, and the site II-specific drug, dansylproline, to the non-defatted HSA and the fatty acid-free HSA have been examined to determine whether the residual fatty acids could affect the binding of these drugs to both albumins. As a result, the fluorescence profiles of these drugs for both albumins were identical (see Results and Discussion). Therefore, we supposed that the fluorescence, as well as the ellipticity change, between HSA and G-HSA observed in this study could be induced by glycosylation.

RESULTS AND DISCUSSION

Site I-Specific Drugs Dansylamide, phenylbutazone and warfarin bind to Sudlow's site I. Figure 1 shows the fluorescence spectra of dansylamide in the presence and absence of G-HSA and HSA. In the presence of both albumins, the fluorescence peak of dansylamide shifted from 550 to 480 nm, which might have been caused by the binding of dansylamide to a highly hydrophobic pocket of site I on the albumin molecules. The fluorescence intensity of dansylamide increased with an increase in albumin concentration reflecting the binding of dansylamide to the albumins.^{9,10)} The changes in the fluorescence maxima are plotted against albumin concentrations as indicated in Fig. 2. The fluorescence of dansylamide in the presence of the fatty acid free HSA was identical to that

Fig. 1. Fluorescence Spectra of Dansyl Amide in the Presence of (A) G-HSA and (B) HSA

Dansylamide concentration was $2 \mu\text{M}$. The albumin concentrations in (A) and (B) were, (1), 0; (2), $1.15 \mu\text{M}$; (3), $2.30 \mu\text{M}$; (4), $3.45 \mu\text{M}$; (5), $4.55 \mu\text{M}$. Excitation wavelength was 335 nm and temperature was 25°C .

Fig. 2. Relative Fluorescence Intensity of Dansylamide at Fluorescence Maxima as a Function of Albumin Concentration

(○), G-HSA; (●), HSA. Other conditions were the same as in Fig. 1.

Fig. 3. CD Spectra of (A) Phenylbutazone-G-HSA and (B) Phenylbutazone-HSA

The albumin concentration was $20 \mu\text{M}$. D/P ratios were, (A): (1), 0; (2), 0.45; (3), 0.90; (4), 3.11; (B): (1), 0; (2), 0.90; (3), 1.79; (4), 3.11. Temperature was 19°C .

Fig. 4. Plots of the Induced Ellipticities of Phenylbutazone-G-HSA and Phenylbutazone-HSA

○, phenylbutazone-G-HSA; ●, phenylbutazone-HSA. Other conditions were the same as in Fig. 3.

in the presence of HSA (data not shown here). There were no appreciable differences in the fluorescence changes between G-HSA and HSA. This result suggests a similar binding pattern of dansylamide to both albumins.

When phenylbutazone binds to HSA, the induced CD appears near 290 nm .¹¹⁾ Figure 3 shows the induced CD spectra caused by phenylbutazone binding to G-HSA and HSA. The induced ellipticities increased with an

increase in molar ratio of drug to protein (D/P). The different ellipticities obtained after the subtraction of that of albumin alone are plotted against D/P, as shown in Fig. 4. The induced ellipticities for phenyl butazone-HSA complexes reached a plateau at D/P ratios of greater than three, while the increase of the ellipticities for phenylbutazone-G-HSA complexes remained at about

Fig. 5. CD Spectra of (A) Warfarin-G-HSA and (B) Warfarin-HSA. The albumin concentration was $20 \mu\text{M}$. D/P ratios were, (A): (1), 0; (2), 2.70; (B): (1), 0; (2), 1.35; (3), 2.70. Temperature was 17°C .

Fig. 6. Plots of the Induced Ellipticities of Warfarin-G-HSA and Warfarin-HSA

○, warfarin-G-HSA; ●, warfarin-HSA. Other conditions were the same as in Fig. 5.

Fig. 7. Fluorescence Spectra of Dansylprolin in the Presence of (A) G-HSA and (B) HSA

Dansylprolin concentration was $2 \mu\text{M}$. The albumin concentrations in (A) were, (1), 0; (2), $3.43 \mu\text{M}$; (3), $6.76 \mu\text{M}$; (4), $10 \mu\text{M}$; (5), $13.1 \mu\text{M}$, and those in (B) were, (1), 0; (2), $0.23 \mu\text{M}$; (3), $0.69 \mu\text{M}$; (4), $1.15 \mu\text{M}$; (5), $1.61 \mu\text{M}$. Temperature was 25°C .

Fig. 8. Relative Fluorescence Intensity of Dansylproline at Fluorescence Maxima as a Function of Albumin Concentration

○, G-HSA; ●, HSA. Other conditions were the same as in Fig. 7.

half that of the HSA complexes. This result indicates that the glycosylation of HSA depresses the induced ellipticity caused by the binding of phenylbutazone to G-HSA.

Warfarin induces CD when it binds to HSA.¹²⁾ Figure 5 shows the induced CD spectra caused by warfarin

Fig. 9. CD Spectra of (A) Ibuprofen-G-HSA and (B) Ibuprofen-HSA

The albumin concentration was 20 μ M. D/P ratios in (A) and (B) were, (1), 0; (2), 7.37. Temperature was 20 $^{\circ}$ C.

Fig. 11. CD Spectra of (A) Flufenamic Acid-G-HSA and (B) Flufenamic Acid-HSA

The albumin concentration was 20 μ M. D/P ratios in (A) and (B) were, (1), 0; (2), 0.66; (3), 1.33; (4), 1.99; (5), 2.64. Temperature was 16 $^{\circ}$ C.

binding to G-HSA. The plots of the induced ellipticities at 310 nm against D/P are shown in Fig. 6. The induced ellipticities of warfarin-G-HSA and HSA complexes increased with an increase in D/P ratio, and reached a plateau at D/P of more than three. The ellipticities of warfarin-G-HSA complexes were larger than those of the HSA complexes. This means that the induced ellipticity caused by warfarin binding to G-HSA may increase somewhat as a result of glycosylation.

Site II Specific Drugs Dansylproline, ibuprofen and flufenamic acid used in this study bind to Sudlow's site II.^{9,10)} Figure 7 shows the fluorescence spectra of dansylproline in the presence and absence of G-HSA and HSA. In the presence of HSA, the fluorescence spectra resembled those of dansylamide (Fig. 1), while in the presence of G-HSA, the fluorescence peaks were observed at wavelengths longer than 500 nm. This result suggests that the hydrophobicity of the binding region of dansylproline in site II on the HSA molecule was reduced by glycosylation. The fluorescence intensity of dansylproline was increased by binding to the albumins, as observed in the case of dansylamide. The change in fluorescence intensity of the dansylproline-HSA complex is about four

Fig. 10. Plots of the Induced Ellipticities of Ibuprofen-G-HSA and Ibuprofen-HSA

○, ibuprofen-G-HSA; ●, ibuprofen-HSA. Other conditions were the same as in Fig. 9.

times stronger than that of the dansylproline-G-HSA complex. The fluorescence of dansylproline in the presence of the fatty acid-free HSA coincided completely with that in the presence of HSA (data not shown here). These results suggest that the binding of dansylproline to Sudlow's site II on HSA is markedly inhibited by glycosylation.

The induced CD is caused by the binding of flufenamic acid to HSA.¹³⁾ The induced CD spectra of flufenamic acid-HSA complexes and flufenamic acid-G-HSA complexes are shown in Fig. 9, and the changes in ellipticities at 300 nm are plotted against D/P ratios in Fig. 10. The induced ellipticities increased in proportion to increases in D/P ratio, and reached a plateau at a D/P ratio greater than three. The maximum ellipticity of HSA complex is about 20% larger than that of G-HSA complex.

Figure 11 shows the induced CD spectra of ibuprofen-albumin complexes. In this case, the induced CD spectra were observed at about 300 nm. Figure 12 shows a change in the induced ellipticities at 295 nm against D/P ratios. The ellipticities of ibuprofen-HSA complexes also increased with an increase in D/P ratio, and reached a plateau at a D/P ratio of more than 15, while those of the ibuprofen-G-HSA complexes were small and reached a

Fig. 12. Plots of the Induced Ellipticities of Flufenamic Acid-G-HSA and Flufenamic Acid-HSA

○, flufenamic acid-G-HSA; ●, flufenamic acid-HSA. Other conditions were the same as in Fig. 11.

TABLE I. Relative Changes in Fluorescence Intensity and the Ellipticity of G-HSA Caused by Site-Specific Drug Binding

	Drugs	Relative changes (%) ^{a)}
Site I specific drugs	Dansylamide	104
	Phenylbutazone	48
	Warfarin	108
Site II specific drugs	Dansylproline	25
	Ibuprofen	20
	Flufenamic acid	83

a) Percent relative changes were calculated from the final fluorescence intensity and the induced ellipticity of G-HSA and HSA as values relative to HSA.

plateau at D/P ratios greater than 5. These results indicate that the induced ellipticity caused by the binding of flufenamic acid and ibuprofen to HSA was diminished by glycosylation.

Relative changes in fluorescence intensity and the induced ellipticity of G-HSA for Sudlow's site I and II-specific drugs are summarized in Table I as values relative to HSA. It seems reasonable to conclude from the values for dansylamide and dansylproline that the glycosylation of HSA may dominantly inhibit dansylproline binding to Sudlow's site II on the HSA molecule. On the other hand, it does not influence on dansylamide binding to Sudlow's site I. The reduction of hydrophobicity in site II may be one of reasons for the low binding ability of G-HSA for site II-specific drugs. The results obtained in this study also support the proposition by Shaklai *et al.*⁶⁾ that the primary glycosylating site, Lys 525, plays a key role in the binding of physiologically important ligands to albumin. On the other hand, CD results indicate that the glycosylation of HSA induces microenvironmental change at the drug binding sites which influence the induced ellipticity caused by site-specific drug binding to HSA. The induced ellipticity might reflect not only the drug binding to the primary strong binding sites (Sudlow's sites I and II) but also to the secondary weak binding sites. The reason for the latter is as follows: (1) The induced ellipticity caused by drug binding reached a plateau at relatively higher D/P ratios (more than three for phenylbutazone, warfarin and flufenamic acid, or more than fifteen for ibuprofen); (2) The drugs used in this study have some secondary weak binding sites.¹⁴⁾ The difference between the magnitude of the induced ellipticity caused by the binding of the same drug to HSA or G-HSA indicates the occurrence of a microenvironmental change at the drug binding sites, and it may reflect the strength of the binding ability of both albumins. In this study, the induced ellipticity caused by the site II-specific drugs was diminished in G-HSA, suggesting a lower binding ability of G-HSA for these drugs. However, the induced ellipticity caused by the site I-specific drugs was different in the different drugs, suggesting a varying influence of glycosylation on the binding ability of HSA for these site I-specific drugs. The secondary possible glycosylating site, Lys 199, which is located near site I, may also play a role in controlling the drug binding to HSA.

In conclusion, it was suggested that the glycosylation of HSA induced microenvironmental changes in the specific drug binding sites, named Sudlow's site I and II as reflected by a fluorescence change as well as a change in the magnitude of the induced ellipticity caused by drug binding. The results of this study may be a useful as a basis for further investigations into determining the effect of glycosylation on the structure and function of HSA.

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