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Study on the Binding of Dicumarol to α_1-Acid Glycoprotein Using Circular Dichroism Spectroscopy

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The interaction of dicumarol with α_1-acid glycoprotein (α_1-AGP) was studied by circular dichroism measurements. A single site having a binding constant of $2.9 \times 10^5\ M^{-1}$ was capable of inducing optical activity in dicumarol. The induced ellipticity of the dicumarol-α_1-AGP complex was decreased by raising the pH from 6.0 to 9.0, reflecting the changes in the molecular species of the drug and the microenvironmental changes in α_1-AGP. The displacement of dicumarol from α_1-AGP by fatty acids and neutral salts suggested a hydrophobic force to be involved in the binding. Interestingly, dicumarol was displaced by acidic drugs that bind to the binding site on human serum albumin called site I, or the warfarin site, whereas site II acidic drugs did not displace any dicumarol. Simple attempts to correlate displacement of dicumarol by *p*-aminobenzoates with the molecular size of a series of *p*-aminobenzoates suggested that the drug binding site is a hydrophobic cleft about 20 Å in depth.

Keywords——dicumarol; α_1-acid glycoprotein; circular dichroism; hydrophobic interaction; drug binding site; displacement

During the last decade, α_1-acid glycoprotein (α_1-AGP) has attracted the attention of pharmaceutical researchers because of its possible significance for the pharmacokinetic pattern of basic drugs.[1] Though the interaction mode of basic drugs with α_1-AGP is not clearly established, recent studies suggest that hydrophobic interaction plays an important role in the binding, rather than electrostatic interaction.[2–4] If this hypothesis is true, anionic drugs may also bind to α_1-AGP by hydrophobic interaction. In fact, recent studies show that some acidic drugs, such as warfarin are bound very strongly to α_1-AGP.[4–6] However, few observations are available about the molecular aspects of the interaction of drugs with α_1-AGP, in sharp contrast to the interaction of drugs with human serum albumin (HSA).[7,8] The present study was undertaken to investigate the binding mode of dicumarol to α_1-AGP and to define the features of the drug binding site on the α_1-AGP molecule, using the circular dichroism (CD) technique.

Experimental

Materials——α_1-AGP (lot No. 36F-9390) was obtained from Sigma Chemical Co. (St. Louis, MO). Dicumarol was obtained from Aldrich Chemical Co. (Milwaukee, WI). For the displacement investigations, potassium warfarin (Eisai Co., Tokyo), phenylbutazone, acenocoumarin (Chiba-Geigy Co., Summit, NJ), ibuprofen, flurbiprofen, pindolol, probenecid (Kaken Pharm. Co., Tokyo), imipramine, methoxypromazine (Yoshitomi Pharm. Co., Fukuoka), diazepam and propranolol (Sumitomo Pharm. Co., Osaka) were used as supplied. All other materials were of reagent grade and all solutions were prepared in deionized and distilled water. *p*-Aminobenzoates were synthesized by the method of Kadaba *et al.*[9] and their structures were confirmed by elemental analysis, melting point determination and nuclear magnetic resonance spectroscopy. All of the buffers used were prepared with sodium

phosphate dibasic and sodium phosphate monobasic. The pH values were checked at 25 °C using a suitably standardized pH meter.

CD Studies——CD measurements were made with a JASCO model J-50A spectrometer (Tokyo), using 1 and 10 mm cells. All α_1-AGP and dicumarol solutions were prepared in phosphate buffer. α_1-AGP solutions of 0.5—2.0×10^{-5} M (M.W. 44100) were used. All solutions were scanned from a wavelength at which no induced optical activity was observed. The induced ellipticity is defined as the ellipticity of the drug–α_1-AGP mixture minus the ellipticity of α_1-AGP alone at the same wavelength, and is expressed in degrees.

Fluorescence Studies——Fluorescence measurements were made with a Hitachi 650-60 fluorescence spectrophotometer (Tokyo). The fluorometric titrations were done as follows: α_1-AGP solutions of 2.0—10×10^{-6} M were titrated by successive additions of drugs (to give a final concentration of 0.2—10×10^{-6} M), and the fluorescence intensity was measured (excitation 290 nm and emission at 340 nm). At the selected wavelength, dicumarol did not contribute to the fluorescence.

Equilibrium Dialysis Studies——Dialysis experiments were performed using a Sanko Plastic dialysis cell (Fukuoka). α_1-AGP solution (2 ml) was poured into one compartment and 2 ml of dicumarol solution into the other. Adsorption of dicumarol onto membranes was negligible. After 12 h of dialysis, the concentration of free dicumarol was assayed by high performance liquid chromatography (HPLC).

Treatment of Binding Data——After values for the fraction of bound dicumarol had been found for all points along the titration curve, the results were plotted according to the Scatchard equation[10]

$$r/D_f = nK - rK$$

where r is the number of mol of dicumarol bound per mol of protein, n is the number of binding sites, K is the binding constant and D_f is the concentration of free dicumarol.

Results and Discussion

Figure 1 shows the induced CD spectra for dicumarol bound to α_1-AGP, with a positive peak at 325 nm and two negative peaks near 270 and 300 nm. The induced CD curves were shown to have a shape independent of concentration. That is, the positions of the peaks did not change and the isosbestic point at 309 nm is maintained throughout the titration. This concentration independence is clearly indicative of homogeneity in the binding process, that is, it suggests that α_1-AGP has a single binding site capable of inducing optical activity in the dicumarol molecule.

Figure 2 shows plots of induced ellipticities at 325 nm against dicumarol concentration at

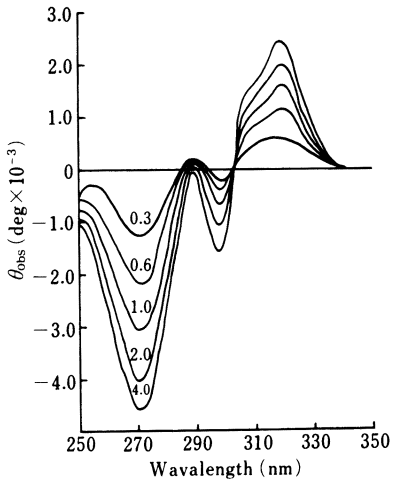

Fig. 1. Observed Ellipticity of Dicumarol–α_1-AGP Complexes at Various Drug-to-Protein Ratios at pH 7.4

[α_1-AGP] = 1.5×10^{-5} M.
Drug-to-protein ratios are shown on the curves.

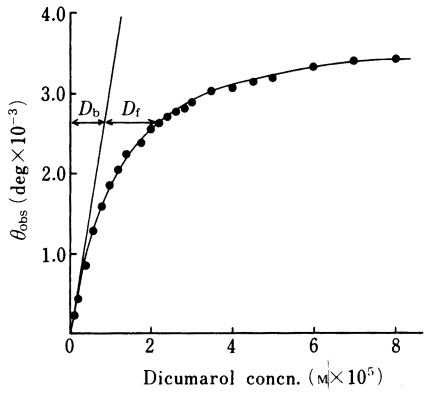

Fig. 2. Estimation of Free and Bound Drugs by the Method of Rosen[11]

Measurements were made at 325 nm. D_b and D_f are concentrations of the bound drug and the free drug, respectively.

a fixed α_1-AGP concentration. All points are the averages of three determinations. It is assumed that the CD curves obtained by subtraction of the spectrum of α_1-AGP alone from those of the drug–α_1-AGP mixtures correspond to the bound drug and contain no significant contribution from the disturbance of the aromatic residues of the protein. Drawing the tangent to the curve at zero drug concentration enables the appropriate intensive factor for the bound drug to be determined.[11] This method of deriving concentrations of free and bound drug only allows precise interpretation in terms of binding constants when a single site is involved. When more than one site contributes to the ellipticity, the method is less precise. The observed induced ellipticity is a function of the amount bound at the individual sites and associated molar ellipticities. If the binding constants of the primary and secondary sites are very different from one another and the ellipticities of the secondary sites are small, then reasonable estimates of the primary binding constants can be obtained. These problems are shared by other spectroscopic methods where heterogeneity of binding is involved.

Figure 3 shows the Scatchard plot, using data derived in the manner described above. The Scatchard plots in Fig. 3 again indicate one binding site for dicumarol to α_1- AGP. Table I shows the binding parameters along with those obtained by the fluorescence quenching technique.[12] The binding of dicumarol causes quenching of the intrinsic fluorescence of α_1-AGP that may arise from tryptophan and/or tyrosine residues. The amount of quenching directly reflects the amount of the drug bound. The binding constant and the number of

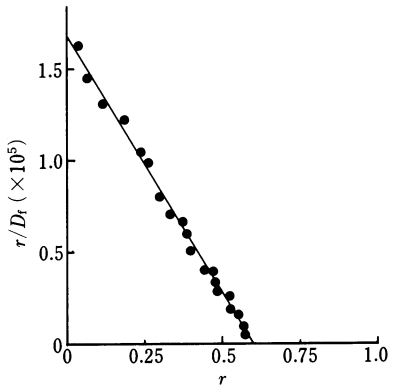

Fig. 3. Scatchard Plots of Dicumarol–α_1-AGP Interaction, Using Data from Fig. 2 (See Text)

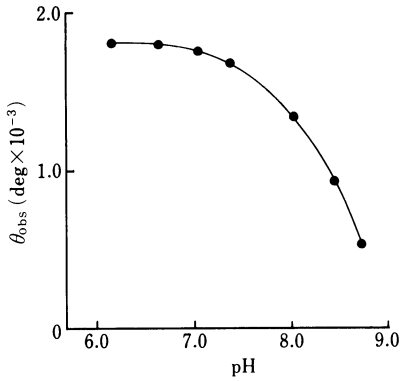

Fig. 4. Effects of pH on Induced Ellipticities for Dicumarol–α_1-AGP Interaction at 25 °C

[dicumarol] = 1.5×10^{-5} M; [α_1-AGP] = 1.5×10^{-5} M.

TABLE I. Binding Parameters of Dicumarol–α_1-AGP Interaction at pH 7.4 and 25 °C

	n	K (M^{-1})
CD	0.6	2.9×10^5
Fluorescence	0.8	6.6×10^5

TABLE II. Displacement of Dicumarol from α_1-AGP by Various Drugs

	Displacement (%)[a]	
	1×10^{-5} M	2×10^{-5} M
Warfarin	58	70
Phenylbutazone	9	14
Ibuprofen	—[b]	—[b]
Diazepam	7	19
Pindolol	9	14
Propranolol	30	65
Imipramine	181[c]	230[c]
Acenocoumarin	13	20
Flurbiprofen	—[b]	—[b]
Probenecid	—[b]	—[b]
Methoxypromazine	73	99

α_1-AGP (1×10^{-5} M) and dicumarol (1×10^{-5} M) were used throughout. The percentage displacement is expressed as the decreased signal height at 325 nm. *a*) Accuracy of 2%. *b*) Less than 2%. *c*) Dicumarol–imipramine–α_1-AGP system showed induced the negative ellipticity at 325 nm.

binding sites found by the CD method are somewhat smaller than those obtained from the fluorescence measurement. The differences may be due simply to the difference of techniques. That is, though quenching may mainly occur when the drug is bound near a tryptophan residue of α_1-AGP which generates the native fluorescence of the protein, small contributions of other factors such as concentration quenching might also exist.

The effect of pH on the induced ellipticity of dicumarol–α_1-AGP complex was examined. As shown in Fig. 4, the ellipticity was decreased as the pH was raised from 6.0 to 9.0. This pH profile of the ellipticity may be due to the change of binding affinity rather than change in the geometry of the binding site as a result of conformational changes because the free concentration of dicumarol (D_f) increased with pH (D_f: 31.4% at pH 6.5; 37.1% at pH 7.4; 70.8% at pH 8.4). The decreased affinity of dicumarol to α_1-AGP can be explained on the basis of changes in the molecular species of the drug and conformational changes of α_1-AGP; dicumarol exists in various chemical forms because the pK_a's of dicumarol are 6.10 and 8.05, respectively.[13] On the other hand, microenvironmental changes in α_1-AGP occur around physiological pH.[14]

In order to find out whether dicumarol shares the binding site with acidic and basic drugs, the effects of several drugs on the induced ellipticities of dicumarol bound to α_1-AGP were investigated. All the drugs had negligible extrinsic Cotton effects under the experimental conditions of Table II. The shape of the CD curve after competition was consistent with that expected for reduced binding of dicumarol. The dicumarol was displaced by the basic drugs except imipramine, particularly by propranolol and methoxypromazine. The enhanced ellipticity by imipramine can be explained in terms of some cooperativity, though the mechanism should be further investigated. It is interesting to note that acidic drugs that bind to the binding site on HSA called site I,[15] or the warfarin site,[16] significantly displaced dicumarol bound to α_1-AGP, whereas none of the acidic site II drugs (ibuprofen, flurbiprofen, probenecid) displaced only dicumarol.

To gain insight into the features of the drug-binding site, the effects of fatty acids and neutral salts on the ellipticity of the complex were examined. Dicumarol was easily displaced from α_1-AGP by oleic acid and stearic acid, whereas the addition of sialic acid and sodium chloride did not cause displacement (not shown), as expected from the previous results.[4] In addition, the thermodynamic parameters determined from a van't Hoff plot gave a positive entropy, which may be taken as evidence of hydrophobic interaction ($\Delta H = -16.2\,\mathrm{kJ \cdot mol^{-1}}$, $\Delta S = 54.3\,\mathrm{J \cdot K^{-1} \cdot mol^{-1}}$). Therefore, hydrophobic interaction seems to play an important role in the binding of the acidic drugs to α_1-AGP.

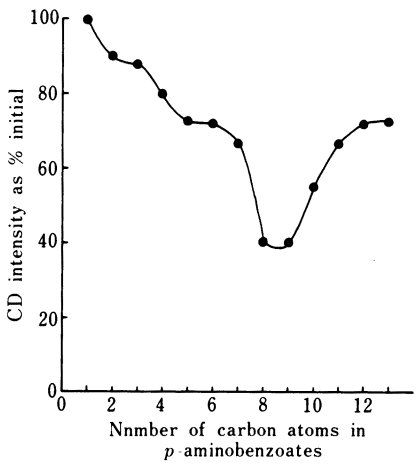

Fig. 5. Effects of *p*-Aminobenzoates on Induced Ellipticities for Dicumarol–α_1-AGP Interaction at 25 °C

[dicumarol] = 1.0×10^{-5} M, [α_1-AGP] = 1.0×10^{-5} M, [*p*-aminobenzoate] = 1.0×10^{-5} M.

Moreover, a displacement study was conducted using a series of *p*-aminobenzoates, developed recently in these laboratories as probes for the warfarin site. Figure 5 shows the changes in the induced ellipticities of the dicumarol–α_1-AGP complex upon the addition of *p*-aminobenzoates at a 1 : 1 ratio of the antagonists to α_1-AGP. Changes in the ellipticity of the complex provided a measure of displacement by *p*-aminobenzoates at the site. The displacement percentage increased with increasing chain length of the alkyl group and reached a maximum with the octyl and nonyl groups. This result supports the above hypothesis that the drug binding site on α_1-AGP in located in a hydrophobic region of α_1-AGP. In addition, the result in Fig. 5 has implications for the molecular size and shape of the drug binding site. If the binding patch can be simply estimated from a model based on standard bond lengths and angles, the Corey–Pauling–Koltun molecular model, the binding site is a hydrophobic cleft about 20 Å deep. Interestingly, this value is consistent with our recent observation that site I on HSA may have a hydrophobic cleft about 19 Å deep.[17] The structural requirements for the drug-binding sites on α_1-AGP are being further characterized in this laboratory.

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