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Binding characteristics of coumarin anticoagulants to human α_1-acid glycoprotein and human serum albumin

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Summary

The binding of a series of coumarin compounds to human α_1-acid glycoprotein (AGP) and to human serum albumin (HSA) was investigated using fluorescence and circular dichroism spectroscopy. The binding parameters, where possible, were determined by means of the intrinsic fluorescence quenching of proteins by coumarin compounds. The binding parameters for the two proteins are almost identical. In addition, the extrinsic Cotton effect generated via binding of coumarin compounds to AGP differed from that in the case of HSA. The possibility was considered that the binding sites of the two proteins might differ in their degree of asymmetry. With the use of physicochemical parameters, the AGP and HSA binding parameters of coumarin compounds were analyzed. Hydrophobic interaction was found to play an important role in binding for both proteins. The size of the 3′-substituent of coumarin compounds also influenced binding to AGP. From the above observations, it was concluded that the binding sites of the coumarin compounds are located within the hydrophobic crevices of the proteins, and that the mode of interaction of coumarin compounds with AGP differs somewhat from that with HSA.

Introduction

It is well known that coumarin compounds, widely used as anticoagulant drugs, bind strongly to their sites on human serum albumin (HSA) (Otagiri et al., 1979; Larsen et al., 1984). These binding sites have been identified with Site 1 on HSA (Sudlow et al., 1975, 1976; Fehske et al., 1979). Recently, we have reported that several coumarin compounds, such as warfarin (Otagiri et al., 1986), phenprocoumon (Otagiri et al., 1987) and dicoumarol (Otagiri et al., 1988) also bind to a common drug binding site on α_1-acid glycoprotein (AGP) with the same affinity as for binding to HSA. In both cases, the driving force for binding appears to be provided by hydrophobic interactions although a detailed explanation concerning the mechanism of binding for either protein system remains to be determined. We have considered this problem from several points of view at the molecular level. Quantitative relationships between physicochemical properties and binding of drugs to protein can be established by multiple regression analysis, since binding usually depends

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upon their physicochemical properties (Hansch and Dunn, 1972; Matsushita et al., 1987). Therefore, in the present study, we applied this method using three physicochemical parameters, namely, the partition coefficient for the influence of hydrophobicity, the pK_a for the electrostatic effect and the van der Waals volume for the steric influence, in order to characterize the binding of a series of coumarin compounds to AGP and HSA. Moreover, comparison of their binding to AGP and HSA was performed.

Experimental

Materials

Human serum albumin (HSA; lot no. 36F-9333) and human α_1-acid glycoprotein (AGP; lot no. 44F-9345) were obtained from Sigma (St. Louis, MO). The molecular weights of AGP and HSA were assumed to be 44 100 and 66 500, respectively. 4-Hydroxycoumarin and dicoumarol were purchased from Tokyo Kasei. Phenprocoumon (Hoffman-La Roche, Nutley, NJ), potassium warfarin (Eisai Co., Tokyo) and acenocoumarin (Ciba-Geigy, Summit, NJ) were used as supplied. Ethyl biscoumacetate and coumetarol were generously donated by Professor L.H.M. Janssen, State University of Utrecht. Coumachlor was a generous gift from Professor S. Awazu, Tokyo College of Pharmacy. All other materials were of reagent grade and all solutions were prepared in deionized and distilled water. All protein and compound solutions were prepared in 0.067 M phosphate buffer (pH 7.4).

Apparatus and methods

Fluorescence measurements were recorded using a Hitachi 650-60 fluorescence spectrophotometer (Tokyo). Fluorometric titrations were carried out as follows: protein solutions of 2.0×10^{-4} M were titrated by successive additions of drug solutions (to give a final concentration of 0.5–15 $\times 10^{-6}$ M) and the fluorescence intensity was determined (excitation at 290 nm; emission at 340 nm) at 25 °C. The intrinsic fluorescence of proteins was corrected for inner filter effects. Circular dichroism (CD) measurements were recorded on a Jasco J-50A recording spectropolarimeter (Tokyo), using a 10 mm cell. 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-protein mixture minus that of the protein alone at the same wavelength and is expressed in degrees.

Data treatment

The fraction of drug bound, X, is usually determined according to Weber and Young (1964)

$$X = \frac{F_0 - F_p}{F_0}$$

where F_p and F_0 denote the fluorescence intensities of protein in a solution with a given concentration of drug and without drug, respectively. After evaluation of the fraction from the curve, the results were analyzed in the form of Scatchard plots (1949)

$$r/D_f = nK - rK$$

where r represents the number of moles of drug bound per mole of protein, n is the number of binding sites, K is the binding constant and D_f is the concentration of free drug.

Multiple regression analysis

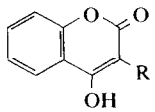
The characteristics of the binding constants were quantitatively examined via multiple regression analysis. The predictor variables included octanol-0.067 M phosphate buffer (pH 7.4), partition coefficient (PC), pK_a (Otagiri et al., 1978) and van der Waals volume (V_w). Correlations and regression equations were calculated on a PC-9801-vm (NEC) personal computer.

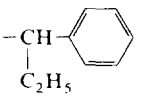
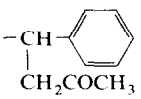
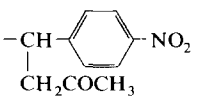
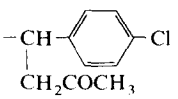
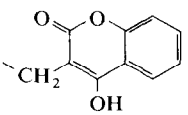
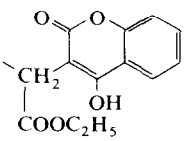
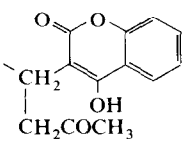
Results and Discussion

Table 1 illustrates the chemical structures and physicochemical parameters of eight coumarin compounds in this study. In the presence of coumarin compounds, the intrinsic fluorescence of AGP and HSA arising from tryptophan was significantly quenched, since the binding sites for

TABLE 1

Chemical structure and physicochemical characteristics of coumarin compounds investigated in the present study



	R	PC [a]	pK_a [a]	V_w [b]
4-Hydroxycoumarin	-H	0.15	4.15	1.28
Phenprocoumon		17.1	4.30	2.44
Warfarin		10.9	5.10	2.62
Acenocoumarin		7.94	5.03	2.81
Coumachlor		16.5	4.90	2.78
Dicoumarol		79.3	6.10 8.05	2.62
Ethyl biscoumacetate		23.3	– 7.52	3.18
Coumetarol		18.0	4.10 9.00	3.01

PC partition coefficient; V_w, van der Waals volume ($\times 10^2$ Å^3).

[a] Values taken from Otagiri et al. (1978).

[b] Calculated from Matsushita et al. (1987).

coumarin compounds are located near the tryptophan residues in the respective proteins. The same phenomena have been reported by Sugiyama et al. (1985) and Friedman et al. (1985). Therefore, the amount of quenching directly reflects the amount of drug bound. As a typical case of binding of coumarin compounds, fluorometric titrations were performed by varying the acenocoumarin concentration at a fixed protein concentration, as shown in Fig. 1. The free and bound concentration of drugs used for the Scatchard plots were calculated according to the method of

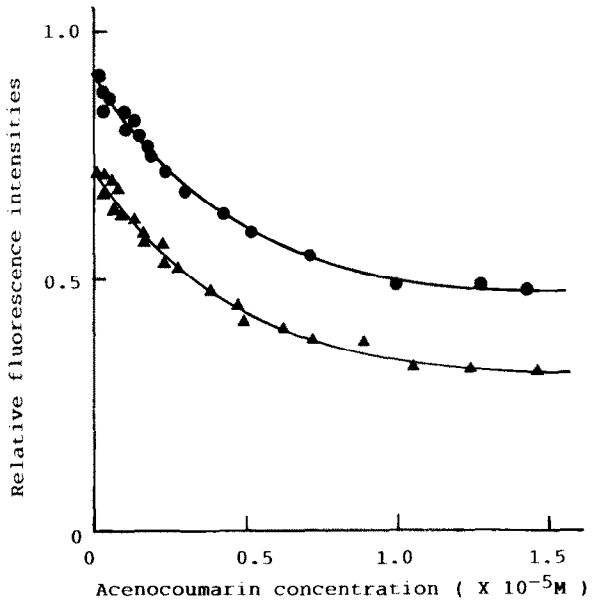


Fig. 1. Plots of relative fluorescence intensities as a function of acenocoumarin concentration for acenocoumarin-AGP and HSA interactions in 0.067 M phosphate buffer (pH 7.4) at 25°C. [AGP] = [HSA] = 2×10^{-6} M, [acenocoumarin] = 0.5–15×10^{-6} M; (▲) acenocoumarin-AGP system, (●) acenocoumarin-HSA system.

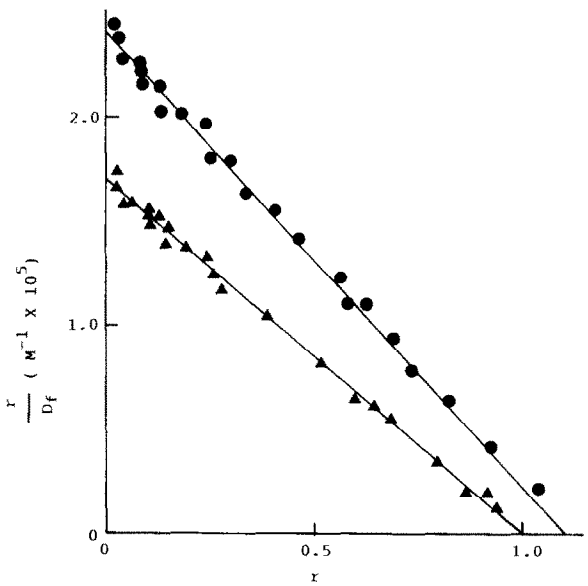


Fig. 2. Scatchard plots of acenocoumarin-AGP and HSA interactions. See Fig. 1 for experimental conditions. (▲) Acenocoumarin-AGP system, (●) acenocoumarin-HSA system.

Scatchard (1949). As a typical case of binding of coumarin compounds, plots of r/D_f vs r for acenocoumarin-protein systems are shown in Fig. 2. The linearity of the Scatchard plots indicates that coumarin compounds bind one class of sites on AGP and HSA. To evaluate the maximum number of binding sites, Job plots were also constructed for coumarin compound-AGP and -HSA systems by maintaining the total concentration of drug and protein at a constant value of 5×10^{-6} M (Job, 1928). A representative example of binding of coumarin compounds is provided by the Job plots for acenocoumarin illustrated in Fig. 3. The inflection points for these plots are near 0.5, the value expected for the formation of a 1:1 complex. The binding parameters for coumarin compound-AGP and -HSA interactions estimated from Scatchard plots are summarized in Table 2. In all systems the value of n was approximately equal to unity. This is in good agreement with the results of the Job plots. The binding constants for the two serum proteins are almost identical and

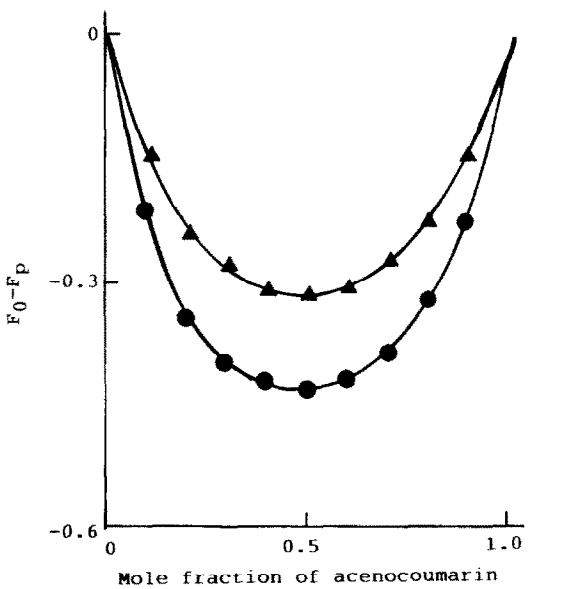


Fig. 3. Job plots of relative fluorescence intensities as a function of mole fraction of acenocoumarin. Total concentration of [serum protein] + [acenocoumarin] was kept constant at 5×10^{-6} M. (▲) Acenocoumarin-AGP system, (●) acenocoumarin-HSA system.

TABLE 2

Binding parameters for coumarin compound-serum protein systems at pH 7.4 and 25°C

	HSA		AGP	
	n	K (M^{-1})	n	K (M^{-1})
4-Hydroxycoumarin	1.0	5.4×10^4	1.3	6.1×10^4
Phenprocoumon	1.0	5.6×10^5	1.0	7.4×10^5
Warfarin	1.3	3.0×10^5	0.9	1.8×10^5
Acenocoumarin	1.1	2.2×10^5	1.0	1.7×10^5
Coumachlor	1.2	4.5×10^5	1.1	2.6×10^5
Dicoumarol	1.3	1.0×10^6	0.8	6.6×10^5
Ethylbiscoumacetate	0.9	5.0×10^5	1.2	2.8×10^5
Coumetarol	1.1	4.5×10^5	1.1	2.6×10^5

are consistent with data in previous reports, despite the latter having been obtained via different methods, such as equilibrium dialysis (Urien et al., 1982), CD (Otagiri et al., 1988) and fluorescence (Otagiri et al., 1986, 1987) spectroscopy. The binding of coumarin compounds to serum proteins generates mono- or biphasic extrinsic Cotton effects. The data show reasonably good agreement for the ultraviolet spectra of coumarin compounds in the presence and absence of serum proteins (not shown). The CD characteristics of coumarin compounds with AGP and HSA are summarized in Table 3. Clearly, the sign and magnitude of the extrinsic Cotton effects of the coumarin compound-AGP systems differ from those of the HSA systems. The sign and magnitude of the extrinsic Cotton effect may depend upon the spatial

TABLE 3

CD characteristics of coumarin compounds with serum proteins

	HSA		AGP	
	λ_{nm}	θ_{obs}	λ_{nm}	θ_{obs}
4-Hydroxycoumarin	290	+1.3	– [a]	
Phenprocoumon	– [a]		305	−1.0
Warfarin	335[s]	+0.4	325[s]	−0.9
	310	+1.1	305	−1.9
			285	−1.3
Acenocoumarin	290	+1.6	305	−1.4
Coumachlor	290	+0.5	285	−0.7
	310	+0.3	305	−1.5
Dicoumarol	300	−5.8	325	+2.0
			300	−0.9
			270	−3.5
Ethyl biscoumacetate	315	+1.1	320	+0.6
	290[s]	+0.6	300	−0.5
			265	−1.9
Coumetarol	320	−1.8	325	+0.5
	290[s]	−0.3	300	−1.3
			265	−2.3

Serum proteins (1.0×10^{-5} M) and coumarin compounds (5×10^{-5} M) were used throughout. λ_{nm}, maximum wavelength of induced CD; θ_{obs}, observed ellipticity (degree$\times10^{-3}$); S, shoulder.

[a] Could not be observed.

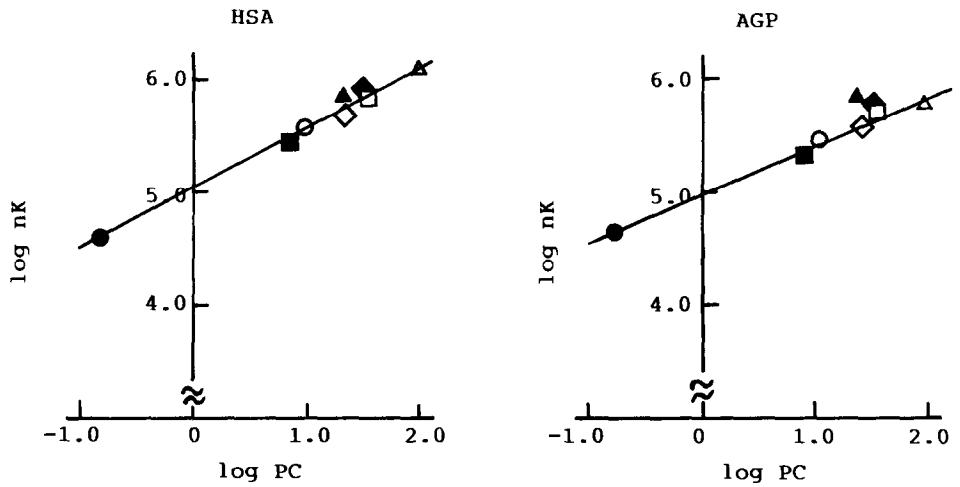


Fig. 4. Relationships between binding parameters of complex and partition coefficient of coumarin compounds. (●) 4-Hydroxycoumarin, (▲) phenprocoumon, (○) warfarin, (■) acenocoumarin, (◆) coumachlor, (△) dicoumarol, (□) ethyl biscoumacetate, (◇) coumetarol.

arrangement and rigidity of the drug-protein complex when comparisons are being made between the interactions of the given drug molecule with similar macromolecules (Chignell, 1968). Therefore, the various spectra in AGP and HSA systems may indicate that the complex is held rigidly in the region near the chromophore responsible for positive or negative contributions to the Cotton effect.

In an attempt to elucidate the binding mechanism of coumarin compounds with serum proteins, we investigated the quantitative relationships between the binding parameters of eight coumarin compounds and their physicochemical parameters using multiple regression analysis. Fig. 4 depicts data on the correlation between the hydrophobic parameters of drugs and the binding parameters of AGP and HSA, respectively. The degree of correlation between the binding constants and log PC is significant in AGP:

$$\log nK = 0.372(\pm 0.090) \log \mathrm{PC} + 5.04(\pm 0.113),$$

$$n = 8,\ r = 0.860,\ s = 0.216 \qquad (1)$$

and HSA:

$$\log nK = 0.505(\pm 0.374) \log \mathrm{PC} + 5.07(\pm 0.047),$$

$$n = 8,\ r = 0.984,\ s = 0.037 \qquad (2)$$

However, no significant correlation was found between the binding constants and pK_a (not shown). Several reports describe a linear relationship between the lipophilicity of drugs and their binding constants to the proteins (Schrier et al., 1965; Terada et al., 1974). In most of those cases, the values of the slopes lie within the range approx. 0.45–0.75 (Helmer et al., 1968; Fujita, 1972). The values obtained for the binding of HSA (Eqn 2) are in accord with this range. On the other hand, a lower value was determined for the binding of AGP (Eqn 1). The difference between the slopes of both systems indicates that the binding of coumarin compounds to AGP is not identical to that with HSA. Thus, the role of hydrophobic interactions is of greater significance in the binding of coumarin compounds to HSA rather than to AGP. Interestingly, in the two parameter equations, a further degree of correlation was found with the combination of log PC and V_w in the case of AGP (Eqn 3):

$$\log nK = 0.608(\pm 0.134) \log \mathrm{PC} - 0.385(\pm 0.185) V_w + 5.80(\pm 0.375).$$

$$n = 8,\ r = 0.935,\ s = 0.115 \qquad (3)$$

It has been shown that binding to AGP depends not only on the hydrophobicity but also upon the size of the 3′-substituent of those compounds. Therefore, the binding sites of coumarin compounds on AGP might be subject to greater steric limitations than in the case of HSA. The results clearly indicate that hydrophobic molecules bind strongly to both proteins but that the microenvironments of their binding sites may differ slightly, e.g., as concerns size and polarity.

In conclusion, the binding site of coumarin compounds is located within the hydrophobic crevice of the proteins and the mode of interaction of coumarin compounds with AGP differs somewhat from that with HSA.

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