Characterization of Drug Binding Sites on α_1-Acid Glycoprotein

Toru MARUYAMA,[a] Masaki OTAGIRI*,[a] and Akira TAKADATE[b]

Faculty of Pharmaceutical Sciences, Kumamoto University,[a] 5–1, Oe-honmachi, Kumamoto 862, Japan and Daiichi College of Pharmaceutical Sciences,[b] 22–1, Tamagawa-cho, Minami-ku, Fukuoka 815, Japan. *Received November 9, 1989*

The classification of drug binding sites on α_1-acid glycoprotein (AGP) was studied by displacement experiments using fluorescent probes. Basic drugs not only displaced basic probes strongly but also acidic probes as well. Acidic probes, on the other hand, were displaced by some acidic drugs such as phenylbutazone and sulfadimethoxine which had no effect on most of the basic probes. This contradiction suggests that the basic drugs do not completely share a binding site with the acidic drugs. The polarity of the basic drug binding site was higher than that of the acidic drug binding site. The negative charges were probably located in or near the former, different from the latter. The basic drug binding site was more sensitive to the conformational change of AGP. It seems that there are particular drug binding sites on the AGP molecule for acidic and basic drugs. However, all the displacement data do not fully support the possibility of two independent drug binding sites. Therefore, it is rather reasonable to consider that these sites are not completely separated but are significantly overlapped and influenced by each other. Accordingly, AGP seems to have one wide and flexible drug binding area.

Keywords drug binding site; α_1-acid glycoprotein; probe; fluorescence; binding site microenvironment

In the last decade during attempts to characterize molecular aspects of the interaction of drugs with α_1-acid glycoprotein (AGP), only one common binding site has been reported for nearly all drugs so far investigated, *e.g.* phenothiazine neuroleptics,[1] β-blocker agents[2] and coumarin anticoagulants.[3,4] Recently, we have found the formation of a ternary complex of AGP with dicumarol and chlorpromazine.[5] This finding easily leads to the suggestion that there are drug binding sites on AGP. Fluorescence spectroscopy is one of the most versatile and sensitive techniques for studying drug–protein interaction[6] since fluorescent probe characteristics are dependent on the environment. For example, Sudlow *et al.*,[7] identified and characterized two specific drug binding sites, site I and site II on human serum albumin (HSA) using dansylamino acids as probes. Moreover, recent fluorescent studies have shown that a high drug affinity site might be located in a hydrophobic area of AGP.[8,9] However, the characterization of the drug binding sites on AGP such as the number of high affinity sites and binding size has not fully been clarified; thus, the present study was undertaken to make such a classification. In addition, the microenvironment of the drug binding sites, including the hydrophobicity and binding size was also examined. Two fluorescent probes with different binding specificity to AGP were used in the present study because probes having several affinity sites might have complicated the data analysis.

Experimental

Materials AGP was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). The AGP gave only one band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Potassium warfarin (WF; Eisai Co., Tokyo), phenylbutazone (Ciba-Geigy Co., Summit, NJ), ibuprofen, flurbiprofen, probenecid (Kaken Pharm. Co., Tokyo), digoxin (Yamanouchi Pharm. Co., Tokyo), sulfadimethoxine (Daiichi Pharm. Co., Tokyo), perphenazine, chlorpromazine, trifluoperazine, imipramine, desipramine (Yoshitomi Pharm. Co., Fukuoka), diazepam, propranolol, pindolol (Sumitomo Pharm. Co., Osaka), diphenhydramine (Kowa Co., Nagoya), salicylic acid (Ono Pharm. Co., Osaka) were used as supplied. Dicumarol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Acenocoumarin was a gift from Prof. L. H. M. Janssen of the University of Utrecht. 7-Anilino-4-methylcoumarin-3-acetic acid (AMCA) and *p*-aminobenzoates were gifts from Prof. S. Goya of Kumamoto University. Dansyl-DL-norleucine (DNSL) was obtained from Sigma Chemical Co. (St. Louis, MO). Auramine O (AO) was obtained from the Merck Co., Ltd. Quinaldine red (QR) was purchased from Tokyo Kasei Kogyo Co., Ltd. AO and QR were recrystallized gently from ethanol–H_2O. Acridine orange-10-dodecyl bromide (AODB) was purchased from Wako Pure Chemical Ind., Ltd. All other materials and organic solvents were of reagent grade and all solutions were prepared in deionized and distilled water. Phosphate buffer of pH 7.4 was prepared with 0.067 M sodium phosphate dibasic and sodium phosphate monobasic.

Apparatus and Methods Fluorescence measurements were made using a Hitachi 650-60 fluorescence spectrophotometer (Tokyo, Japan). The excitation and emission wavelengths of probes are listed in Table I. Fluorometric titrations were carried out as follows; protein solutions of 2.0×10^{-6} M were titrated by successive additions of probe solutions (to give a final concentration of $0.5—15 \times 10^{-6}$ M) and the fluorescence intensity was measured at 25 °C. When necessary, the fluorescence intensities were correceted for both dilution and inner filter effect by the method reported by Chignell.[6] Circular dichroism (CD) measurements were made on a Jasco model J-50A spectrometer (Tokyo, Japan), using 10 mm cells. The induced ellipticity is defined as the ellipticity of the probe–AGP mixture minus the ellipticity of the AGP alone at the same wavelength and is expressed in degrees.

Data Treatment The fraction of probe bound, X, is usually determind by Weber and Young[10]

$$X = \frac{F_P - F_0}{F_b - F_0} \quad (1)$$

where F_P and F_0 are the fluorescence intensities of a given concentration of probe in a solution with and without protein, respectively, and F_b is the fluorescence of the same concentration of fully bound probe. After obtaining values for the fraction of bound probe for all points along the titration curve, the results were plotted according to the Scatchard equation[11]:

$$r/D_f = nK - rK \quad (2)$$

where r is the number of moles of probe 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 probe.

Results and Discussion

Classification of Drug Binding Sites on AGP The binding parameters of probes with AGP are summarized in Table I. All the probes bound to one tight binding site on AGP. The binding parameters for WF and AO are in excellent agreements with those reported in the literature.[9,12] Table II shows the displacement of 6 probes by acidic and basic drugs. All basic drugs inhibited the binding of basic probes

TABLE I. Binding Parameters of Probe–AGP Interactions at 25 °C

	Binding parameter	Excitation and emission wavelengths
Acidic probe		
WF	$n=0.8$, $K=2.2\times10^5$	320:390
	($n=0.82$, $K=2.2\times10^5$)[a]	
DNSL	$n=1.4$, $K=2.6\times10^4$	360:510
AMCA	$n=1.2$, $K=5.5\times10^4$	365:460
Basic probe		
AO	$n=1.0$, $K=8.3\times10^4$	450:510
	($n=0.79$, $K=4.5\times10^4$)[b]	
QR	$n=1.1$, $K=4.2\times10^5$	495:590
AODB	$n=1.2$, $K=2.6\times10^6$	470:530

Values in parenthesis from the literature (*a*) from ref. 12); *b*) from ref. 9).

TABLE II. Displacement of Probe from AGP by Drug

	Displacement (%)[a]					
	Acidic probe			Basic probe		
	WF	DNSL	AMCA	AO	QR	AODB
Acidic drug						
Sulfadimethoxine	14	12	11	—[b]	—[b]	—[b]
Phenylbutazone	21	15	—[b]	—[b]	—[b]	11
Acenocoumarin	45	32	19	24	41	13
Dicumarol	57	39	17	—[b]	—[b]	—[b]
Ibuprofen	0	0	0	0	0	0
Salicylic acid	0	0	0	0	0	0
Probenecid	0	0	0	0	0	0
Flurbiprofen	0	0	0	0	0	0
Digoxin	0	0	0	0	0	0
Basic drug						
Perphenazine	67	38	35	69	63	47
Chlorpromazine	78	69	54	85	68	62
Trifluoperazine	63	44	42	73	62	55
Diazepam	25	19	21	24	21	14
Propranolol	58	46	17	42	39	31
Pindolol	51	22	15	38	51	23
Imipramine	38	19	20	24	19	15
Desipramine	21	17	12	18	15	16
Diphenhydramine	54	49	38	52	43	36

The following concentrations were used; AGP, 3 μM; probe, 3 μM; drug 9 μM. *a*) The accuracy of displacement percentage is less than 2%. *b*) Drug causes slight increase in fluorescence of probe.

to AGP. Interestingly, these basic drugs also lowered the fluorescent intensities of acidic probe–AGP systems. The reduced fluorescence of WF–AGP in the presence of basic drugs can be explained on the basis of displacement, as expected from the drug–AGP interaction reported previously.[3] These results easily lead to the suggestion that the basic drugs share the binding site with acidic drugs.

On the other hand, the inhibition results by acidic drugs contradict this presumption. For example, four acidic drugs, including sulfadimethoxine, which bound to AGP significantly displaced only acidic probes; however, four other acidic drugs with a carboxylic group did not cause any displacement, depending upon the weak binding to AGP rather than binding to different sites. Moreover, the binding of basic probes to AGP was not inhibited by acidic drugs except for acenocoumarin. The degree of displacement of probe by acidic drugs was weaker than that of basic drugs expected from the results of drug–AGP interaction.[13] In contrast to basic drugs, the inhibition behaviors by acidic

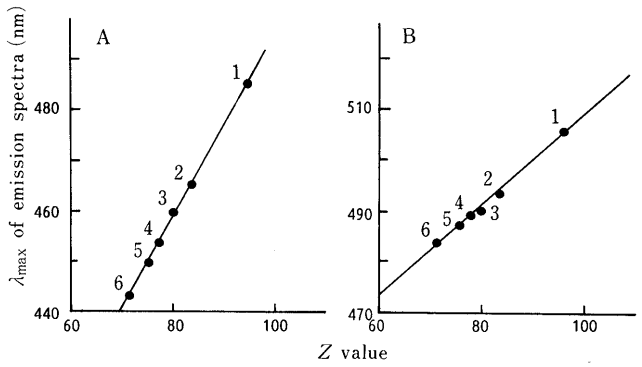

Fig. 1. Emission Maximum of AMCA(A) and AO(B) as a Function of Z Values

The concentrations of probes were 10 μM. Solvents were water (1), methanol (2), ethanol (3), *n*-butanol (4), isopropanol (5), acetonitrile (6). The correlation coefficients were 0.972 (A) and 0.941 (B).

TABLE III. Estimated Z Values of Binding Sites from Emission Maxima of Bound Probes

	Z value	
Acidic probe		
WF	—[a]	
DNSL	78	80
AMCA	81	
Basic probe		
AO	102	
QR	100	100
AODB	98	

a) Could not be estimated.

drugs suggest possibility of the presence of several drug binding sites.

Therefore, it is reasonable to consider from all displacement data that AGP has one wide binding area including some specific binding sites. So, attention was directed towards the nature of those binding sites.

Polarity of Binding Sites The solvent-dependent emission maximum wavelength (λ_{max}) of probe can be used to estimate the polarity of the binding site. Turner and Brand[14] estimated the Z value of 1-anilinonaphthalene-7- sulfonate binding sites for 20 proteins as a polarity scale. We also adopted Z value as a measure of polarity scale in this study.

Figure 1 shows the λ_{max} of AMCA and AO plotted as a function of Z values. There are good correlations between λ_{max} and the Z value in both systems. The Z values of the binding sites were estimated from plots with λ_{max} as listed in Table III. In the case of WF, we could not estimate the binding site polarity, because the shift of λ_{max} depending on solvent polarity was small. The Z values for the basic drug binding site were larger than those of the acidic drug binding site. These larger values may be due to ionic interaction of basic drugs with AGP, although the reason for this unexpected result is not yet fully understood. These limited data suggest that the hydrophobicity of the acidic drug binding site is higher than that of the basic drug binding site.

Optimal Size of Binding Sites Structure–activity relationships were investigated to define the structural requirements for drug binding sites on AGP. These approaches are based upon the idea that molecular interac-

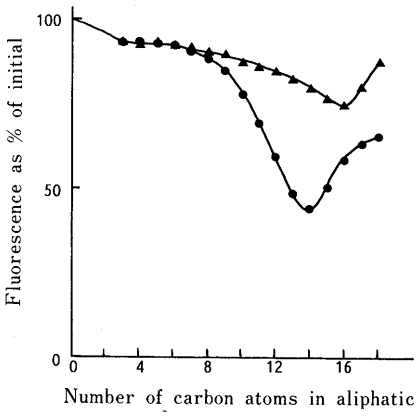

Fig. 2. Effect of Alkylamines on Fluorescence for WF and QR–AGP Interactions at 25 °C

▲, WF; ●, QR. [probe] = 3 μM, [AGP] = 3 μM, [alkylamine] = 6 μM.

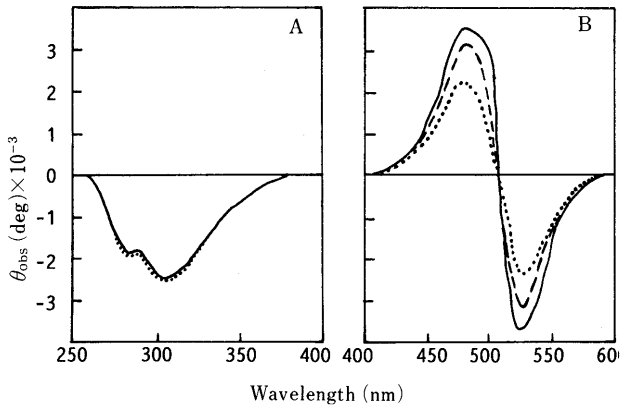

Fig. 3. Effect of CsCl on Induced Ellipticities for WF(A) and AODB(B)–AGP Interactions at 25 °C

CsCl concentration: ——; without CsCl, – – – –; 0.01 M, · · · ·; 1.0 M [probe] = 30 μM, [AGP] = 30 μM.

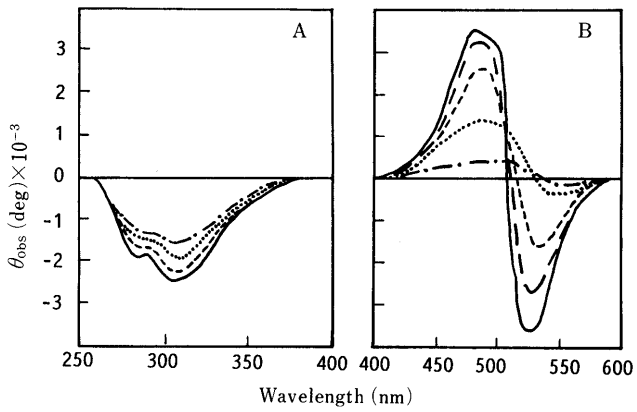

Fig. 4. Effect of Guanidine Hydrochloride on Induced Ellipticities for WF(A) and AODB(B)–AGP Interactions at 25 °C

Guanidine hydrochloride (GH) concentration: ——, without GH; – – – –. 0.5 M; – – – – –, 1.0 M · · · ·, 3.0 M; –·–·–, 4.5 M, [probe] = 30 μM, [AGP] = 30 μM.

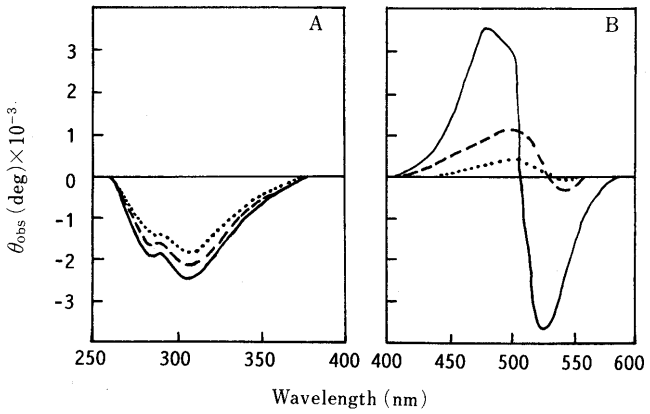

Fig. 5. Effect of KSCN on Induced Ellipticities for WF(A) and AODB(B)–AGP Interactions at 25 °C

KSCN concentration: ——, without KSCN; – – – –, 0.03 M; · · · ·, 3.0 M, [probe] = 30 μM, [AGP] = 30 μM.

tion is determined fundamentally by molecular size, shape and charge distribution.[15] Recently, the drug binding at site II on HSA and the dicumarol binding site on AGP were studied using these techniques.[16,17] In this work, the same displacement experiment was conducted using aliphatic amines which strongly displaced the basic as well as acidic probes.

Figure 2 shows the changes in the fluorescence of WF or QR bound to AGP with the addition of aliphatic amines. Aliphatic amine, C-12 to C-17 caused inhibition of WF and QR binding to AGP. Particularly, C-16 and C-14 amines exhibited the maximum inhibition of WF and QR–AGP systems, respectively. Similar inhibition results were obtaind for *p*-aminobenzoates. These differences of the inhibitory effects observed for WF and QR systems can be explained by the differences in size of the binding sites on AGP, although further investigation is necessary on this matter.

Effect of Neutral Salts on the Binding of Probes To study the localization of charge in the drug binding sites, the effects of CsCl and CsBr on the extrinsic Cotton effects of WF and AODB–AGP complexes were examined. As can be seen in Fig.3, the CD intensity of the AODB–AGP complex was decreased and the isobestic point at 507 nm was maintained in the presence of CsCl, while there was no change in WF–AGP system. Addition of CsBr gave the same results as CsCl, whereas NaCl did not change in induced ellipticities. We also confirmed from CD measurements that CsCl did not affect the conformation of AGP. Thus, the changes in the ellipticities of the basic probe–AGP complexes may be interpreted as displacement by Cs^+ from their binding sites. This result leads to the idea that the binding of basic drugs to AGP may involve electrostatic interaction. In fact, some investigators have proposed that part of the binding of basic drugs to AGP is due to the contribution of electrostatic interaction.[18,19]

It should be noted that the negative charges may distribute in or near the basic drug binding site, although we could not distinguish from the present limited data whether these charges arise from either peptide or sialic acids which were negatively charged at the terminal of glycan chains in AGP. Schley recently reported that the piperazine side chain may cause ionic interactions with the glutamic acids 177 and 178 in the AGP molecule.[1] The involvement of the charges at the drug binding site can be elucidated using desialylated AGP or chemically modified AGP. Investigation on this matter is currently under way.

Effect of Protein Denaturants on the Binding of Probes The effects of guanidine hydrochloride and KSCN, protein denaturants, on the induced Cotton effects of WF

and AODB–AGP complexes were examined. As shown in Figs. 4 and 5, the induced ellipticities of the two AGP complexes were decreased with increasing concentrations of both denaturants. Especially, the induced CD of AODB–AGP system markedly reduced with shift of λ_{max}. As judged from the CD data, these changes may be due to the reversible transition from a β conformation to a random structure of AGP.[20] Therefore, the changes on the induced CD of probe–AGP complexes may be explained on the basis of conformational change of AGP rather than displacement by denaturation. The results may indicate that the basic drug binding site is more sensitive to the conformational change of AGP than the acidic drug binding site.

In conclusion, there are particular drug binding sites for acidic and basic drugs having different characters on an AGP molecule. However, these sites are not completely separated, but rather may be significantly overlapped and may be influenced by each other. Accordingly, AGP seems to have a wide and flexible binding area.

Acknowledgement This work was supported in part by a Grant-in-Aid from the Takeda Science Foundation.

References

1) J. Schley, *J. Pharm. Pharmacol.*, **39**, 132 (1987).
2) F. Brunner and W. E. Müller, *J. Pharm. Pharmacol.*, **39**, 986 (1987).
3) S. Urien, E. Albengres, R. Zini and J. P. Tillement, *Biochem. Pharmacol.*, **31**, 3687 (1982).
4) M. Otagiri, T. Maruyama, T. Imai and Y. Imamura, *J. Pharm. Sci.*, **76**, 646 (1987).
5) M. Otagiri, R. Yamamichi, T. Miyoshi, T. Maruyama and J. H. Perrin, submitted.
6) C. F. Chignell, "Methods in Pharmacology," Vol. 2, Appleton-Century-Crofts, New York, 1972, p. 33.
7) G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, **11**, 824 (1975).
8) S. El-Gamal, U. Wollert and W. E. Müller, *J. Pharm. Sci.*, **72**, 204 (1983).
9) Y. Sugiyama, Y. Suzuki, Y. Sawada, S. Kawasaki, T. Beppu, T. Iga and M. Hanano, *Biochem. Pharmacol.*, **34**, 821 (1985).
10) G. Weber and L. B. Young, *J. Biol. Chem.*, **239**, 1415 (1964).
11) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
12) M. Otagiri, T. Maruyama, T. Imai, A. Suenaga and Y. Imamura, *J. Pharm. Pharmacol.*, **39**, 416 (1987).
13) J. M. H. Kremer, J. Wilting and L. H. M. Janssen, *Pharmacol. Rev.*, **40**, 1 (1988).
14) D. C. Turner and L. Brand, *Biochemistry*, **7**, 3381 (1968).
15) R. Ganellin, *J. Med. Chem.*, **24**, 913 (1981).
16) M. Otagiri, R. Yamamichi, T. Imai, Y. Imamura and A. Takadate, *Chem. Pharm. Bull.*, **36**, 4958 (1988).
17) S. Wanwimolruk, D. J. Birkett and P. M. Brooks, *Mol. Pharmacol.*, **24**, 458, (1983).
18) M. L. Friedman, K. T. Schlueter, T. L. Kirley and H. B. Halsall, *Biochem. J.*, **232**, 863 (1985).
19) M. Otagiri, R. Yamamichi, T. Maruyama, T. Imai, A. Suenaga, Y. Imamura and K. Kimachi, *Pharm. Res.*, **6**, 156 (1989).
20) K. Yamagami and K. Schmid, *J. Biol. Chem.*, **242**, 4176 (1967).