Characterization of Binding Sites for Sulfadimethoxine and Its Major Metabolite, *N*⁴-Acetylsulfadimethoxine, on Human and Rabbit Serum Albumin

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In order to gain an understanding of protein binding of sulfadimethoxine (SDM) and its major metabolite, *N*⁴-acetylsulfadimethoxine (*N*⁴-AcSDM), the binding of SDM and *N*⁴-AcSDM to human and rabbit serum albumin (HSA and RSA) was investigated using circular dichroism (CD), fluorescence and dialysis techniques. The CD spectral characteristics of the compounds bound to the albumins suggested that the drug-binding sites on the HSA and RSA had somewhat different asymmetries. The binding constants for SDM-HSA and -RSA interaction were smaller than those for *N*⁴-AcSDM. Two specific drug-binding sites were found on RSA, similarly to HSA, from the results of competitive displacement using fluorescence probes. Moreover, SDM and *N*⁴-AcSDM were found to share the same first binding site on the albumins. It can be presumed from the displacement data with a series of *p*-aminobenzoates that the characteristics of the binding sites (such as depth and width of the hydrophobic cleft) for SDM and *N*⁴-AcSDM on RSA may be almost the same, but the characteristics of these drug-binding sites on HSA may be somewhat different.

Keywords human serum albumin; rabbit serum albumin; circular dichroism; fluorescence; equilibrium dialysis; binding site; binding specificity

The binding of sulfa drugs to serum albumin has been studied by several investigators¹⁻³⁾ because of their high degree of binding to albumin. In previous papers,⁴⁻⁶⁾ the effect of phenylbutazone on the *in vivo* serum protein binding of sulfadimethoxine (SDM) was investigated in humans and some animals, including rabbits, and the interaction mechanism in rabbits was found to be different from those in humans and other animals. That is, in the rabbit, phenylbutazone indirectly reduced the *in vivo* serum protein binding of SDM through its displacing effect on *N*⁴-acetylsulfadimethoxine (*N*⁴-AcSDM), the major metabolite, although phenylbutazone did not reduce the *in vitro* binding of SDM to rabbit serum. On the other hand, phenylbutazone had little displacing effect on the *in vivo* and *in vitro* binding of SDM to human serum. Furthermore, *N*⁴-AcSDM hardly lowered the *in vitro* binding of SDM to human serum while *N*⁴-AcSDM markedly reduced it in rabbit serum. These SDM-serum protein binding features can be explained on the basis of albumin-SDM interaction, leading us to expect that SDM may share the same binding site with *N*⁴-AcSDM on rabbit serum albumin (RSA) while it may not do so in human serum albumin (HSA). It is thus of interest to determine whether or not SDM and *N*⁴-AcSDM do share a primary binding site on HSA and RSA. However, the drug binding sites on RSA have not been investigated, although the specific drug binding sites on HSA have been established as site I and site II, in other words, as the warfarin and benzodiazepine sites.⁷⁻¹⁰⁾ Thus, in order to gain a better understanding of the protein binding of SDM and its major metabolite, *N*⁴-AcSDM, in humans and rabbits, we attempted to define the characteristics of the drug binding sites on HSA and RSA using circular dichroism (CD), fluorescence and dialysis techniques.

Experimental

Materials HSA fraction V (lot No. 84F-9399) and RSA fraction V (lot No. 13F-9360) were obtained from Sigma Chemical Co. (St. Louis, MO). SDM was supplied by Daiichi Pharmaceutical Co. (Tokyo). *N*⁴-AcSDM was synthesized according to the method of Uno and Ueda.¹¹⁾

Phenylbutazone was supplied by Ciba-Geigy Co. (Summit, NJ). *p*-Aminobenzoic acid alkyl esters (methyl-decyl) were synthesized by the method of Kadaba *et al.*¹²⁾ and their structures were confirmed by elemental analysis, melting point determination and nuclear magnetic resonance (NMR). All other materials were of reagent grade and all albumin and drug solutions were prepared in deionized and distilled water. *p*-Aminobenzoic acid alkyl esters were dissolved in methanol prior to their addition to albumins. The methanol concentration was kept constant at 0.5% in all test solutions of albumins, even when *p*-aminobenzoic acid esters were absent. The associations of *p*-aminobenzoic acid alkyl esters were negligible under these experimental conditions because the calibration curves for *p*-aminobenzoic acid alkyl esters follow Beer's law over the concentration ranges (0.1×10^{-5} — 1.0×10^{-5} M) used.

Apparatus and Methods The CD measurements were made on a Jasco J-50A recording spectropolarimeter (Tokyo) using 10 mm cells at 25 °C. The induced ellipticity is defined as the ellipticity of the drug-albumin mixture minus the ellipticity of the albumin alone at the same wavelength and is expressed in degrees. Fluorescence measurements were made using a Hitachi 650-60 fluorescence spectrophotometer (Tokyo). The excitation wavelength was 340—360 nm, and emission was read at 390—490 nm.

Equilibrium Dialysis Measurements Dialysis experiments were performed at pH 7.4 and 25 °C using a Sanko Plastic dialysis cell (Fukuoka). HSA solution (2 ml) containing SDM or *N*⁴-AcSDM was poured into one compartment and 2 ml of buffer solution into the other. Adsorption of the drug on the membrane was negligible. After 12 h of dialysis, the free concentration of the drug was assayed by high performance liquid chromatography (HPLC). SDM and *N*⁴-AcSDM concentrations used ranged from 0.6×10^{-5} M to 36×10^{-5} M and the HSA concentration was 6×10^{-5} M.

Results and Discussion

Figure 1 shows the CD spectra of SDM and *N*⁴-AcSDM bound to HSA and RSA. Each drug seemed to experience a different asymmetric environment on the albumins, having unique CD characteristics. That is, SDM showed a positive peak around 248 nm and a negative peak around 271 nm, on binding to HSA or RSA. The two peaks in the induced CD curves probably result from excitation within both the sulfanilamide and diazine portions of the SDM. On the other hand, *N*⁴-AcSDM showed a positive peak around 257 nm and a positive shoulder around 275 nm. According to the symmetry rule,¹³⁾ the sign of the induced Cotton effect is governed by the configuration of the asymmetric center and the spatial relationship to the perturbed chro-

Fig. 1. CD Spectra of SDM and N^4 -AcSDM in the Presence of HSA and RSA at pH 7.4 and 25°C

[albumin]=[drug] = 1.5×10^{-5} M. —, SDM; ----, N^4 -AcSDM.

TABLE I. Binding Parameters of SDM and N^4 -AcSDM to HSA and RSA at pH 7.4 and 25°C Obtained by CD Method

	n_1	K_1 ($M^{-1} \times 10^{-5}$)	nK (SDM)/ nK (N^4 -AcSDM)
HSA			0.66
SDM	1.2	3.0	
N^4 -AcSDM	0.7	7.8	
RSA			0.69
SDM	0.9	6.0	
N^4 -AcSDM	0.7	11.2	

TABLE II. Binding Parameters of SDM and N^4 -AcSDM to HSA and RSA at pH 7.4 and 25°C Obtained by Dialysis Equilibrium Method

	n_1	K_1 ($M^{-1} \times 10^{-5}$)	n_2	K_2 ($M^{-1} \times 10^{-4}$)
HSA				
SDM	1.3	0.9	1.9	0.6
N^4 -AcSDM	0.7	3.4	2.3	1.4
RSA				
SDM	1.2	1.8	2.1	1.6
N^4 -AcSDM	1.0	6.0	3.0	5.0

mophore. The differences in the spectra of SDM and N^4 -AcSDM bound to the albumins suggest that the binding sites of the two drugs on the albumins are somewhat different.

These CD signals were large enough to be quantitatively investigated. The binding parameters were calculated by the method of Rosen.¹⁴⁾ As shown in Table I, the binding constants of N^4 -AcSDM to HSA and RSA are larger than those of SDM. The binding constants for N^4 -AcSDM obtained by equilibrium dialysis were also large compared with those for SDM. However, the Scatchard plots obtained by the dialysis method showed curvature and so the data were analyzed assuming two independent classes of binding sites with a nonlinear least-squares curve-fitting procedure.¹⁵⁾ The primary binding constants obtained from the CD data for all systems are in reasonable agreement with those obtained by dialysis (Table II), suggesting that the CD technique detects only the primary binding sites which generate the induced ellipticities. This clearly indicates that the CD method may be used to simply estimate the characteristics of the high-affinity drug-binding sites on the albumins.

It is well-known that some specific drug-binding sites are present on HSA, site I and site II, which are also called the warfarin site and the benzodiazepine site, respectively.⁷⁻¹⁰⁾

TABLE III. Binding Parameters for Probe-Protein Systems at pH 7.4 and 25°C

Probe	Protein					
	HSA			RSA		
	n	K (M^{-1})	Binding ratio ^{a)}	n	K (M^{-1})	Binding ratio ^{a)}
Warfarin	1.0	2.0×10^5	1:1	1.2	9.5×10^4	1:1
Dansyl-L-proline	1.4	1.4×10^5	1:1	1.4	7.8×10^4	1:1

a) These values were obtained from Job's plots.

TABLE IV. Displacement of Probes from RSA by Drugs

Drug	Probe	
	Warfarin	Dansyl-L-proline
Phenylbutazone	+++	0
Acenocoumarin	+++	0
Chlorothiazide	+++	0
Ibuprofen	+	+++
Ketoprofen	+	+++
Fenbufen	+	+++

+, ++ and +++ mean the degree of displacement of the probes (10% < + < 30%, 30% < ++ < 60%, 60% < +++). RSA (4×10^{-6} M), probe (4×10^{-6} M) and drug (1.2×10^{-5} M) were used throughout the investigation. Data are the average of four experiments.

However, few observations are available about the drug-binding sites on other albumins including RSA. So, the fluorescence technique was also used in the current work according to the method of Sudlow *et al.*,⁷⁾ in order to find out whether RSA has specific sites for drug binding, like HSA, or not. Warfarin and dansyl-L-proline, the probes studied here, are referred to as site I and site II drugs, respectively. As shown in Table III, the two probes were each bound to a single site on RSA, suggesting that these probes are useful for monitoring the drug binding sites on the RSA. If probes having several high affinity sites were used for the displacement experiments, the data analysis would be complicated.

The results of Table IV indicate that the probes studied can be divided into two groups, as expected from the results obtained by Sudlow *et al.*⁷⁾ For example, warfarin was strongly displaced by phenylbutazone, acenocoumarin and chlorothiazide, whereas dansyl-L-proline was not significantly displaced by these drugs. In sharp contrast, dansyl-L-proline was strongly displaced by ibuprofen, ketoprofen and fenbufen, while warfarin showed small displacement by these drugs. This clearly suggests that two specific binding sites on RSA, such as site I and site II for HSA, are present, although a detailed study would still be desirable.

Thus, in order to identify the binding sites of SDM and N^4 -AcSDM on HSA and RSA, competitive displacements were carried out using the above fluorescent probes. As shown in Fig. 2, both SDM and N^4 -AcSDM significantly displaced warfarin. However, dansyl-L-proline was not initially displaced up to a drug-to-albumin ratio of 1.0, but then was displaced. HSA and RSA have two classes of binding sites for SDM and N^4 -AcSDM, that is, high and low affinity sites (Table II). Therefore, the initial plateau region observed for dansyl-L-proline in Fig. 2 can be

Fig. 2. SDM and N^4 -AcSDM-Induced Changes in Fluorescence of Probe Bound to HSA and RSA at pH 7.4 and 25°C

(a) SDM-HSA system. (b) SDM-RSA system. (c) N^4 -AcSDM-HSA system. (d) N^4 -AcSDM-RSA system. [albumin] = 6×10^{-6} M. [probe] = 6×10^{-6} M. ●, warfarin (site I marker); ○, dansyl-L-proline (site II marker).

Fig. 3. Effect of N^4 -AcSDM on Induced Ellipticity of SDM-Albumin Complex at pH 7.4 and 25°C

[albumin] = 1×10^{-5} M. [SDM] = 1×10^{-5} M. ○, HSA; △, RSA. Measurements were made at 300 nm.

explained as follows: SDM and N^4 -AcSDM were bound mainly to the site I on the albumins, and with increasing drug concentrations, the drugs were bound to site II. This suggests that SDM and N^4 -AcSDM share the first binding site, site I on HSA and RSA.

Moreover, this hypothesis is supported by the CD experiments shown in Fig. 3. In this case, although the CD spectra of SDM and N^4 -AcSDM with albumins almost overlap as indicated in Fig. 1, fortunately the N^4 -AcSDM-albumin complexes showed little induced ellipticities at wavelengths longer than 300 nm under these experimental conditions. As shown in Fig. 3, N^4 -AcSDM significantly displaced SDM from the two albumins, particularly from RSA, suggesting that the drug and its main metabolite do share the same binding site on HSA and RSA. The differences in displacement data observed for HSA and RSA can be explained on the basis of binding affinity and/or the structural requirements of the drug binding sites on the two albumins. If the difference in Fig. 3 depends upon the magnitude of the binding constants, displacement for the HSA system with the high ratio of binding affinity

Fig. 4. Effect of p -Aminobenzoates on Induced Ellipticities of Complexes of SDM and N^4 -AcSDM with Albumin at pH 7.4 and 25°C

[albumin] = 1×10^{-5} M. [SDM] = [N^4 -AcSDM] = 1×10^{-5} M. [p -aminobenzoate] = 1×10^{-5} M. ○, SDM; ●, N^4 -AcSDM.

($nK(\text{SDM})/nK(N^4\text{-AcSDM})$) should be large, compared with that for RSA. However, the results are the opposite. It therefore seems to be reasonable to explain the results on the basis of the specificity of the binding sites including depth and width of binding areas, rather than binding affinities.

So, in order to determine the specificity (width, depth and hydrophobicity of the binding site clefts) of binding to the high-affinity sites of SDM and N^4 -AcSDM, a displacement study was conducted, using a series of p -aminobenzoates, probes for the warfarin site found recently at these laboratories.¹⁶⁾ Figure 4 shows the changes in the induced ellipticities of the complexes of SDM and N^4 -AcSDM with albumins upon the addition of p -aminobenzoates at a 1:1 ratio of the displacers to albumins. Change in the ellipticity of the complex provided a measure of displacement percentage at the site. The displacement percentage rose with increasing chain length of the alkyl group, and then reached a maximum at the heptyl group for the SDM-HSA system and the octyl group for N^4 -AcSDM. In the case of RSA, the maximum inhibitory effect was observed at p -aminohexylbenzoate. The displacement percentage observed for SDM are bigger than those for N^4 -AcSDM, depending upon the magnitude of the binding constants. The small differences in the maximum inhibitory effects of SDM and N^4 -AcSDM may reflect the specificity of the drug-binding sites on the HSA and RSA. That is, the characteristics of the binding sites for SDM and N^4 -AcSDM on RSA may be almost the same, but the binding site clefts for SDM and N^4 -AcSDM on HSA are somewhat different. Further characterization of these drug-binding sites is currently under way, using peptic fragments of HSA and RSA.

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