Interaction Between Two Dicarboxylate Endogenous Substances, Bilirubin and an Uremic Toxin, 3-Carboxy-4-Methyl-5-Propyl-2-Furanpropanoic Acid, on Human Serum Albumin

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Purpose. Two dicarboxylate endogenous substances, bilirubin (BR) and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), have a very high affinity to human serum albumin (HSA). This study was undertaken to clarify the existence of a dicarboxylate binding site on HSA.

Methods. Chemical modification, pH dependent binding and X-ray crystallographic analysis were performed to characterize these dicarboxylate binding sites.

Results. It was found the binding behavior for dicarboxylates was different from typical site I ligands such as warfarin (WF) and phenylbutazone (PB) and that electrostatic interaction was an important factor for their binding to HSA. Moreover, His residues were considered to play an important role in pH dependent binding of dicarboxylic acids but in a different manner from the site I ligands. X-ray crystallography of CMPF and BR revealed the distances between the two carboxyl groups in their chemical structures were 5.854 Å and 9.979 Å, respectively. This difference may be reflected in pH dependent binding. Using fluorescent probe displacement, we attempted to identify the binding site for monocarboxylate derivatives of CMPF and investigated the role of individual carboxyl group in the recognition of the binding site. The results suggested two carboxyl groups were important for the specific binding of CMPF to site I.

Conclusions. The binding site for dicarboxylic acids is located in subdomain IIA, which includes site I, on the HSA molecule. Electrostatic interaction is an important driving force for binding to HSA.

KEY WORDS: human serum albumin; uremic toxin; bilirubin; dicarboxylate; electrostatic interaction.

INTRODUCTION

In renal disease state, it is well known endogenous substances, generally called "uremic toxins" accumulate in plasma. Indoxyl sulfate, indole-3-acetic acid, hippuric acid, and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) have been determined as uremic toxins (1). Among these compounds, CMPF is thought to deserve the most attention, because it is not removed by hemodialysis and its concentrations remain high for at least 90 days even after a successful kidney transplantation (2). Its plasma concentration reaches about 400 μM (3) in disease state. In addition, it has very high affinity ($K = 1.3 \times 10^7\ M^{-1}$, $n = 1$) to human serum albumin (HSA) (3) and is largely responsible for the reduced binding of many drugs (especially site I drugs, such as warfarin or furosemide) in uremic serum. The urinary excretion of CMPF by a normal male was reported to be about 2 mg/day (4). CMPF has also been implicated in anemia, neurological symptoms (5), alteration of thyroid function (6), and is also likely to inhibit active tubular secretion (7).

Bilirubin (BR), the amphiphilic and cytotoxic yellow-orange pigment that causes jaundice, is an unsymmetrically-substituted tetrapyrrole dicarboxylic acid found characteristically in mammals. It is produced in copious quantities by catabolism of heme, principally from the hemoglobin of red blood cells. The pigment forms a tightly bound association complex with HSA ($K = 5.5 \times 10^7\ M^{-1}$, $n = 1$) (8) and inhibits the binding of warfarin (WF, site I ligand) but not for benzodiazepines (site II ligands) (9). Normal human metabolism generates 300 mg/day/individual of bilirubin through the daily breakdown of about 10^{11} red blood cell. Impaired secretion of bilirubin may indicate *inter alia*, pathologic liver conditions, e.g., cirrhosis or hepatitis, blockage of the common bile duct (as in gallstones), or bilirubin glucuronyl transferase insufficiency (as in neonatal jaundice).

It should be noted both CMPF and BR have a very high affinity for HSA and inhibit the binding of site I ligands. So far, site I recognized only bulky ligands with or without one dissociable group such as warfarin and phenylbutazone. However, if this specific binding site could recognize the dicarboxylate, the hydrophilic crevice in this subdomain might contain at least two cationic sites, which contributed largely to the recognition of CMPF and BR, the dicarboxylate endogenous substances. In fact, suramin, a drug used in the treatment of human trypanosomiasis, which is a polyanion with six sulfonic acid groups, also binds to subdomain IIA(10).

In this report, we investigated the effects of chemical modification of HSA and pH alteration binding of these two compounds, and compared with those of typical site I ligands, WF and phenylbutazone (PB) to prove whether or not subdomain IIA recognizes the dicarboxylate. In addition, we synthesized the monocarboxylate derivatives of CMPF and investigated the role of each carboxyl group of CMPF on the binding to HSA.

MATERIALS AND METHODS

Materials

WF was a gift from the Eisai Co. (Tokyo, Japan). PB was obtained from Nacalai Tesque Inc. (Kyoto, Japan). BR and dansylsarcosine (DNSS) were purchased from Sigma Chemical Co. (St. Louis, Missouri). HSA was a gift from the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted with activated charcoal in solution at 0°C, acidified with H_2SO_4 to pH 3 and then freeze-dried (11). The molecular mass of HSA was assumed to be 66500 Da. HSA gave only

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one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. CMPF was synthesized according to the method of Costigan *et al.* (12) and the structure of the compound was assigned on the basis of IR, NMR, and mass spectral data analysis. The purity of the compound was determined by HPLC. Using the method of Pfordt *et al.* (13), 3-methoxycarbonyl-4-methyl-5-(1-propenyl)-2-propylfuran (1a) and 3-ethoxycarbonyl-4-methyl-5-(1-propenyl)-2-(2-ethoxycarbonyl)ethylfuran (1b) were synthesized. 3-Carboxy-2,5-dipropyl-4-methylfuran (A) was synthesized utilizing 1a as follows. Namely, hydrogenation of 1a with 10% Pd-C in ethanol followed by saponification gave 2a (A) quantitatively ⟨IR; 2962, 1674, 1568 cm^{-1}, ^{1}H-NMR (d, $CDCl_3$); 0.91 (3H, t, J = 7.3 Hz), 1.54–1.76 (4H, m), 2.07 (3H, s), 2.51 (2H, t, J = 7.3 Hz), 2.94 (2H, t, J = 7.3Hz), MS (EI); 210 (M^+)⟩. On the other hand, 2-(2-carboxy)ethyl-3,4-dimethyl-5-propylfuran (B) was utilizing 1b as follows. Hydrogenation of 1b followed by saponification gave 3-ethoxycarbonyl-2-(2-carboxy)ethyl-4-methyl-5-propylfuran (2b) in 80% yield. Reduction of 2b with diisobutylaluminum hydride gave 2-(2-carboxy)ethyl-3-hydroxymethyl-4-methyl-5-propylfuran (3b) in 93% yield. Finally, the treatment of 3b with TMSI in acetonitrile afforded 3c (B) in 80% yield ⟨IR; 2962, 1713, 1599 cm^{-1}, ^{1}H-NMR (d, $CDCl_3$); 0.90 (3H, t, J = 7.3Hz), 1.51–1.64 (2H,m), 1.83 (3H, s), 2.46 (2H, t, J = 7.3 Hz), 2.64 (2H, t, J = 6.9 Hz), 2.85 (2H, t, J = 6.9 Hz), MS (EI); 210 (M^+)⟩. All other chemicals were of analytical grade. All the buffers used were prepared with sodium phosphate dibasic and phosphate monobasic salts.

Determination of Free Concentration of WF, PB, and CMPF

The unbound concentrations of WF, PB, and CMPF were estimated by ultrafiltration. The filtrate was injected onto a LiChrosorb RP-18 column. The mobile phase consisted of 0.2 M sodium acetate buffer (pH 4.5)/acetonitrile (35:65) for WF, 30:70 for PB and flow rate was 1 mL/min. The fluorescence detector for WF was set at 300 nm for excitation and 400 nm for emission. The UV detector for PB was set at 270 nm. The unbound CMPF concentration was estimated by the previously established HPLC method (3). Calibration correlating concentrations to peak area ratios were performed daily to avoid interday variation.

Peroxidase Method

The unbound fraction of BR was estimated by peroxidase method (14). 3 mL of BR-albumin solution was placed in a 1 cm cell in the spectrophotometer with thermostatic cell holder at 25°C. 5 μL of freshly diluted hydrogen peroxide 35 mM was added and the light absorption was recorded as a function of time at constant wavelength, 455 nm, for 3 min. Peroxidase solution, 100 μL in water, was added. A final peroxidase concentration was chosen so $t_{0.2}$, the time taken for oxidation of the initial 0.2 of the total amount of BR, was longer than 2 min to keep within the range where the velocity is proportional to the enzyme concentration, and shorter than 10 min, for stability. The relative free fraction was calculated from the following equation:

$$\text{relative free fraction } (\%) = \frac{C_{f,m}}{C_{f,n}} \times 100 = \frac{t_{0.2,n}}{t_{0.2,m}} \times 100 \quad (1)$$

where $C_{f,n}$ and $C_{f,m}$ represent the free concentration of BR in the presence of native and modified HSA, respectively. $t_{0.2,n}$ and $t_{0.2,m}$ represent $t_{0.2}$ for native and modified HSA, respectively.

Fluorescence Quenching Titration

To estimate the binding of BR in different pH, we used fluorescence quenching titration (15). 5 or 10 μL of the BR solution was added to the albumin solution (3 mL). In most cases the region of interest was within the BR/HSA molar ratio of 0 to 1.0. At least 20 points were obtained in the region of interest. The solution was mixed by inversion three times. Fluorescence was then measured. At the end of the titration, the absorption of the titration solution was determined at 294 nm and 343 nm. These readings were used for the inner filter effect correction. The correction for the inner filter effect and data analysis were performed according to the procedures of Levine (15).

Fluorescence Probe Displacement

To identify the binding sites of CMPF monocarboxylate derivatives, we used fluorescent probe displacement (3). The intrinsic fluorescence of HSA was obtained at 290 nm. Within the limits of sensitivity, an excitation wavelength was chosen to ensure the least possible absorption of incidental light. Emission wavelengths were chosen to give the maximum fluorescence of the probe bound to HSA, with insignificant fluorescence in the buffer. The probes having either a single binding site or two sites, with widely separated dissociation constants were chosen. We used WF and DNSS as the site I and II probe, respectively. The probe to HSA ratio was kept at 1:10 in order to minimize the nonspecific binding of probes. The percentage of probe displacement was determined according to the method of Sudlow *et al.* (16):

$$\frac{F_1 - F_2}{F_1} \times 100 \quad (2)$$

where F_1 and F_2 represent the fluorescence of the probe plus HSA, with and without the drug, respectively.

Preparation of HSA Derivatives

Trp-Modified HSA (HNB-HSA)

The single Trp residue of HSA was modified by 2-hydroxy-5-nitrobenzyl bromide (HNB) following the procedure of Fehske *et al.* (17). 500 mg HSA was dissolved in 100 mL of 10 M urea adjusted to pH 4.4 by acetic acid. 1.0 mL of methanol was added to 8.0 mL of this solution. HNB was added (1100 fold molar excess of HSA) and the reaction mixture was shaken occasionally. After 2 h, the insoluble hydrolyzed reagent was separated by centrifugation. The supernatant was dialyzed against water for 60 h and then lyophilized. The degree of

modification was determined using ultraviolet absorption, as shown below.

$$\text{modified (\%)} = \frac{A_{410} \times 66500 \times 0.498}{13800\,(A_{280} - 0.167 \times A_{410})} \quad (3)$$

Tyr-Modified HSA (TNM-HSA)

The Tyr residues modification was performed at room temperature according to the procedure of Fehske *et al.* (18). HSA was dissolved in 0.05 M Tris buffer adjusted to pH 8.0. 10 molar excess tetranitromethane (TNM) dissolved in ethanol were added to the HSA solution. The degree of modification was calculated from the following equation:

$$\text{modified (\%)} = \frac{A_{428}}{4100 \times c \times m} \times 100 \quad (4)$$

where c is the protein concentration and m is the number of Tyr residues on HSA molecule. Out of the 18 Tyr residues of HSA, only 3 were modified.

Lys-Modified HSA (SA-HSA)

Succinic anhydride (SA) catalyzed succinylation of HSA was carried out according to the procedure of Gounaris and Perlmann (19). To the mixture of HSA (500 mg) and 20 mL of 0.1 M sodium chloride solution adjusted to pH 8.0 with 0.5 M $NaHCO_3$, SA (5 mg) was slowly added at 15°C and the pH was maintained between 7.5 and 8.5 by 1 N NaOH. 30 min after the last addition of SA, the reaction mixture was passed through a Sephadex G-25 column. Protein fractions were collected and dialyzed against water for 60 h and then lyophilized. The unreacted Lys residues were determined by trinitrobenzene sulfonic acid procedure of Haynes *et al.* (20). Out of 59 Lys residues, only 5 were modified.

His-Modified HSA (DEP-HSA)

The His residues were modified by diethylpyrocarbonate (DEP) according to the procedure of Roosemont *et al.* (21). 500 mg of HSA was dissolved in 50 mL acetate buffer (pH 6.5, 100 mM), then DEP in ethanol was added to the HSA solution. The ratio of DEP to HSA was 23. The mixture was stirred for 20 min at room temperature and dialyzed against water and lyophilized. The number of His residues modified was calculated using the following equation:

$$\text{modified (\%)} = \frac{A_{240} \times \dfrac{3\text{ mL}}{\text{mL of test solution}}}{\Delta\varepsilon \times c \times m} \times 100 \quad (5)$$

where $\Delta\varepsilon$ is the differential molar absorptivity for His at pH 6.0, c is the protein concentration and m is the number of His residues on HSA. Only 3 His residues out of 18 were modified.

Secondary structures of all modified HSAs estimated by CD were not significantly different from that of native HSA.

X-Ray Structural Determination of CMPF

A yellow prismatic crystal of $C_{12}H_{16}O_5$ having approximate dimensions of 0.20 × 0.20 × 0.30 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Mo-Kα radiation and a 12 kW rotating anode generator. Cell constants and an orientation matrix for data collection were obtained from a least-squares refinement using the setting angles of 25 carefully centered reflections in the range $20.16 < 2\theta < 23.83°$ corresponded to a primitive monoclinic cell with dimensions: a = 4.505 (5) Å; b = 15.925 (4) Å; c = 16.982 (4) Å; β = 92.04 (6)°; Volume = 1217 (1) $Å^3$. For Z = 4 and F.W. = 240.26, the calculated density is 1.31 g/cm^3. The systematic absence of

$$h01{:}l \neq 2n$$

$$0k0{:}k \neq 2n$$

uniquely determine the space group to be:

$$P21/c\ (\#14).$$

The data were collected at a temperature of 20 ± 1°C using the ω-2θ scan technique to a maximum 2θ value of 55.0°. Omega scans of several intense reflections made before data collection, had an average width at half-height of 0.24° with a take-off angle of 6.0°. Scans of (1.00 + 0.30 tan θ)° were made at a speed of 16.0°/min (in omega). The weak reflections ($I < 10.0\sigma(I)$) were rescanned (maximum of 3 scans) and the counts were accumulated to ensure good counting stastitics. Stationary background counts were recorded on each side of the reflection. The ratio of peak counting time to background counting time was 2:1. The diameter of the incident beam collimator was 1.0 mm, the crystal to detector distance was 235 mm, and the computer controlled detector aperture was set to 9.0 × 13.0 mm (horizontal × vertical).

RESULTS

Interaction Between CMPF and BR

Sakai *et al.* (3) showed that CMPF competitively inhibits the binding of WF to HSA. These results suggest CMPF binds to site I (in subdomain IIA) on HSA. On the other hand, BR also inhibits the binding of the ligands bound to site I, such as WF and PB, but it does not inhibit the binding of L-tryptophane and benzodiazepines that bind to site II (9). CMPF and BR have also much higher affinity to HSA than the ligands bound to site I (Table 1). Therefore, to confirm whether CMPF and BR share the same binding site on HSA, we studied the interaction between CMPF and BR. Klotz plot analysis indicated that CMPF inhibited the binding of BR competitively (Fig. 2).

Effects of the Chemical Modification of HSA on the Binding of Site I Ligands and Dicarboxylic Acids.

CMPF and BR have a very high affinity to HSA. This high affinity is thought to be derived from not only "the hydrophobic

Table 1. Binding Parameters of WF, PB, CMPF, and BR

	n	K (× 10^5 M^{-1})
WF	1.0	3.5
PB	1.0	2.5
CMPF	1.0	130
BR	1.0	550

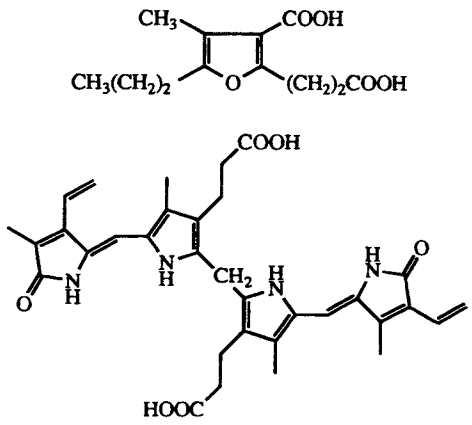

Fig. 1. Chemical structures of CMPF and BR.

interaction" but also largely "the electrostatic interaction" between these ligands and HSA. Both CMPF and BR have two carboxyl groups in their structures, thus, they are named as "dicarboxylic acids". It is possible these carboxyl groups contribute to the high affinity of CMPF and BR to HSA. To prove this hypothesis, we compared the binding of these two dicarboxylates and typical site I ligands, WF and PB with chemically modified HSA. Chemical modification of the lone Trp residue of HSA, 214-Trp, led to the decrease binding of WF but did not affect the binding of PB and dicarboxylic acids (Fig. 3, column B). The modification of Tyr residues decreased the binding of dicarboxylic acids significantly than those of site I ligands (Fig. 3, column C). Interestingly, the chemical modification of basic amino acid residues, such as Lys and His residues, did not affect the binding of site I ligands, but decreased the binding of dicarboxylate remarkably (Fig. 3, column D and E). These results clearly suggested electrostatic interaction is important for the binding of dicarboxylate to HSA.

Effect of pH on the Binding of Site I Ligands and Dicarboxylate

HSA is known to undergo conformational change between pH 6 and 9, the N-B transition. This conformational change affects the microenvironment of the binding sites and then causes the change of binding of various ligands to HSA. Therefore, to investigate the binding microenvironments of site I ligands and dicarboxylates, we studied the effect of pH on the binding of both groups of ligands. The bindings of WF and PB increase while those of CMPF and BR decrease with a rising pH (Fig. 4). These results indicated the microenvironment of the binding region of dicarboxylate is different from that of site I ligands.

It is generally considered that His residues play an important role in the N-B transition. We investigated the effects of the chemical modification of His residues on the bindings of site I ligands and dicarboxylates in various pH. As shown in Fig. 5, interestingly, chemical modification of His gave little pH effect on WF binding whereas the decrease CMPF binding with increasing pH was enhanced. Further, pH independence of WF binding for DEP-HSA system was also confirmed by the fluorescence characteristics of HSA-WF complex (data not shown).

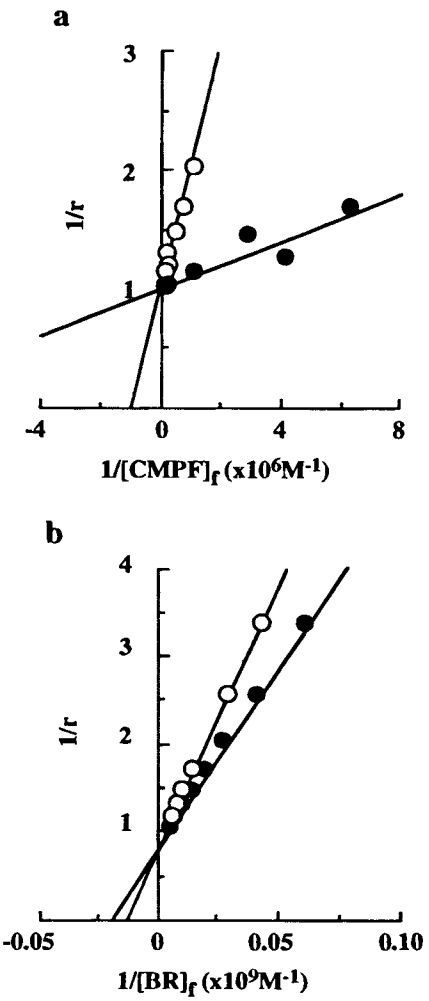

Fig. 2. CMPF (a) and BR (b) binding to HSA in the presence of another ligand at pH 7.4 and 25°C. (a) Binding of CMPF to HSA (200 μM) in the absence (●) and presence (○) of BR (400 μM). (b) Binding of BR to HSA (30 μM) in the absence (●) and presence (○) of CMPF (60 μM).

Distance Between Two Carboxyl Groups of CMPF and BR

As mentioned above, the electrostatic interaction between Lys and His residues of HSA and the carboxyl groups of dicarboxylate might contribute to their binding. In an electrostatic interaction, the distance or/and orientation between basic amino acid residues and carboxyl groups is important. Thus, we examined the distances between the two carboxyl groups of CMPF and BR using x-ray crystallography. As shown in Fig. 6, the conformation of BR is "ridge-tile form" while the distance between the carbon atoms of the carboxyl groups is 9.979 Å and that of CMPF is 5.854 Å.

Role of the Two Carboxyl Groups on the Binding of CMPF to HSA

The chemical modification study suggested the carboxyl groups of dicarboxylate interact with basic amino acid residues of HSA electrostatically. Here, we investigated the binding of

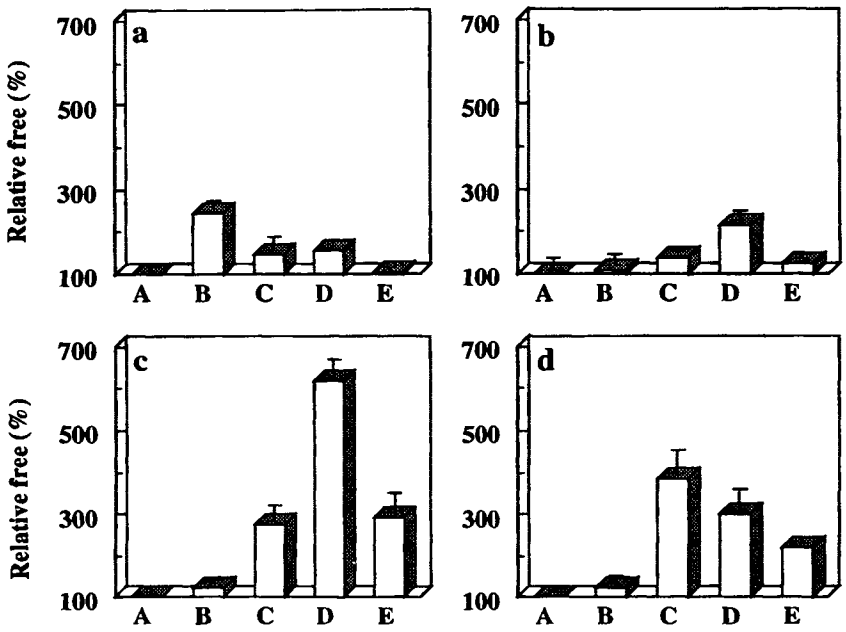

Fig. 3. Binding of WF (a), PB (b), CMPF (c), and BR (d) to native and modified HSA at pH 7.4 and 25°C. A: native HSA, B: HNB-HSA, C: TNM-HSA, D: SA-HSA, E: DEP-HSA. The following concentrations were used; [HSAs] = 30 μM, [ligands] = 30 μM.

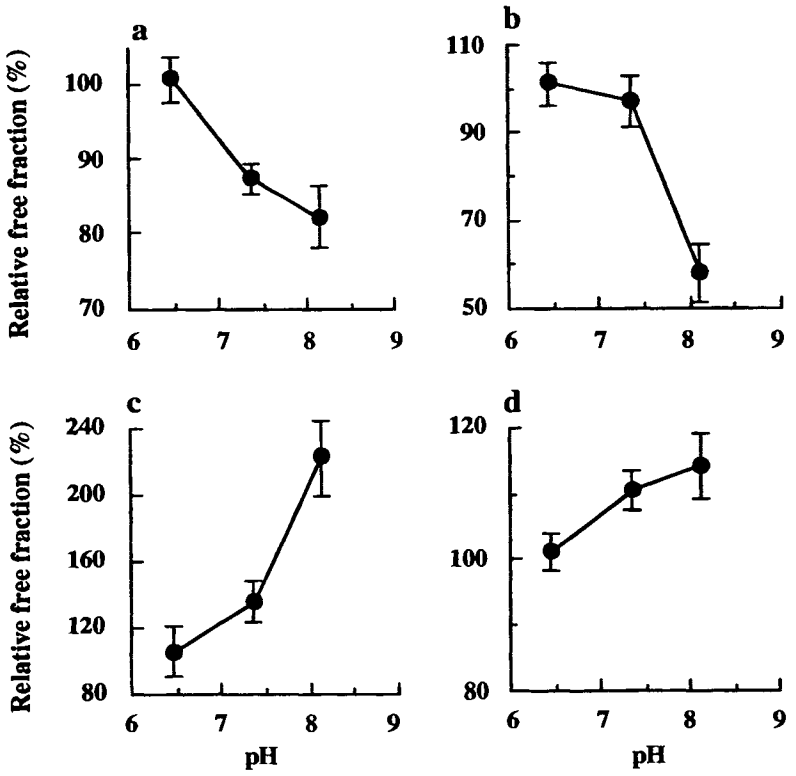

Fig. 4. Effect of pH on the binding of WF (a), PB (b), CMPF (c) and BR (d) at 25°C. The following concentrations were used; [HSA] = 30 μM, [ligands] = 30 μM.

the monocarboxylated CMPF derivatives, 4-methyl-2,5-dipropyl-3-furancarboxylic acid (CMPF2-CH_3) and 3,4-dimethyl-5-propyl-2-furanpropanoic acid (CMPF3-CH_3) to HSA using fluorescence probe displacement in order to elucidate the role of the two carboxyl groups on the binding of CMPF to site I on HSA. WF and DNSS were used as the site I and the site II probes, respectively. CMPF displaced WF but not DNSS as reported previously (Fig. 7), so it binds to site I specifically.

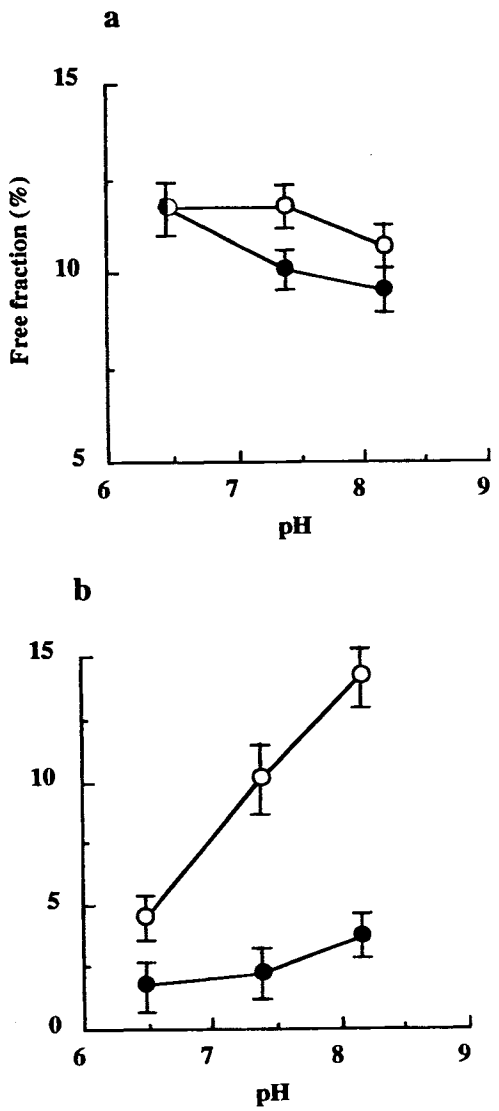

Fig. 5. Effects of pH on WF (a) and CMPF (b) bound to native (●) and DEP-HSA (○) at 25°C. The following concentrations were used; [HSA] = 30 μM, [ligands] = 30 μM.

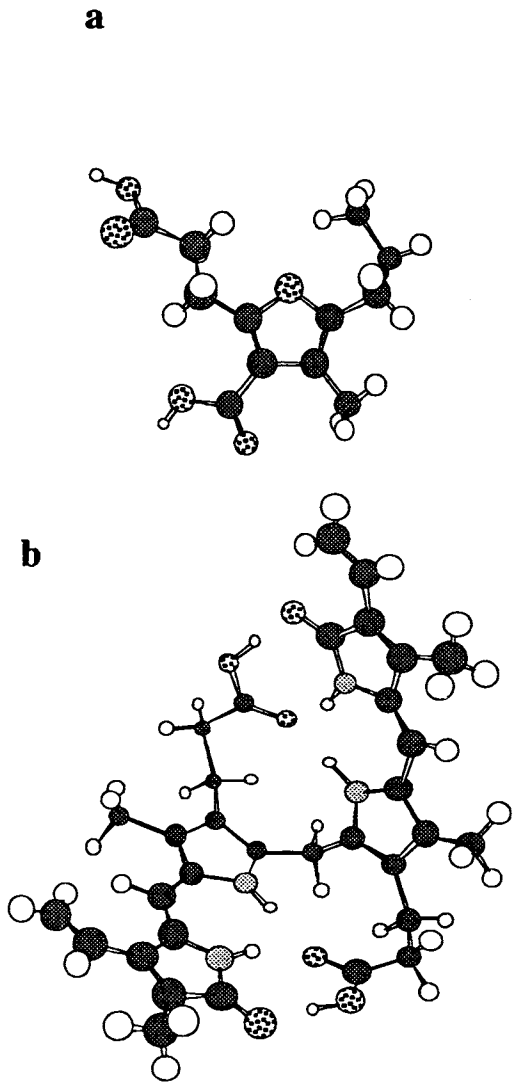

Fig. 6. Ball and stick conformational representations of CMPF (a) and BR (b).

Interestingly, CMPF2-CH_3 displaced both probes while CMPF3-CH_3 displaced only DNSS, the site II probe. And in preliminary experiment, we obtained the binding tendency of CMPF, CMPF2-CH_3, and CMPF3-CH_3 (CMPF2-CH_3 > CMPF > CMPF3-CH_3, unpublished data). These results indicated CMPF3-CH_3 binds to site II specifically and CMPF2-CH_3 binds to both site I and site II. It is therefore considered the two carboxyl groups of CMPF play an important role in the binding to site I specifically. In an ultracentrifugation study, similar results were obtained (data not shown).

DISCUSSION

Both CMPF and BR have very high affinity to HSA, in comparison to other dicarboxylates such as methotrexate and cromoglicate. In Fig. 2, Klotz plot analysis indicated CMPF and BR share the same common binding site on HSA. Sakai *et al.* (3) suggested CMPF inhibits the binding of WF competitively. BR also inhibits the binding of WF. Therefore, these endogenous substances are considered to bind to subdomain IIA where site I exists. Why do these endogenous substances have high affinity to HSA? It is no doubt the high affinity of these dicarboxylate to HSA is due to strong interaction between the two carboxyl groups as well as the bulky hydrophobic parts of the ligands with specific amino acid residues and hydrophobic crevice in HSA. First, we investigated the effect of chemical modification of HSA on the binding of site I ligands (WF and PB) and the dicarboxylates. When the aromatic residues, such as Trp or Tyr were modified, the binding of WF decreased with Trp modification and those of dicarboxylate also decreased with Tyr modification. When the basic amino acid residues, such as Lys or His were modified, only the binding of the dicarboxylates decreased. Aki *et al.* (22) reported using isothermal titration microcalorimetry, hydrophobic interaction was shown to be very important for the binding of WF to HSA, characterized by a small negative molar enthalpy change and minor or positive values of molar change of entropy. On the other hand, Berde *et al.* (23) indicated from the result of temperature dependence of affinity of BR to HSA, the interaction between BR and HSA

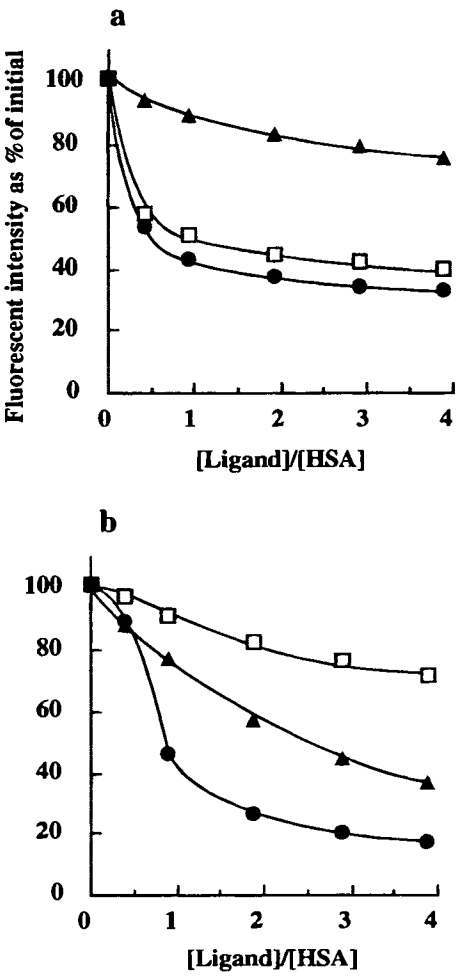

Fig. 7. Effects of furan compounds on the fluorescence intensities of WF (a) and DNSS (b) bound to HSA at pH 7.4 and 25°C. Furan compounds used in this experiment were CMPF (□), CMPF2-CH_3 (●) and CMPF3-CH_3 (▲). The following concentrations were used; [HSA] = 20 μM, [probes] = 10 μM.

was entropy-driven. Therefore, electrostatic interaction seems to be important.

It is also possible His residues are important for the binding of dicarboxylate. In N-B transition, the binding of dicarboxylate decreased when pH increased from 6.5 to 8.2. This change seemed to be caused by His residues because most of the pKa values of His residues are within this pH region. In pH 6.5, the His residues involved in dicarboxylate binding protonate, and then may interact favorably with the dicarboxylate anions. On the other hand, in pH 8.2, the His residues deprotonate and the interaction with dicarboxylate anion may become weaken. The pKa values for the carboxyl groups of CMPF are 3.2 and 3.6 (12), and those of BR are 4.2 and 4.9 (24). Thus, in this experimental condition, CMPF and BR exist essentially as dianion form. As His residues may also be associated with the conformational change in N-B transition, the microenvironmental change of the His residues may also affect the binding of dicarboxylate. This speculation was supported by the result of Fig. 5 using His-modified HSA (DEP-HSA). WF had almost the same affinity for DEP-HSA as native HSA at pH 6.5 but increase of binding to DEP-HSA was not observed, different from that for native HSA. This may be due to the degree of induced conformational change becoming smaller after modification of His residues. CMPF had lower affinity for DEP-HSA than native HSA, particularly in the B-form.

The effects of chemical modification and the degree of the pH dependent binding of CMPF and BR show almost the same tendency, but is slightly different. We then examined the configuration of CMPF and BR, especially the distance between two carboxyl groups. The distance between the carboxyl groups of CMPF is 5.854 Å and that of BR is 9.979 Å. From the results of chemical modification experiment and X-ray crystallography of the dicarboxylates, the amino acid residues involved in electrostatic interaction with the carboxyl groups seem to be 240-Lys (25) and 242-His (8). Modification of Lys causes the decrease of BR binding but the degree of decrease is smaller than that of CMPF. Also, recently, Curry *et al.* have determined the crystal structure of HSA complexed with five molecules of myristate at 2.5 Å resolution (26). 257-Arg was found to interact with the carboxylate of myristate bound to subdomain IIA. However, arginyl residues modification of HSA did not affect the binding of BR (27). These results suggest electrostatic interaction contributes to the binding of CMPF and BR, depending upon the distance between Lys or His residue and the carboxyl group of these dicarboxylates. The hydrophobic surface of BR is larger than that of CMPF, considering from the molecular structures of the two ligands. BR is an unsymmetrically-substituted tetrapyrrole dicarboxylic acid whereas CMPF is a furan dicarboxylic acid (Fig. 1). In chemical modification experiment, the effect of Tyr modification on the binding of BR is larger than of any other amino acid residue modification but that of CMPF is not. In addition, pH dependent binding of BR is much less than that of CMPF. These results clearly suggest the hydrophobic interaction for binding of BR is larger than that of CMPF.

Using monocarboxylated CMPF derivatives, we investigate the role of two carboxyl groups on the binding of CMPF. CMPF3-CH_3 displaces DNSS, suggesting CMPF3-CH_3 binds to site II only. Ketoprofen, ibuprofen, and pranoprofen, the arylpropionate non-steroidal inflammatory drugs, have hydrophobic moiety and carboxyl group distal to the hydrophobic moiety, and bind to site II. CMPF3-CH_3 has the same structural properties, therefore is thought to bind to site II only. Interestingly, CMPF2-CH_3 displaces both WF and DNSS. Mefenamate and flufenamate, which have carboxyl group directly connected to the hydrophobic core, bind to site I and II (28). When the ratio of CMPF2-CH_3 concentration to HSA concentration is less than 1, CMPF2-CH_3 displaces WF preferentially than DNSS. From the fact that WF and DNSS have almost the same affinity for HSA (WF:3.5 × 10^5 M^{-1}, DNSS:8.3 × 10^5 M^{-1}), this results suggests CMPF2-CH_3 binds to site I preferentially. Jacobsen *et al.* (29) reported two molecules of xanthobilirubic acid, which has dipyrrolic structure, bind to the same binding site for BR and this report suggested two carboxyl groups are important in the binding of BR to HSA.

Meredith *et al.* (30) reported the binding sites for dicarboxylate on bovine serum albumin. In their report, they used long chain (LCDCAs) and medium chain dicarboxylic acids (MCDCAs), formed after ω-oxidation of long chain and medium chain fatty acids, respectively, as dicarboxylate. While LCDCAs bind to subdomain IIC, IIIAB and IIAB with high affinity, MCDCAs bind to subdomain IIAB with low affinity.

Subdomain IIAB includes site I, so LCDCAs and MCDCAs bind to site I.

In conclusion, the present study indicated CMPF, a uremic toxins, inhibits the serum protein binding of BR and the binding site for dicarboxylate exists in site I on HSA molecule. This finding suggests in renal failure treatment, it may be possible that drugs which possess two carboxyl groups and proper hydrophobicity including methotrexate compete with CMPF in albumin binding and progress the uremic syndrome by increasing the free concentration of CMPF.

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