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Esterase-like Activity of Human Serum Albumin. VII.[1] Reaction with *p*-Nitrophenyl 4-Guanidinobenzoate

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The reaction of *p*-nitrophenyl 4-guanidinobenzoate (NPGB) with human serum albumin (HSA) was examined kinetically at various pH's and 25 °C. The Michaelis constant (K_s in M) and the catalytic rate constant (k_2 in s^{-1}) were determined. The ratio of k_2 to k_0 (hydrolysis rate constant of NPGB in s^{-1}) at pH 7.4 was 75.6, indicating the esterase-like activity of HSA. The effects of the reversible binding of site-specific drugs and the chemical modification by site-specific reagents on the HSA activity indicated that HSA has multiple reactive sites towards NPGB. Results of the reaction in the presence of excess NPGB over HSA also suggested the existence of multiple active sites. The pH-profile for k_2 showed inflection points at about pH 6.0 and pH 10.0, suggesting the involvement of groups with pK_a's of 6.0 and 10.0 in HSA.

Keywords human serum albumin; esterase-like activity; *p*-nitrophenyl 4-guanidinobenzoate; kinetics; Michaelis constant; pH profile; drug binding; competitive inhibition; chemical modification; site-specific

It has been reported that human serum albumin (HSA) possesses esterase-like activity towards esters,[1-4] amides,[5] and phosphates.[6] 4′-Methylumbelliferyl 4-guanidinobenzoate (MUGB) and aryl guanidinobenzoates are used as active site titrant for many serine proteases (*e.g.*, trypsin) on the basis of the formation of stable 4-guanidinobenzoyl-linked enzymes.[7,8] These guanidinobenzoates also are candidates for contraceptive drugs because they are inhibitors of acrosin (a serine protease), which has an essential function in the fertilization process.[8,9] Schwartz demonstrated that MUGB is degraded in plasma and amniotic fluid and that albumin may be responsible for this degradation.[10] It has been reported recently[11] that HSA enhanced the hydrolysis rate of MUGB by a factor of 46.6 at pH 7.4 and 25 °C, and that only one histidine residue was critical for the activity among the 16 histidine residues in HSA. Since the mechanism involved still seems to be unclear, we examined further the reaction of *p*-nitrophenyl 4-guanidinobenzoate (NPGB) with HSA. NPGB was selected as a model substrate, since NPGB has higher solubility in water than MUGB and the reaction can be easily followed spectrophotometrically. The degradation of NPGB in a HSA solution was accelerated by a factor of 75.6 at pH 7.4, and HSA had multiple reactive sites towards NPGB. The kinetics and mechanism of the reactions are described herein.

Experimental

Materials HSA (Sigma Chem. Co., Fraction V, lots 16F-9344 and 16F-9633) was used after purification by Chen's method.[12,13] The molecular weight of HSA was assumed to be 69000 and the concentration was determined by use of molar absorptivity ($\varepsilon = 3.66 \times 10^4\,M^{-1}\,cm^{-1}$) at 278 nm.[13,14] NPGB hydrochloride was initially synthesized by the dicyclohexylcarbodiimide (DCC)-assisted condensation of *p*-nitrophenol (NP) and 4-guanidinobenzoic acid (GB) hydrochloride in dry pyridine and dimethylformamide (1 : 1).[7,8] Later, NPGB·HCl was purchased from Sigma Chem. Co. Cinnamoylimidazole (CI), MUGB, and diethylpyrocarbonate (DEP) were obtained from Sigma Chem. Co. Phenylbutazone (PB) was purchased from Aldrich Chem. Co. Clofibric acid (CA) and 5-nitroaspirin (NA) were the same as used in the previous study.[15] All other chemicals obtained commercially were of a reagent grade.

Ultraviolet (UV) absorption spectroscopy was carried out with a Shimadzu UV-260 spectrophotometer and a Hitachi UV-124 spectrophotometer.

Kinetic Runs The buffer systems used were as follows: pH 5.0, 0.2 M acetate; pH 6.0—8.0, 0.067 M phosphate; pH 9.0, 0.1 M phosphate–0.05 M borate; pH 10.0—11.0, 0.05 M borate. Ionic strength was adjusted to 0.2 with NaCl. The reaction temperature was 25 °C.

The reactions of NPGB (1.00×10^{-5} M) with HSA (an excess concentration over NPGB) in the presence and absence of a drug were followed spectrophotometrically by monitoring the release of *p*-nitrophenol (NP) at 320 nm from pH 5.0 to 6.0 and at 400 nm from pH 7.0 to 11.0. The pseudo first-order rate constant (k_{obs}) was determined from a plot of log ($A_\infty - A_t$) *versus* time, where A_∞ and A_t are the absorbances at the completion of the reaction and at time *t*, respectively.

The reactions in the presence of excess NPGB (2.5×10^{-5} M to 1.0×10^{-4} M) over HSA (5.0×10^{-6} M) were also followed spectrophotometrically.

Effects of Chemical Modification of HSA on the Reaction Rate with NPGB HSA (5.00×10^{-5} M) was modified with equimolar CI, NA, and MUGB before the reaction with NPGB (1.00×10^{-5} M). The modification of HSA was followed spectrophotometrically at 330, 370, and 360 nm for CI,[5] NA,[15] and MUGB,[11] respectively. After the completion of the modification (no change in absorbance occurred), 15 μl of 2.00×10^{-3} M NPGB in water was added to 3 ml of the reaction solution containing modified HSA and modifier. The rate of NP release was followed at 400 nm, and the pseudo first-order rate constant (k^{m}_{obs}) was calculated.

Ethoxycarbonylation of HSA with DEP was carried out by the method described previously.[6] The effects of the modification of HSA on the reaction rate with NPGB were examined in a way similar to that in the previous study.[6]

Results and Discussion

Figure 1a shows the effects of HSA concentration on k_{obs} in its reaction with NPGB. The concentration with subscript 0 in this paper always indicates the initial concentration. The k_{obs} value increases asymptotically with the concentration of HSA, suggesting the reaction pathway shown in Chart 1. Figure 1b shows a typical Lineweaver–Burk (double reciprocal) plot[1,16] for the data shown in Fig. 1a. The K_s and k_2 values are obtainable from the slope and intercept. The values of k_2, K_s, and k_0 at pH 7.4 and 25 °C were estimated to be $4.44 \times 10^{-4}\,s^{-1}$, 8.13×10^{-4} M, and $5.87 \times 10^{-6}\,s^{-1}$, respectively. The ratio of k_2 to k_0 is 75.6, indicating an esterase-like activity of HSA towards NPGB.

$$\text{NPGB} + \text{HSA} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{NPGB}\cdot\text{HSA} \xrightarrow{k_2} \text{NP} + \text{acyl-HSA (or GB + HSA)}$$

$$\text{NPGB} + \text{HSA} \xrightarrow{k_0} \text{NP} + \text{GB}$$

Chart 1

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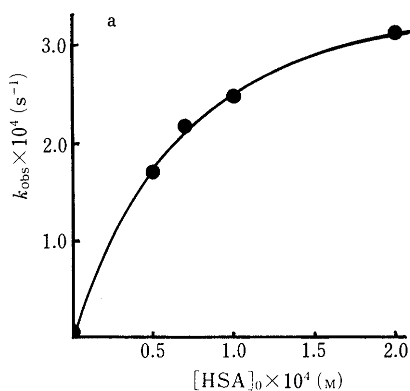

Fig. 1a. Effect of HSA Concentration on the Rate of *p*-Nitrophenol Release

pH 7.4, 0.067 M phosphate buffer ($\mu = 0.2$ with NaCl) at 25 °C; $[NPGB]_0 = 1.00 \times 10^{-5}$ M.

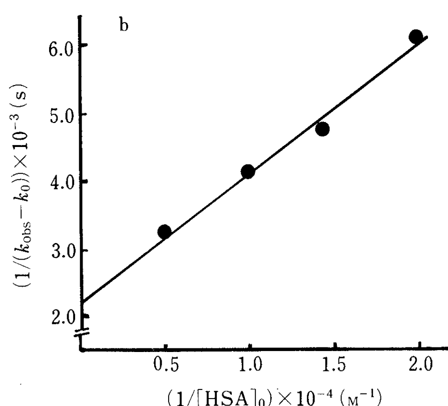

Fig. 1b. Plot of $1/(k_{obs} - k_0)$ *versus* $1/[HSA]_0$

Data from Fig. 1a.

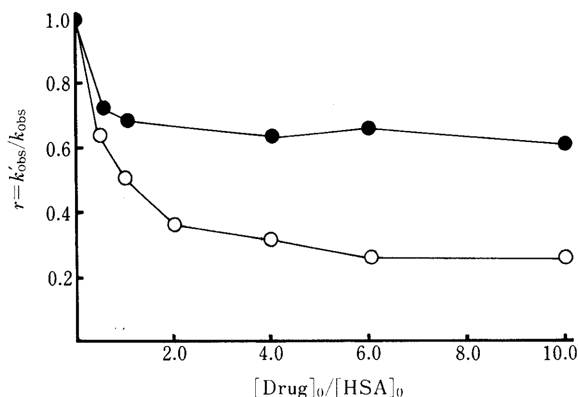

Fig. 2. Effect of CA and PB on the Reaction Rate of NPGB with HSA at pH 7.4 and 25 °C

●, CA; ○, PB; $[NPGB]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; $k_{obs} = 1.69 \times 10^{-4}$ s^{-1}.

To localize the reactive site(s) towards NPGB, the effects of some drugs, whose binding sites on HSA are known already, on k_{obs} were examined. Figure 2 shows the results for CA and PB. In this figure, k'_{obs} on the ordinate represents the rate constant in the presence of the drug, and r is the ratio of k'_{obs} to k'_{obs}. Both CA and PB inhibit the reaction of NPGB with HSA. It is well known that CA strongly binds to the R site alone and PB binds primarily to the U site and secondarily to the R site.[15,17] These inhibitions shown in Fig. 2 could not be interpreted as simple competitive inhibition, because in the case of competitive inhibition, the r value at a large excess of CA over HSA, for example, should be 0.0347 ($k_0/k_{obs} = 5.87 \times 10^{-6}/1.69 \times 10^{-4}$). Two possibilities can be considered for the inhibition. One is non-competitive inhibition or mixed-type inhibition[18] by CA, that is, the complex CA·HSA still has reactivity towards NPGB less than that of the native (uncomplexed) HSA. The other is that HSA has multiple reactive sites towards NPGB and that the R site is only one site out of many (the contribution of the R site activity to the total activity of HSA may only be about 30% ($r = 0.7$ in the excess CA over HSA)).

In order to determine which mechanism is proper, the effects of chemical modification of the reactive sites on the reaction rate with NPGB were examined, and further, the reaction in the presence of excess NPGB over HSA was carried out. It has been reported that CI and NA acylate tyrosine-411 residue of the R site[5] and lysine-199 residue of the U site,[15] respectively. MUGB[11] and DEP[6] modify the histidine residue. Table I shows the results of the effects of chemical modification. The residual activities listed in Table I imply the existence of multiple reactive sites on HSA.

Figure 3 shows the effect of ethoxycarbonylation by

TABLE I. Effects of Chemical Modification on the Reaction Rate of NPGB with HSA at 25 °C[a]

Reagent	Modified site	Residual activity (%)[b]	Ref.
CI	Tyrosine-411 (R site)	83.0	5
NA	Lysine-199 (U site)	106.5	15
MUGB	Histidine residue	67.9	11

a) HSA (5.00×10^{-5} M) was modified with the equimolar reagent before the reaction with NPGB (1.00×10^{-5} M). *b*) Residual activity (%) $= \{(k^m_{obs} - k_0)/(k_{obs} - k_0)\} \times 100$, where k^m_{obs} is the pseudo first-order rate constant for the reaction of NPGB with the modifier-treated HSA as described in the experimental section.

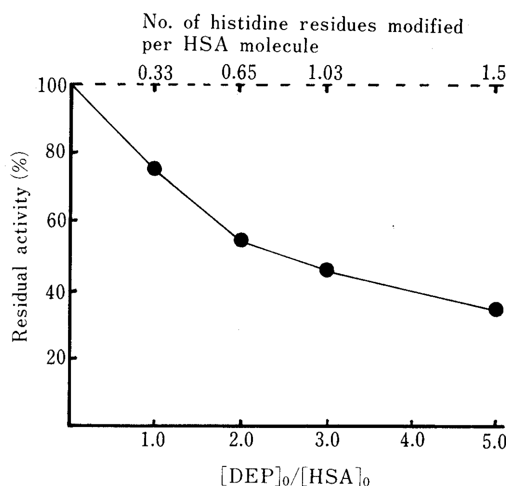

Fig. 3. Effects of Ethoxycarbonylation on the Reaction Rate of NPGB with HSA

pH 7.4 phosphate buffer containing 0.5% (v/v) ethanol at 25 °C; $[NPGB]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M.

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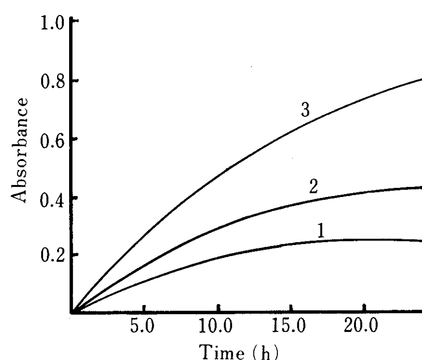

Fig. 4. Time Courses for the Reaction of Excessive NPGB with HSA at pH 7.4 and 25 °C

Concentration of HSA was 5.00×10^{-6} M; 1, 2.5×10^{-5} M of NPGB; 2, 5.00×10^{-5} M of NPGB; 3, 1.00×10^{-4} M of NPGB.

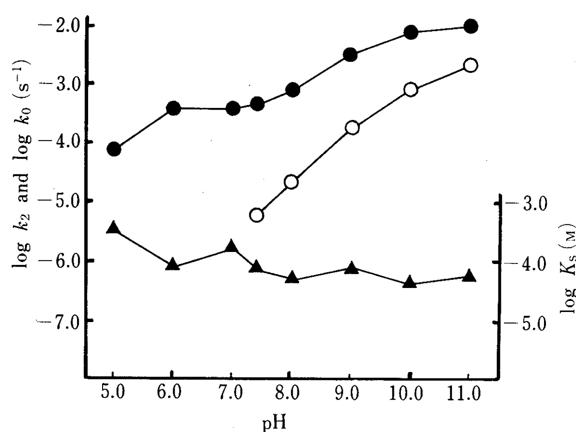

Fig. 5. The pH Profiles of the Kinetic Parameters for the Reactions of NPGB with HSA at 25 °C

●, k_2; ○, k_0; ▲, K_s.

DEP on the reaction rate with NPGB. The lower scale of abscissa shows the ratio of the initial concentration of DEP employed to that of HSA, and the upper scale is the number of histidine residues modified per HSA molecule, which was calculated according to the method described in the previous paper.[6] When about 1 and 1.5 mol of histidine residues per mol of HSA molecule were modified by DEP, the residual activities were about 45% and 35%, respectively. These results indicate that the imidazole group(s) of histidine residues also is (are) the catalytic group(s) towards NPGB, and that there should be at least two reactive histidine residues because of the existence of about 35% residual activity remaining after the modification of about 1.5 mol of histidine per mol of HSA.

Figure 4 shows the time courses for the reactions of excessive NPGB with HSA. The absorbance of about 0.05 on ordinate in Fig. 4 corresponds to one mol of *p*-nitrophenol (NP, 5.00×10^{-6} M) released from the reaction with NPGB per mol of HSA (5.00×10^{-6} M). There seems to be no heterogeneity in the reactivities towards NPGB, that is, all sites on HSA appear to be homogeneous. These results again indicate that HSA has multiple reactive sites towards NPGB.[19]

The pH profiles of the kinetic parameters for the reaction are shown in Fig. 5. With the pH profile of k_0, the deviation from a slope of +1 in the alkaline region may be due to the deprotonation of the guanidino group of NPGB ($pK_a = 12$[11]). The pH profile of k_2 suggests the involvement of two ionizable catalytic groups with pK_a's of about 6 and 10 in the reaction. It may be reasonable to consider that the catalytic group with a pK_a of 6 would be the imidazole group of histidine residue and that the group with the pK_a of 10 the hydroxy group of tyrosine or the amino group of lysine residue, because HSA has multiple reactive sites towards NPGB as described above. The K_s value at pH 5 is larger than those at neutral and alkaline pH's; that is, binding of NPGB to the reactive site at pH 5 is weaker than those at the neutral regions. The imidazole group in the reactive site may play an important role in the binding of the substrate.

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19) Similarly to the case of NPGB, except for an existence of 5% (v/v) methanol as the solubilizer of MUGB, we examined the time courses for the reactions of excess MUGB (4.00×10^{-5}, 3.00×10^{-5}, and 2.00×10^{-5} M) with HSA (5.00×10^{-6} M). The results indicated that HSA seems to possess at least 3 reactive sites towards MUGB.

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