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Esterase-like Activity of Human Serum Albumin. VI.[1] Reaction with *p*-Nitrophenyl Glycinate

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The reaction of *p*-nitrophenyl glycinate (NPG, $pK_a = 7.1$) with human serum albumin (HSA) was investigated kinetically at various pH's and 25 °C. The Michaelis constant (K_S/m in M, where m is the number of reactive sites) and the catalytic rate constant (k_2 in s^{-1}) were determined. The affinity of the cationic substrate ($NPGH^+$) to the reactive site is lower than that of the neutral substrate (NPG). The pH profile of k_2 is sigmoidal, like that of k_0, indicating ionization of the reaction species at pH 6—7. The effect of chemical modification of histidine residues by diethylpyrocarbonate on the reaction rate with NPG and the deuterium oxide isotope effect on the reaction rate of HSA with NPG indicate the reaction of NPG·HSA complex with OH^- rather than the nucleophilic reaction (formation of glycinated HSA) of the histidine imidazole group(s) with NPG. Results on the reaction in the presence of excess NPG over HSA suggest that HSA has non-specific multiple reactive sites towards NPG. The reactive sites were considered to be relatively exposed on the surface of the HSA molecule and to be sensitive to conformational change.

Keywords——human serum albumin; esterase-like activity; *p*-nitrophenyl glycinate; kinetics; pH–rate profile; Michaelis–Menten type complex; chemical modification; deuterium isotope effect; drug binding

In the previous papers[1-5] it was reported that human serum albumin (HSA) has esterase-like activity towards esters,[2-4] amides,[5] and phosphate.[1] Although degradation of meclofenoxate (MFX; dimethylaminoethyl *p*-chlorophenoxyacetate; $pK_a = 8.2$) in HSA solution was accelerated in the acidic region below pH 7.0 (protonated form of MFX, $MFXH^+$), in the basic region (neutral form of MFX) the degradation was retarded.[6] As a part of our continuing studies on the esterase-like activity of HSA, the reaction of *p*-nitrophenyl glycinate (NPG) with HSA was investigated kinetically in order to elucidate the effect of the protonation of the substrate ($NPGH^+$) on the reaction rate. NPG was selected as a model substrate, since the pK_a value of its conjugated acid ($NPGH^+$) is 7.1[7,8] and the reaction can be easily monitored spectrophotometrically. The degradation of NPG in HSA solution was accelerated over the whole pH region. The kinetics and mechanism of the reaction are described in this paper.

Experimental

Materials and Apparatus——HSA (Sigma Chem. Co., Fraction V, lots 85F-9351, 105F-9328, and 16F-9344) was used after purification by Chen's method.[9,10] Assuming a molecular weight of 69000 for HSA, its concentration was determined by use of the molar absorptivity ($\varepsilon = 3.66 \times 10^4\ M^{-1}cm^{-1}$) at 278 nm.[10,11] NPG hydrobromide was prepared from *N*-benzyloxycarbonyl *p*-nitrophenyl glycinate according to the method of Hay and Main[7]: mp 208—214 °C (lit. 212 °C[7] and 213 °C[8]). Phenylbutazone (PB) and clofibric acid (CA) were the same as those used in the previous studies.[12] Diethylpyrocarbonate (DEP) was purchased from Aldrich Chem. Co. (lot 02720HP) and was used without further purification. All other chemicals obtained commercially were of reagent grade.

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

Shimadzu UV-260 spectrophotometer. A stopped-flow spectrophotometer (Otsuka Denshi RA-401) was used for measurement of the fast reaction. The pH value was measured with a Hitachi-Horiba F-7_{LC} pH meter.

Kinetic Runs——The buffer systems used were as follows: pH 4.0—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 NPG (1.00×10^{-5} M) with HSA (an excess concentration compared with NPG) in the presence and absence of a drug were followed spectrophotometrically by monitoring the release of *p*-nitrophenol at 320 nm from pH 4.0 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 ($A_\infty - A_t$) against 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 NPG (1.00×10^{-5} and 2.00×10^{-5} M) over HSA (2.00×10^{-6} M) were also followed spectrophotometrically at 400 nm.

The reaction of NPG (1.00×10^{-5} M) with HSA (5.00×10^{-5} M) was also carried out in deuterium oxide. pD was adjusted with concentrated NaOD in D_2O and the pD value was estimated from pD = pH-meter reading + 0.4.[13]

Determination of Kinetic Parameters for the Reaction of NPG with HSA——The reaction in the presene of HSA over NPG (1.00×10^{-5} M) can be expressed as shown in Chart 1. In Chart 1, P and NPG·HSA are *p*-nitrophenol and

$$\begin{array}{ccccc} & & \overset{K_S}{\rightleftharpoons} & & \overset{k_2}{\longrightarrow} \\ \text{NPG} + \text{HSA} & & & \text{NPG}\cdot\text{HSA} & \longrightarrow \text{P} + \text{acyl-HSA} \\ & \Big\downarrow k_0 & & & \text{(or glycine + HSA)} \\ \text{P} + \text{glycine} & & & & \end{array}$$

Chart 1

the Michaelis–Menten type complex between NPG and HSA, respectively. Acyl-HSA is HSA acylated with NPG (that is, glycinated HSA). K_S is the dissociation constant of NPG·HSA. The rate constants of NPG·HSA and NPG are represented by k_2 and k_0, respectively. According to Chart 1, the k_{obs} value determined experimentally can be represented by Eq. 1.[2,3]

$$k_{obs} = \frac{k_0 + (k_2/K_S)[\text{HSA}]_0}{1 + (1/K_S)[\text{HSA}]_0} \tag{1}$$

where $[\text{HSA}]_0$ is the initial concentration of HSA. The K_S and k_2 values can be calculated from the slope and intercept of the double-reciprocal plot based on Eq. 2.[2,3]

$$\frac{1}{k_{obs} - k_0} = \frac{K_S}{(k_2 - k_0)} \cdot \frac{1}{[\text{HSA}]_0} + \frac{1}{k_2 - k_0} \tag{2}$$

When there exist m reactive sites on HSA and the reactivities of the sites towards NPG are assumed to be identical with each other (see Results and Discussion), the k_{obs} value determined experimentally is represented by Eq. 3.[14,15]

$$k_{obs} = \frac{k_0 + (mk_2/K_S)[\text{HSA}]_0}{1 + (m/K_S)[\text{HSA}]_0} \tag{3}$$

Equation 3 can be rearranged to Eq. 4 for the double-reciprocal form.

$$\frac{1}{k_{obs} - k_0} = \frac{K_S}{m(k_2 - k_0)} \cdot \frac{1}{[\text{HSA}]_0} + \frac{1}{k_2 - k_0} \tag{4}$$

Applying the same k_{obs} values to both Eqs. 2 and 4 for the determination of the kinetic parameters, the dissociation constants obtained are different (that is, K_S and K_S/m, respectively), but the catalytic rate constant k_2 is identical.

Time courses of the reactions in the presence of excess NPG (1.00×10^{-5} and 2.00×10^{-5} M) over HSA (2.00×10^{-6} M) were analyzed as follows. The absorbance at time t (A_t) may be given by Eq. 5.

$$A_t = \varepsilon_P[\text{P}]_0(1 - \exp(-k_0 t)) + \varepsilon_P[\text{HSA}]_0 \sum_{i=1}^{N} (n_i(1 - \exp(-k_i' t))) \tag{5}$$

where ε_p is the average value of molar absorptivity of *p*-nitrophenol ($(9.47 \pm 0.11) \times 10^3\ \text{M}^{-1}\,\text{cm}^{-1}$ at pH 7.4) in the presence of HSA (2.00×10^{-6} M). $[\text{P}]_0$ is the *p*-nitrophenol concentration produced by the spontaneous reaction

($k_0 = (9.51 \pm 0.90) \times 10^{-3}\,s^{-1}$). N is the number of classes of independent reactive sites and each class, i, possesses n_i sites with a pseudo first-order rate constant k_i'. In other words, n_i is the number of the reactive sites having the rate constant k_i'. The non-linear least-squares method (MULTI®[16]) was applied to Eq. 5 for the determinations of $[P]_0$, N, n_i, and k_i'.

Modification of HSA with DEP——Ethoxycarbonylation of HSA with DEP was carried out by a method similar to that reported previously.[1] The effects of the modification of HSA on the reaction rate with NPG were investigated in the same way as in the previous study.[1]

Results and Discussion

Reaction of NPG with HSA

The effects of the HSA concentration on k_{obs} for the reaction with NPG are shown in Fig. 1a. The k_{obs} value increases hyperbolically with the concentration of HSA, suggesting saturation kinetics for the reaction as shown in Chart 1. Figure 1b shows the plot based on Eq. 2; the K_s and k_2 values are obtainable from the slope and intercept. The ratio of k_2 to k_0 at pH 7.0 is 5.7, indicating an esterase-like activity of HSA towards substrate NPG having the pK_a value of 7.1.[7]

To localize the reactive site(s) towards NPG, the effects of some drugs, whose binding sites of HSA are known, on k_{obs} were examined. Figure 2 shows the results for PB and CA, which bind primarily to the II and R sites, respectively.[12,17-20] In Fig. 2, k_{obs}^{drug} on the ordinate represents the rate constant in the presence of a drug and r is the ratio of k_{obs}^{drug} to k_{obs}. PB accelerates the reaction (r in Fig. 2 is from 1.1 to 1.2), suggesting a conformational change of the reactive site of HSA towards NPG. The conformational change also implies that the activity of the reactive site towards NPG in PB·HSA is larger than that in HSA itself.

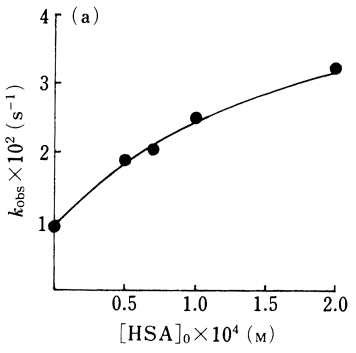

Fig. 1(a). Effect of HSA Concentration on the Rate of *p*-Nitrophenol Release

pH 7.0, 0.067 M phosphate buffer ($\mu = 0.2$ with NaCl) at 25 °C; $[NPG]_0 = 1.00 \times 10^{-5}$ M.

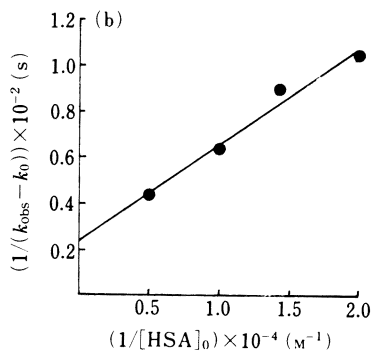

Fig. 1(b). Plot of $1/(k_{obs} - k_0)$ *versus* $1/[HSA]_0$

Data from Fig. 1(a).

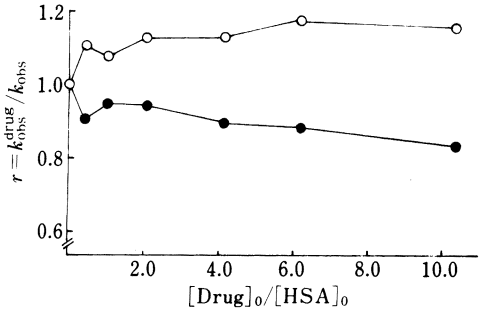

Fig. 2. Effect of PB and CA on the Reaction Rate of NPG with HSA at pH 7.4 and 25 °C

○, PB; ●, CA.

$[NPG]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; $k_{obs} = 2.07 \times 10^{-2}\,s^{-1}$.

TABLE I. Estimated Parameters in Eq. 5 for the Reactions in the Presence of Excess NPG over HSA[a]

$[NPG]_0 \times 10^5$ (M)	A_∞[b] $\times 10^2$	$\varepsilon_P[P]_0 \times 10^2$	$[P]_0/[HSA]_0$	n_1	$k'_1 \times 10^2$ (s^{-1})
1.00	9.47±0.05[c]	5.57±0.78[c]	2.91±0.37[c]	2.10±0.42[c]	2.26±0.27[c]
2.00	18.9±0.3[c]	7.86±1.30[c]	4.14±0.66[c]	6.03±0.68[c]	1.96±0.03[c]

a) pH 7.4 and at 25 C; $[HSA]_0 = 2.00 \times 10^{-6}$ M. *b*) A_∞ is the absorbance at infinite time. *c*) Mean ± standard deviation from 4 experimental runs.

On the other hand, CA inhibits the reaction of NPG with HSA by 10% to 20%, that is, r in Fig. 4 is from 0.9 to 0.8. This inhibition could not be interpreted as a simple competitive inhibition, because for competitive inhibition the r value at a large excess of CA over HSA should be 0.459 ($k_0/k_{obs} = 9.51 \times 10^{-3}/2.07 \times 10^{-2}$). Two possibilities can be considered for the inhibition caused by CA. One is a conformational change of the reactive site of HSA towards NPG, similarly to the case of PB, but CA·HSA has less reactivity towards NPG than HSA itself (that is, a mixed-type inhibition). The other is that HSA has multiple reactive sites towards NPG and the CA binding site (R site) is one of the reactive sites on HSA (the contribution of the R site activity to the total activity of HSA may be only from 10% to 20%). In order to examine whether multiple reactive sites towards NPG exist on HSA or not, the reaction in the presence of excess NPG over HSA was carried out. The data were analyzed according to Eq. 5 by use of a non-linear least-squares method (MULTI®[16]). The analysis indicated that one class ($N=1$ in Eq. 5) of the activities (k'_1) is enough to characterize the reaction of HSA with NPG, and the results are summarized in Table I. The numbers of the reactive sites (n_1) are 2.12 and 6.03 in the presence of 5-fold and 10-fold NPG over HSA, respectively. These results suggest that HSA has non-specific multiple reactive sites towards NPG.

The pH Profiles of Kinetic Parameters for the Reaction of NPG with HSA

Figure 3 shows the pH profiles of k_0, k_2, and K_S/m. The profile of k_0 would be explained by the reactions of protonated ($NPGH^+$) and free (unprotonated, NPG) substrates with hydroxide ion (OH^-) in the acid and alkaline region, respectively (Chart 2).[7,8] In Chart 2, k_0^C and k_0^F are the second-order rate constants of cationic and free forms of NPG, respectively. According to Chart 2, k_0 is represented by Eq. 6.

$$k_0 = \frac{k_0^C[OH^-]}{(K_a/[H^+])+1} + \frac{k_0^F[OH^-]}{1+([H^+]/K_a)} \qquad (6)$$

The parameters in Eq. 6 and Chart 2 were determined using the pH-profile of k_0, Eq. 6, and the ionic product of water K_w (assumed to be 1×10^{-14} M^2) and are listed in Table II. Since the effects of buffer concentration of k_0 were not corrected, the parameters estimated may be only approximate and apparent values. The pK_a value estimated kinetically is somewhat different from pK_a 7.1 ($\mu=0.1$ with KNO_3 at 25 °C) determined potentiometrically by Hay and Basak.[8] The solid curve for k_0 depicted in Fig. 3 is the calculated curve based on Eq. 6 and the values in Table II.

Although the number (m) of the reactive sites towards NPG is unknown in the presence of an excess HSA over NPG, the K_S/m value in dimension M may be a measure of binding affinity of the substrate to the HSA reactive site. The K_S/m values in the acid region are larger than those in the alkaline region, suggesting that the binding of $NPGH^+$ to HSA is weaker than that of NPG ($pK_a = 7.1$[7,8]).

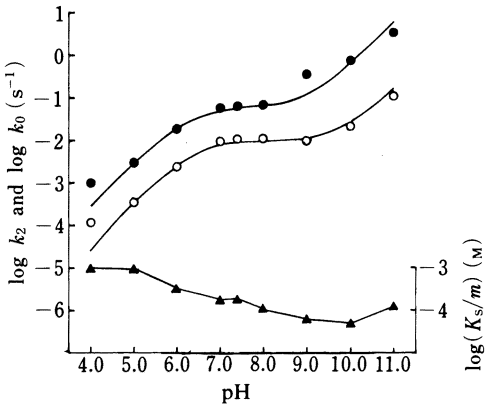

Fig. 3. The pH Profiles of K_S/m, k_2, and k_0 for the Reaction of NPG with HSA at 25 °C

▲, K_S/m; ●, k_2; ○, k_0.
The solid curves for k_0 and k_2 were simulated on the basis of Eqs. 6 and 7, respectively, using the parameters determined (see the text).

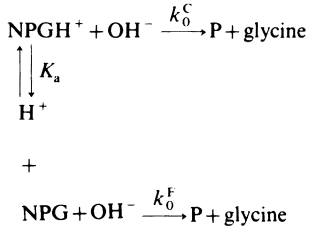

Chart 2

TABLE II. Kinetic Parameters for the Reaction of NPG in the Absence and Presence of HSA at 25 °C

Absence (Eq. 6)		Presence (Eq. 7)	
k_0^C in $M^{-1}s^{-1}$	3.47×10^5	k_2^C in $M^{-1}s^{-1}$	2.97×10^6
k_0^F in $M^{-1}s^{-1}$	1.42×10^2	k_2^F in $M^{-1}s^{-1}$	5.12×10^3
K_a in M	3.73×10^{-7}	K'_a in M	5.39×10^{-7}
(pK_a)	(6.43)	(pK'_a)	(6.27)

The shape of the log k_2–pH profile is sigmoidal, like that of k_0, indicating ionization of the reaction species at pH 6—7. There are two possibilities to account for this profile. One is that the imidazole group ($pK_a = 6-7$) of histidine residues on HSA participates in the reaction with NPG (not NPGH$^+$, see Chart 3). In Chart 3, $K_{a,1}$ and $K_{a,2}$ are the dissociation constants of the cationic and neutral imidazole groups, respectively. The intrinsic rate constants based on the neutral and anionic imidazole groups are represented by k_N and k_A, respectively.

The other possibility is that NPGH$^+$·HSA and NPG·HSA complexes react with hydroxide ion (OH$^-$) in the acid and alkaline regions, respectively (Chart 4). In Chart 4, K_S^C and K_S^F are the dissociation constants of the Michaelis–Menten type complexes of NPGH$^+$·HSA and NPG·HSA, respectively. The rate constants for the reactions of NPGH$^+$·HSA and NPG·HSA with OH$^-$ are represented by k_2^C and k_2^F, respectively. K'_a is the protolytic dissociation constant of NPGH$^+$·HSA to NPG·HSA and H$^+$. Examinations of the effect of ethoxycarbonylation of histidine residues of HSA by DEP on the reaction rate with NPG and also of the deuterium oxide isotope effect on the rate may permit a choice between the two possibilities.

Figure 4 shows the effect of the ethoxycarbonylation by DEP on the reaction rate with NPG. The abscissa shows the ratio of the initial concentration of DEP employed to that of HSA (lower scale), and also the number of histidine residues modified per HSA molecule (upper scale). On the ordinate, k_{obs}^m is the pseudo first-order rate constant for the reaction of NPG with the DEP-treated HSA as described in the experimental section. The modification of histidine residues of HSA accelerates the reaction with NPG, indicating that the imidazole group is not the catalytic group towards NPG. The acceleration may imply that the reactive site towards NPG is sensitive to the conformational change of HSA.

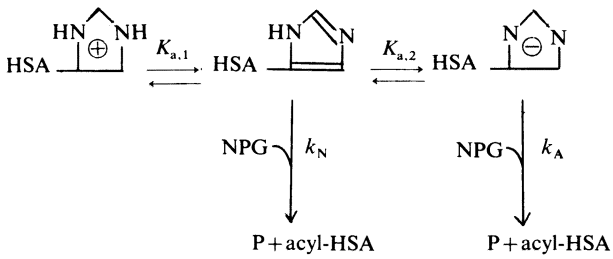

Chart 3

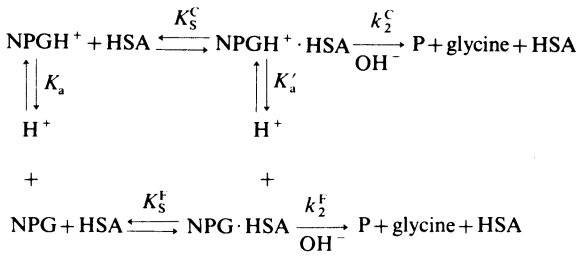

Chart 4

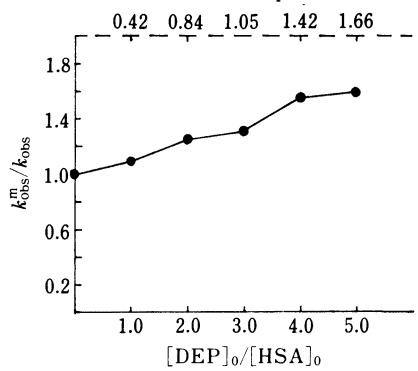

Fig. 4. Effects of Ethoxycarbonylation on the Reaction Rate of NPG with HSA

pH 7.4 phosphate buffer containing 0.5% (v/v) ethanol at 25 °C; $[NPG]_0 = 1.00 \times 10^{-5}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; $k_{obs} = 2.07 \times 10^{-2}\,s^{-1}$; lower scale on abscissa, $[DEP]_0/[HSA]_0$; upper scale, number of histidine residues modified per HSA molecule.

In deuterium oxide at pD 7.4, the pseudo first-order rate constant for the reaction ($k_{obs}^{D_2O} = 8.94 \times 10^{-3}\,s^{-1}$) was 1/3.6 of that in water at pH 7.4 ($k_{obs} = 3.22 \times 10^{-2}\,s^{-1}$). This deuterium effect indicates that the water molecule plays a role in the reaction of HSA with NPG. Therefore, general (specific) acid or base catalysis rather than nucleophilic catalysis predominates in the reaction,[21] supporting the reaction mechanism shown in Chart 4 rather than that in Chart 3. According to Chart 4, k_2 represented in Chart 1 can be expressed by Eq. 7, as in the case of k_0.

$$k_2 = \frac{k_2^C[OH^-]}{(K_a'/[H^+])+1} + \frac{k_2^F[OH^-]}{1+([H^+]/K_a')} \qquad (7)$$

From the pH-profile of log k_2 shown in Fig. 3, each parameter in Eq. 7 was estimated and is listed in Table II. The solid curve for k_2 in Fig. 3 was calculated from Eq. 7 using the parameters listed in Table II. It was found that the cationic complex (k_2^C) reacts with OH^- about 600-fold more quickly than the neutral complex (k_2^F). The reaction of the cationic complex with OH^- (k_2^C) is 8.6-fold faster than that of $NPGH^+$ with OH^- (k_0^C) and k_2^F is 36-fold larger than k_0^F.

In conclusion, HSA has non-specific multiple reactive sites towards NPG. The degradation of NPG was accelerated by the reaction of the Michaelis–Menten type complex between NPG and HSA with hydroxide ion (OH^-). The reactions of the cationic complex and the neutral complex with OH^- are 8.6-fold and 36-fold faster than those of $NPGH^+$ and NPG in the absence of HSA, respectively. The reactive site of HSA toward NPG is considered

to be sensitive to conformational change caused by chemical modification with DEP and drug bindings, and also to be relatively exposed on the surface of the HSA molecule because of the easy reaction of OH^- with NPG·HSA complexes.

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15) More generally, k_{obs} can be represented by Eq. 8.

$$k_{obs}=\frac{k_0+[\mathrm{HSA}]_0\sum_{i=1}^{n}(k_i''/K_i)}{1+[\mathrm{HSA}]_0\sum_{i=1}^{n}(1/K_i)} \qquad (8)$$

where K_i is the dissociation constant of the NPG·HSA complex having the rate constant k_i''.
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