Esterase-Like Activity of Human Serum Albumin. VIII.[1] Reaction with Amino Acid *p*-Nitrophenyl Esters

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The reactions of human serum albumin (HSA) with optically active amino acid *p*-nitrophenyl esters (substrate, S) were examined kinetically at 25 °C. The rate data were analyzed in terms of a mechanism involving 1 : 1 complexing (S·HSA) between S and HSA. The dissociation constant (K_S in M) and the catalytic rate constant (k_2 in s^{-1}) of S·HSA were determined. Among ten substrates examined, the reactions with *N*-carbobenzoxy-D(L)-alanine *p*-nitrophenyl esters (*N*-CBZ-D(L)-AlaNP) were most accelerated by HSA. Results of the reaction in the presence of excess *N*-CBZ-D(L)-AlaNP over HSA indicated the existence of one strong reactive site on HSA. The effects of the reversible binding of the site-specific drug and the chemical modification by site-specific reagents on the HSA activity showed that the reactive site towards *N*-CBZ-D(L)-AlaNP is the R site located near tyrosine-411 residue of HSA.

Keywords human serum albumin; esterase-like activity; amino acid *p*-nitrophenyl ester; dissociation constant; chemical modification; drug binding; kinetics; clofibric acid; phenylbutazone

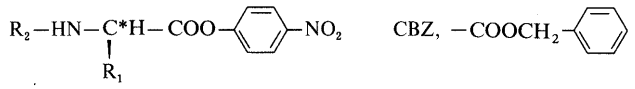
Introduction

It has been reported that human serum albumin (HSA) possesses esterase-like activity towards esters,[1-4] amides,[5] and phosphates.[6] A previous study[7] showed that HSA accelerates the hydrolysis of *p*-nitrophenyl glycinate (GlyNP) due to non-specific reactive sites. As a part of our continuing studies on the esterase-like activity of HSA, the reactions of amino acid *p*-nitrophenyl esters (Table I) with HSA were investigated kinetically, and the results are described herein. The reactions of bovine serum albumin (BSA) with these esters have been reported recently.[8]

Experimental

Materials HSA (Sigma Chem. Co., fraction V, lots 16F-9344, 36F-9333, and 76F-9353) was used after purification by Chen's method.[9] 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.[10,11] The substrates used are listed in Table I. *N*-CBZ-GlyNP (Tokyo Kasei, lot AW01), *N*-CBZ-L-AlaNP (Bachem, lot R3946), *N*-CBZ-D-PheNP (Sigma, lot 104F-0339), and *N*-CBZ-L-PheNP (Sigma, lot 45F-0840) were purchased commercially. *N*-CBZ-D-AlaNP was synthesized from D-α-alanine (Ala) and *p*-nitrophenol (NP) according to the method of Izumiya.[12] GlyNP, D(L)-AlaNP, and D(L)-PheNP were prepared from *N*-CBZ-GlyNP, *N*-CBZ-D(L)-AlaNP, and *N*-CBZ-D(L)-PheNP, respectively, according to the method of Hay and Main.[13] *N-trans*-Cinnamoylimidazole (CI, US Biochem. Co., Ltd., lot 44400) and phenylbutazone (PB, Aldrich, lot 04023AM) were obtained commercially. 5-Nitroaspirin (NA) and clofibric acid (CA) were synthesized by the methods of Ciampa[14] and Jones,[15] respectively. All other chemicals obtained commercially were of a reagent grade.

TABLE I. Amino Acid *p*-Nitrophenyl Esters Used



Substrate	R_1	R_2
GlyNP	H	H
N-CBZ-GlyNP	H	CBZ
D(L)-AlaNP	CH_3	H
N-CBZ-D(L)-AlaNP	CH_3	CBZ
D(L)-PheNP	$CH_2C_6H_5$	H
N-CBZ-D(L)-PheNP	$CH_2C_6H_5$	CBZ

N-CBZ-GlyNP, *N*-carbobenzoxy-glycine *p*-nitrophenyl ester; D(L)-AlaNP, D(L)-alanine *p*-nitrophenyl ester; *N*-CBZ-D(L)-AlaNP, *N*-carbobenzoxy-D(L)-alanine *p*-nitrophenyl ester; D(L)-PheNP, D(L)-phenylalanine *p*-nitrophenyl ester; *N*-CBZ-D(L)-PheNP, *N*-carbobenzoxy-D(L)-phenylalanine *p*-nitrophenyl ester.

Ultraviolet (UV) absorption spectroscopy was carried out with a Shimadzu UV-260 spectrophotometer and a Hitachi UV-124 spectrophotometer. A stopped-flow spectrophotometer (Otsuka Denshi RA-401) was used for measurement of the fast reaction.

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 medium contained 0.5% (v/v) acetonitrile unless otherwise noticed. The reaction temperature was 25 °C.

The reactions of substrate (9.95×10^{-6} M) with HSA (an excess concentration over the substrate) in the presence and absence of a drug were followed spectrophotometrically by monitoring the release of NP at 320 nm from pH 5.0 to 6.0 and at 370 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 reaction in the presence of excess *N*-CBZ-AlaNP (5.00×10^{-5} M) over HSA (1.00×10^{-5} M) was also followed with the stopped-flow apparatus and the UV spectrophotometers.

Effects of Chemical Modification of HSA on the Reaction Rate with *N*-CBZ-AlaNP HSA (5.00×10^{-5} M) was modified with CI (2.50×10^{-4} M) before the reaction with *N*-CBZ-AlaNP (9.95×10^{-6} M). The modification of HSA was followed spectrophotometrically at 330 nm. After the completion of the modification (no change in absorbance occurred), 15 μl of 2.00×10^{-3} M *N*-CBZ-AlaNP in acetonitrile was added to 3 ml of the reaction solution containing the modified HSA and the modifier. The rate of NP release was followed at 400 nm, and the pseudo first-order rate constant (k^m_{obs}) was calculated.

Acetylation of HSA with NA was carried out according to a slightly modified method[11] of Hawkins *et al.*[16] A mixture of HSA (1.00×10^{-4} M) and NA (5.00×10^{-4} M) in pH 7.4 buffer was incubated at 25 °C for 80 min. The mixture was dialyzed at 4 °C for 48 h against multiple changes of 8 M urea and then for 24 h against pH 7.4 buffer. In control experiments, NA was omitted; an HSA solution (control-I, 1.00×10^{-4} M) and also a mixture (control-II) of HSA (1.00×10^{-4} M) and 5-nitrosalicylic acid (5.00×10^{-4} M) were treated as above. These HSA solutions (1.00×10^{-4} M) were reacted with *N*-CBZ-D-AlaNP (1.99×10^{-5} M), and the k^m_{obs} values were determined.

Results and Discussion

Reaction of Amino Acid *p*-Nitrophenyl Esters with HSA The effects of the HSA concentration on k_{obs} in its reaction with D-AlaNP are shown in Fig. 1. The concentration with subscript 0 in this paper always indicates the initial concentration. The k_{obs} value increases hyperbolically with the concentration of HSA, suggesting saturation kinetics for the reaction as shown in Chart 1. In Chart 1, S·HSA is the 1 : 1 complex between S (substrate) and HSA, acyl-HSA is HSA acylated with S, K_S is the dissociation

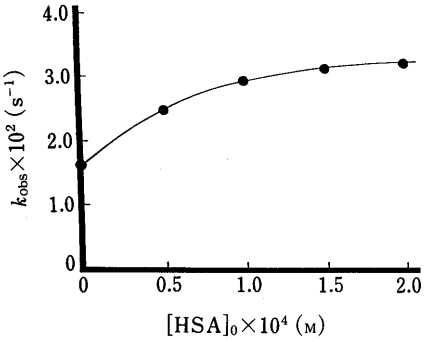


Fig. 1. Effects of HSA Concentration on the Reaction Rate with D-AlaNP

pH 7.4 1/15 M phosphate buffer ($\mu=0.2$) at 25 °C; $[D\text{-AlaNP}]_0=9.95\times10^{-6}$ M.

$$S+HSA \underset{}{\overset{K_S}{\rightleftharpoons}} S\cdot HSA \xrightarrow{k_2} NP+\text{acyl-HSA (or amino acid + HSA)}$$
$$\Big\downarrow k_0$$
$$NP+\text{amino acid}$$

Chart 1

Table II. Kinetic Parameters for the Reaction of Amino Acid *p*-Nitrophenyl Esters with HSA[a]

Substrate	k_2 (s^{-1})	K_S (M)	k_0 (s^{-1})	k_2/k_0
GlyNP[7]	6.09×10^{-2}	1.95×10^{-4}	9.70×10^{-3}	6.28
N-CBZ-GlyNP	7.56×10^{-2}	1.20×10^{-5}	2.42×10^{-4}	3.12×10^{2}
L-AlaNP	9.44×10^{-2}	4.42×10^{-4}	1.45×10^{-2}	6.51
N-CBZ-L-AlaNP	4.79×10^{-1}	$<1.00\times10^{-5}$ [b]	8.45×10^{-5}	5.67×10^{3}
D-AlaNP	3.97×10^{-2}	8.66×10^{-5}	1.62×10^{-2}	2.45
N-CBZ-D-AlaNP	1.12	$<1.00\times10^{-5}$ [b]	9.26×10^{-5}	1.21×10^{4}
L-PheNP	4.23×10^{-2}	1.82×10^{-4}	5.01×10^{-3}	8.44
N-CBZ-L-PheNP	1.08×10^{-1}	$<1.00\times10^{-5}$ [b]	1.03×10^{-4}	1.05×10^{3}
D-PheNP	3.99×10^{-2}	3.08×10^{-4}	4.23×10^{-3}	9.43
N-CBZ-D-PheNP	6.10×10^{-2}	$<1.00\times10^{-5}$ [b]	1.23×10^{-4}	4.96×10^{2}

a) pH 7.4 1/15 M phosphate buffer ($\mu=0.2$) containing 0.5% (v/v) acetonitrile at 25 °C; $[S]_0=9.95\times10^{-6}$ M. b) Could not be determined accurately under the experimental conditions employed because of the small K_s values.[11,21]

constant of S·HSA, k_2 is the first-order rate constant of S·HSA degradation, and k_0 is the first-order hydrolysis rate constant of S. According to Chart 1, the k_{obs} values determined experimentally can be represented by Eq. 1.[2,17-20]

$$k_{obs}=\frac{k_0K_S+k_2\,[HSA]_0}{K_S+[HSA]_0} \quad (1)$$

The K_S and k_2 values are calculated from the intercept and slope of the double-reciprocal plot based on Eq. 2.[2,17]

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

Table II lists the kinetic parameters thus obtained for the reaction, along with the values obtained previously.[7] The k_2/k_0 values for the esters having a CBZ group are larger than those without a CBZ group. The values of K_S for the CBZ-contained esters are smaller than those for the plain esters. The CBZ group protects a protonation of the amino group of the esters and enhances the hydrophobicity of the esters. The esterase-like active sites of HSA seem to be hydrophobic[4,7] and thus the differences in the reactivities

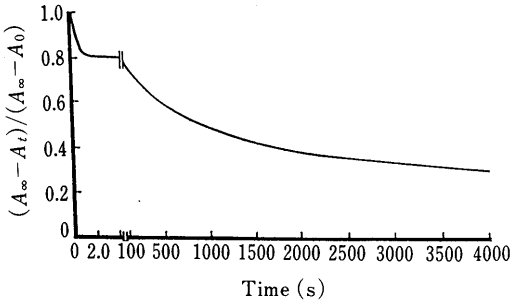


Fig. 2. Plot of $(A_\infty-A_t)/(A_\infty-A_0)$ *versus* t

A_∞, A_t, and A_0 are the absorbances at the completion of the reaction, time t, and time 0, respectively; $[N\text{-CBZ-D-AlaNP}]_0=5.00\times10^{-5}$ M; $[HSA]_0=1.00\times10^{-5}$ M; pH 7.4 1/15 M phosphate buffer ($\mu=0.2$) at 25 °C.

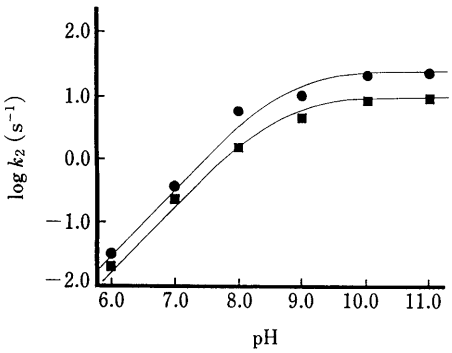


Fig. 3. Plots of $\log k_2$ *versus* pH at 25 °C

●, *N*-CBZ-D-AlaNP; ■, *N*-CBZ-L-AlaNP; $[N\text{-CBZ-AlaNP}]_0=9.95\times10^{-6}$ M; $[HSA]_0=5.00\times10^{-5}$ M.

(k_2 and K_S) between the esters with and without the CBZ group come from the difference in the hydrophobicity of the esters.

Reaction of *N*-CBZ-AlaNP with HSA Since the reactions with *N*-CBZ-AlaNP were most accelerated by HSA among 10 substrates in Table II, the reactive sites of HSA for *N*-CBZ-L(D)-AlaNP were characterized. Figure 2 shows the time course of the reaction of excessive *N*-CBZ-D-AlaNP with HSA. In this figure, A_∞, A_t, and A_0 are the absorbances at 400 nm at the completion of the reaction, at times t and zero, respectively. The initial rapid and the following slow reactions are found in Fig. 2. The reaction in the initial part corresponds closely to the reaction with 1 mol of HSA per mole of the substrate, *i.e.*, $(5.00\times10^{-5}$ M of substrate -1.00×10^{-5} M of HSA)/5.00×10^{-5} M of substrate = 0.8. These results suggest that only one strong reactive site towards *N*-CBZ-D-AlaNP is present in HSA. Similar results (only one primarily reactive site) were obtained for the L-enantiomer.

Figure 3 shows the pH-profiles of k_2 for both substrates (*N*-CBZ-D(L)-AlaNP). The $\log k_2$ values increase linearly with pH up to about 8, giving the slopes of about 1. Above pH 10, k_2 becomes approximately independent of pH. The pK_a values of the catalytic group were estimated as about 8.8 and 8.7 from these profiles for D- and L-enantiomers, respectively. These values are close to the value (9.5) of the hydroxy group of tyrosine residue[2,5] and of the amino group of lysine residue.[21]

Reactive Sites on HSA for the Reaction with *N*-CBZ-D(L)-AlaNP To localize the reactive site towards the substrates, the effects of chemical modification of the reactive site on

Table III. Effects of Acylation of HSA by CI on the Reaction Rate with *N*-CBZ-AlaNP[a]

HSA	k^{m}_{obs} (s^{-1})	
	D-Enantiomer	L-Enantiomer
Control-I HSA[b]	1.17 ± 0.11	$(5.23 \pm 0.16) \times 10^{-1}$
Control-II HSA[c]	$(3.17 \pm 0.14) \times 10^{-1}$	$(2.35 \pm 0.10) \times 10^{-1}$
Cinnamoyl-HSA[d]	$(1.02 \pm 0.11) \times 10^{-2}$	$(1.85 \pm 0.10) \times 10^{-2}$

a) Experiments were carried out 3—4 times. *b*) pH 7.4 1/15 M phosphate buffer ($\mu = 0.2$) containing 0.5% (v/v) acetonitrile at 25 °C; $[N\text{-CBZ-AlaNP}]_0 = 9.95 \times 10^{-6}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M. *c*) The reaction of *N*-CBZ-AlaNP (9.95×10^{-6} M) with HSA (5.00×10^{-5} M) was carried out in the presences of cinnamic acid (2.50×10^{-4} M) and imidazole (2.50×10^{-4} M). *d*) HSA (5.00×10^{-5} M) was reacted with CI (2.50×10^{-4} M) prior to the use for the reaction with *N*-CBZ-AlaNP (9.95×10^{-6} M).

Table IV. Effects of Acylation of HSA by NA on the Reaction Rate with *N*-CBZ-AlaNP[a]

HSA[b]	k^{m}_{obs} (s^{-1})[c] D-Enantiomer
Control-I HSA	1.16 ± 0.06
Control-II HSA	$(9.10 \pm 0.36) \times 10^{-1}$
Acetyl-HSA	1.10 ± 0.14

a) Experiments were carried out 3—4 times. *b*) See the experimental section. *c*) pH 7.4 1/15 M phosphate buffer ($\mu = 0.2$) at 25 °C; $[N\text{-CBZ-AlaNP}]_0 = 1.99 \times 10^{-5}$ M; $[HSA]_0 = 1.00 \times 10^{-4}$ M.

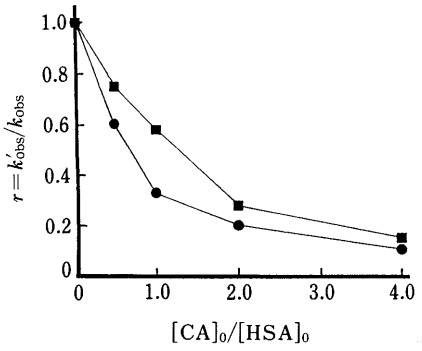


Fig. 4. Effects of CA on the Reaction Rate of *N*-CBZ-AlaNP with HSA

●, D-enantiomer; ■, L-enantiomer; $[N\text{-CBZ-AlaNP}]_0 = 9.95 \times 10^{-6}$ M; $[HSA]_0 = 5.00 \times 10^{-5}$ M; pH 7.4 1/15 M phosphate buffer ($\mu = 0.2$) at 25 °C; k_{obs} for D-enantiomer = $1.17\ s^{-1}$; k_{obs} for L-enantiomer = $0.523\ s^{-1}$.

the reaction rate with the substrates were examined. It has been reported that CI[5] and NA[21] acylate tyrosine-411 residue of the R site[5,22] and lysine-199 residue of the U-site,[21,22] respectively. Tables III and IV show the results of the effects of the chemical modifications. The k^{m}_{obs} values for the reactions of the substrates with cinnamoyl-HSA (Table III) were about 1/100 and 1/30 of the values for the reactions of D- and L-enantiomer with control-HSA, respectively, indicating the reactive sites towards the substrates to be the R site. There seems to be no significant change in k^{m}_{obs} between the control-HSAs and the acetyl-HSA (Table IV).

To further localize the reactive site towards *N*-CBZ-AlaNP, the effects of some drugs, whose binding sites on HSA are already known, on k_{obs} were examined. Figure 4 shows the results for CA which binds to the R site.[22,23] In this figure k'_{obs} on the ordinate is the rate constant in the presence of CA. CA inhibits the reactions of both substrates with HSA, indicating the reactive site towards these substrates to be the R site.

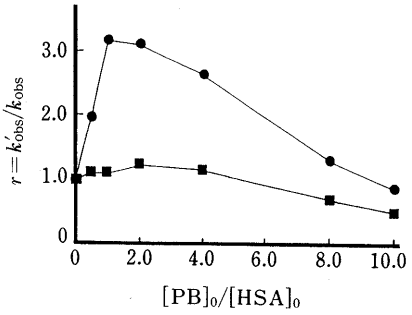


Fig. 5. Effects of PB on the Reaction Rate of *N*-CBZ-AlaNP with HSA

●, D-enantiomer; ■, L-enantiomer. The experimental conditions are the same as those in Fig. 4.

Figure 5 shows the effects of PB, which binds primarily to the U site and secondarily to the R site, on the reaction rate with *N*-CBZ-AlaNP. The binding of PB to HSA accelerates the reaction with D-enantiomer and has apparently no effect on the reaction with L-enantiomer. The acceleration for D-enantiomer may be due to a conformational change of the R site induced by PB binding.[24] The conformational change discriminates sterically the D- and L-enantiomers. PB inhibits the reaction of HSA with *p*-nitrophenyl acetate (NPA)[22] which is a typical substrate for the R site. The reason for the difference in the effects of PB on the reaction rates with these three substrates (the D- and L-enantiomers and NPA) is now under study.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and also by funds from the Uehara Memorial Foundation.

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18) Since a referee recommended the derivation of Eq. 1 from the reaction scheme shown in Chart 1, its derivation is briefly described here. The details are referred to the references.[19,20] According to the reaction scheme, the rate of NP formation is given by

$$d[NP]/dt = -d([S]+[S\cdot HSA])/dt$$
$$= -d[S]_T/dt = k_0[S]+k_2[S\cdot HSA] \quad (3)$$

where $[S]_T$ is the residual total substrate concentration at time t. The observed first-order rate constant, k_{obs}, is given by

$$k_{obs} = (-d[S]_T/dt)/[S]_T = k_0F_u + k_2F_c = k_0 + (k_2 - k_0)F_c \quad (4)$$

where F_u and F_c are the fractions of the reactant which are uncomplexed and complexed at time t, respectively.

The equilibrium condition for the 1 : 1 complex formation involving the reactant is given by Eq. 5.

$$K_S = [S][HSA]/[S\cdot HSA]$$
$$= ([S]_0 - [S\cdot HSA])([HSA]_0 - [S\cdot HSA])/[S\cdot HSA] \quad (5)$$

Under the conditions where $[HSA]_0 \gg [S]_0$, Eq. 5 simplifies to

$$K_S = ([S]_0 - [S\cdot HSA])[HSA]_0/[S\cdot HSA] = (1-F_c)[HSA]_0/F_c \quad (6)$$

Combination of Eqs. 4 and 6, with elimination of F_c, leads Eq. 1 in the text.
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