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Degradation of 4′-Methylumbelliferyl 4-Guanidinobenzoate Catalyzed by Human Serum Albumin

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The degradation of 4′-methylumbelliferyl 4-guanidinobenzoate (MUGB) was investigated in the presence of human serum albumin (HSA). HSA enhanced the rate of hydrolysis by a factor of 46.6 at pH 7.4. The pH-profile for the degradation of MUGB·HSA complex showed an inflection point at about pH 9.0, suggesting the involvement of a group with a pK_a of about 9. Inactivation of HSA with diethylpyrocarbonate (a histidine-specific reagent), and concomitant reactivation by hydroxylamine, indicated that histidine residues are involved in the esterase-like activity. Statistical analysis of the residual activity remaining in the partially modified HSA showed that only one histidine residue was critical for the activity among the 16 modifiable residues. In comparison with the HSA-catalyzed degradation of gabexate mesilate (GM) containing a guanidino group, the effects of site-specific ligands (warfarin, phenylbutazone and clofibric acid) on the HSA activity for MUGB degradation were examined. The results suggested that the active site for MUGB degradation is located far from the U-site and is different from the active site for GM degradation.

Keywords——4′-methylumbelliferyl 4-guanidinobenzoate; drug stability; albumin; esterase-like activity; protein binding; kinetics

It has been shown that human serum albumin (HSA) has a nonspecific esterase-like activity towards some model substrates[1] various ester and amide drugs such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea,[2] gabexate mesilate (GM),[3] meclofenoxate hydrochloride.[4] Also, HSA degrades disulfiram through its sulfhydryl group (Cys-34).[5] Such HSA-catalyzed degradations can be regarded as an important aspect of the drug disposition in the circulation which is strongly related to the subsequent physiological activity.

4′-Methylumbelliferyl 4-guanidinobenzoate (MUGB) is used as an active-site titrant for many serine proteases, *e.g.*, trypsin, and thrombin due to the formation of stable *p*-guanidinobenzoyl-linked enzymes.[6] MUGB also binds with acrosin, one of the serine proteases, forming the acyl-enzyme in which serine residue is involved.[7] Acrosin is associated with the sperm acrosome and has an essential function in the fertilization process. The addition of acrosin inhibitors (MUGB and other aryl-guanidinobenzoates) has been shown to prevent fertilization both *in vitro* ($I_{50}=9.8\times10^{-10}$ M in mouse[7]) and *in vivo* in the rabbit, rodent, and primate.[8]

Shwartz demonstrated that MUGB is degraded in plasma and amniotic fluid, and albumin may be responsible for this degradation.[9] Since the mechanism involved is poorly understood, we examined the degradation behavior of MUGB in purified HSA and in chemically-modified HSA in order to elucidate possible active residues.

Experimental

Materials——HSA (Fraction V, lot no. 85 F-9351) was purchased from Sigma Chemicals, and was used after purification by the method of Chen.[10] The molecular weight of HSA was assumed to be 69000 and the concentration was determined from the absorption at 278 nm ($E^{0.1\%}_{1\,cm}=0.531$).[1] 4′-Methylumbelliferyl 4-guanidinobenzoate

(MUGB), diethylpyrocarbonate (DEP) and diisopropyl fluorophosphate (DFP) were obtained from Sigma. 2,4,6-Trinitrobenzenesulfonic acid sodium salt (TNBS) and hydroxylamine hydrochloride were purchased from Wako Chemical Co. Gabexate mesilate (GM, Ono Pharmaceutical Co.), potassium warfarin (WF, Eisai Co.), phenylbutazone (PB, Japan Ciba-Geigy Co.) were gifts from the respective manufactures. Clofibric acid (CA) was the same as used in a previous study.[11]

Measurement of MUGB and GM Degradation——MUGB stock solution (15 μl) was added to 3 ml of buffer solution or HSA solution preincubated in a cell (25 $\pm$ 0.2 °C) secured in a spectrophotometer (UV-260, Shimadzu). The degradation of the MUGB was followed by monitoring the appearance of 4-methylumbelliferone (MU) at 360 nm. The concentration of HSA was kept more than 5-fold in excess of that of MUGB (1.0×10^{-5} M), so that the MUGB would react preferentially with a primary reactive site on the HSA.[1,12] For pH-profiles studies, Sörensen buffer (pH 6.5—8.5) and Kolthoff buffer (pH 9.0—11.0) were used as reaction media. The ionic strength was adjusted to 0.2 with NaCl. The temperature was kept at 25 °C. The degradation of GM was followed by high-performance liquid chromatography (HPLC). The chromatographic conditions were as described previously.[3] The effects of specific ligands (WF, PB and CA) were investigated after adding them to the reaction mixture.

Effect of Trinitrobenzenesulfonate (TNBS) and DFP[13,14]——After trinitrophenylation of HSA by TNBS (HSA, 1.0×10^{-4} M; TNBS, 5.0×10^{-5} M), the activity of the modified HSA towards MUGB was measured. DFP (20-fold molar excess of HSA) was reacted with HSA at pH 7.4, 25 °C. Then, after 14 h, the activity of HSA was measured.

Inactivation of HSA with DEP and Reactivation with Hydroxylamine——Carbethoxylation of histidine residues in HSA was carried out by the method described previously.[3] The number of histidine residues modified (m) was calculated from the absorbance at 242 nm ($\varepsilon = 3.3 \times 10^3\ M^{-1}cm^{-1}$). The residual activity of the modified HSA was determined after separation through a Sephadex G-25 column (1.0 cm i.d. $\times$ 20 cm). The procedure for deacylation of the *N*-carbethoxyhistidine by 0.5 M hydroxylamine hydrochloride was as described elsewhere.[3]

Results and Discussion

The degradation of MUGB in the presence of HSA followed first-order kinetics, and the pseudo-first-order rate constant (k_{obs}) increased asymptotically with increasing HSA concentration (Fig. 1a). The dependency of k_{obs} on the HSA concentration was analyzed on the basis of Michaelis–Menten type kinetics involving the formation of HSA–drug complex (Chart 1).[12] Where MUGB·HSA is the Michaelis–Menten type complex, GB-HSA is *p*-guanidinobenzoyl-HSA, GB and MU represent *p*-guanidinobenzoic acid and 4-methylumbelliferone, k_0 and k_2 are the pseudo-first-order rate constants for the degradation of MUGB and

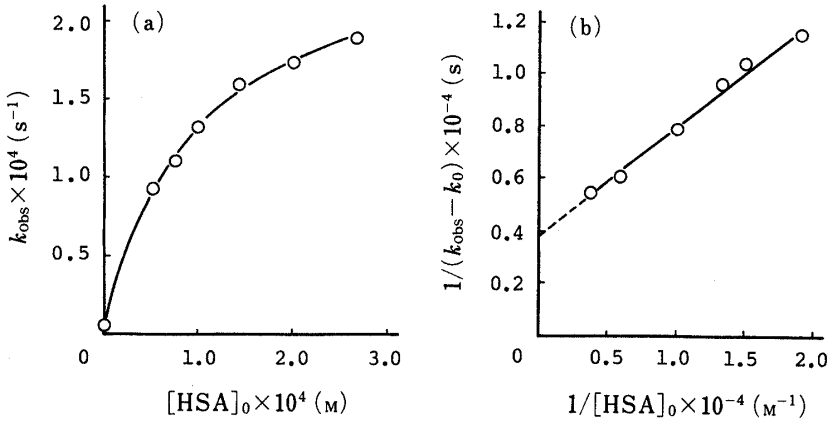

Fig. 1. Effect of HSA Concentration on the Degradation of MUGB
MUGB, 1.0×10^{-5} M at pH 7.4 and 25 °C.

$$\begin{array}{ccccc} \text{MUGB} + \text{HSA} & \overset{K_s}{\rightleftharpoons} & \text{MUGB}\cdot\text{HSA} & \xrightarrow{k_2} & \text{GB-HSA} + \text{MU} \\ \downarrow k_0 & & & & \downarrow k_3 \\ \text{MU} + \text{GB} & & & & \text{GB} + \text{HSA} \end{array}$$

Chart 1

the complex, respectively. K_s is the dissociation constant of the complex. Assuming that the concentration of HSA is in excess of the substrate, the k_{obs} may be expressed as follows[12]:

$$k_{obs}=\frac{k_0K_s+k_2[HSA]_0}{K_s+[HSA]_0} \quad (1)$$

where $[HSA]_0$ is the initial concentration of HSA. Eq. 1 can be rearranged to the following double reciprocal form:

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

Figure 1b shows a typical linear plot according to Eq. 2 for the dependency of k_{obs} on the concentration of HSA at pH 7.4. The values of k_0, k_2 and K_s were estimated to be $5.77\times10^{-6}s^{-1}$, $2.69\times10^{-4}s^{-1}$ and 1.10×10^{-4} M, respectively, indicating that the MUGB·HSA complex decomposes almost 47 times faster than the free form of MUGB.

Figure 2 shows pH profiles for k_0, k_2 and K_s. The K_s value became smaller with increasing pH. At pH 10.5, MUGB binds with HSA 35-fold more effectively than at pH 6.5. The difference is apparently too large to be regarded as the contribution of the N-B transition,[13] so suggesting the presence of an anionic binding center on HSA with increasing pH. Concerning the high pK_a value (12—13) of the guanidino group, it is assumed that the electrostatic interaction between negatively charged groups on the protein surface and a cationic center of the drug is primarily involved in addition to a possible hydrophobic interaction.

Kuwata *et al.* reported that at alkaline pH and at low ionic strength, mercaptalbumin isomerizes slowly to the aged form (A-form) by an intramolecular SH/S–S exchange reaction.[14] We investigated the esterase-like activity of HSA at a constant high ionic strength (0.2), so that N-A isomerization is unlikely to occur in the course of degradation of MUGB.[14] It has been reported, however, that commercial HSA contains a small amount of A-form, and the HSA activity observed in the present study might be regarded as the total activity of HSA with microheterogeneity.[15]

The pH profile of log k_2 revealed an inflection point at about pH 9.0, suggesting that either lysine or tyrosine residue might be involved in the esterase-like activity of HSA. However, these residues have no relevance to the esterase-like activity of HSA since no effect was found on the activity by pretreatment with TNBS or DFP. TNBS is a specific reagent for

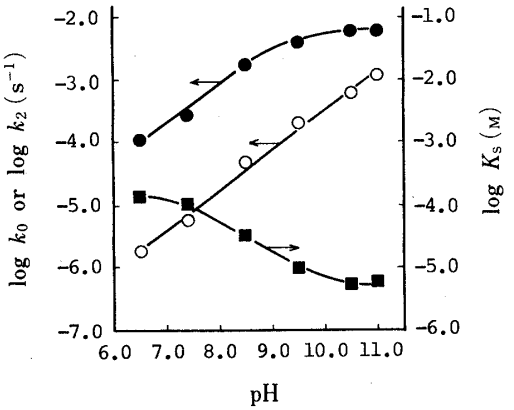

Fig. 2. pH Dependence of log k_0, log k_2 and log K_s for the Degradation of MUGB at 25 °C
○, k_0; ●, k_2; ■, K_s.

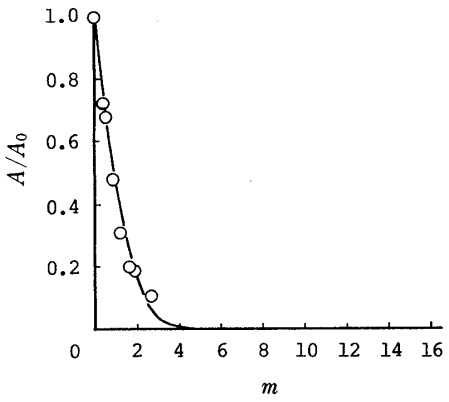

Fig. 3. Plot of the Residual HSA Activity (A/A_0) against the Number of Residues Modified (m)
The solid line was calculated from Eq. 3 with $n=16$, $p=i=1$ and $\alpha=0.044$.

lysine residues (in particular, Lys-199 is most reactive[16]) and DFP induces diisopropylphosphorylation of the reactive Tyr-411[17] in HSA.

To examine the possibility of involvement of histidine residues, HSA was modified by DEP, which has been widely used as a histidine-acylating reagent for proteins.[18,19] Figure 3 shows the relationship between the residual activity of HSA (A/A_0) and the number of histidine residues modified (m), where A is the k_{obs} of DEP-modified HSA and A_0 is the k_{obs} of intact HSA. The activity was linearly decreased when the number of modified residues was less than 2, and gradually diminished with increasing m. However, extrapolation of the fraction of activity remaining from the first-phase reaction to zero activity does not always provide an accurate estimate of the number of essential residues.[20,21] The statistical analysis of Tsou[20] was therefore used to calculate the number of essential histidine residues responsible for the activity of HSA. The method was based on the assumption that all 16 histidine residues present in one HSA molecule were modifiable, and stepwise modification of the essential residues results in complete inactivation.

The relationship between the number of histidine residues modified (m) and the residual activity of HSA (A/A_0) can be derived from:

$$m=n(1-x)=n-p(A/A_0)^{1/i}-(n-p)(A/A_0)^{\alpha/i} \quad (3)$$

where it is assumed that HSA has n modifiable residues among which p residues including i essential residues react with the reagent at a pseudo-first-order rate constant (k_1) and $n-p$ residues which are not essential react at $k_2(=\alpha k_1)$. The fraction of unmodified residues is $x(=(n-m)/n)$. Eq. 3 can be rewritten in the form of

$$\log[nx/(A/A_0)^{1/i}-p]=\log(n-p)+((\alpha-1)/i)\log(A/A_0) \quad (4)$$

Figure 4 shows a plot of the left-hand component in Eq. 4 against $\log(A/A_0)$ were $n=16$ and $p=i=1$. Only this condition yielded a good linear fit for the experimental data. Thus, the results indicated that only one histidine residue is most likely to be critical for the catalytic function. The value of $\alpha\,(=k_2/k_1)$ estimated from the slope was 0.044. The solid line in Fig. 3 was calculated by substituting the parameters obtained into Eq. 3, which gives a good agreement with the experimental data.

Hydroxylamine is a reagent which can remove the carbethoxy groups from DEP-treated histidine, tyrosine and serine.[18,19] The activity of HSA which was inactivated by DEP to 11% of that of intact HSA was fully restored by treatment with hydroxylamine within 3 h (Table I). A similar recovery of activity was observed for GM degradation by DEP modified HSA.[3] Considering the effect of DFP mentioned above, and the absence of reactive serine(s),[16] these

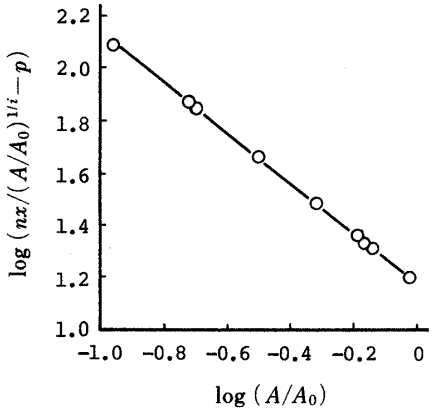

Fig. 4. Tsou's Analysis of the Data According to Eq. 4

TABLE I. Restoration of Inactivated HSA with Hydroxylamine

Incubation time (h)	Activity[a] (%)
0	12
0.5	71
1.0	89
3.0	100

a) k_{obs} (treated with NH_2OH)/k_{obs} (intact HSA) × 100.

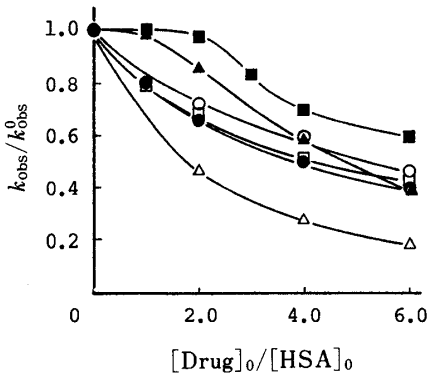

Fig. 5. Effects of Various Drugs on the Degradation of MUGB and GM at pH 7.4 and 25 °C

Closed symbols, MUGB; open symbols, GM. △, Warfarin (WF); □, phenylbutazone (PB); ○, clofibric acid (CA).

results support the involvement of histidine residues.

While the pK_a of the imidazole ring of histidine generally falls in the range of 6—7, the apparent pK_a of 9.0 obtained from Fig. 2 raises the question of whether this value should be assigned to the histidine residue. It is worth commenting here, therefore, that such an apparently high pK_a would not be unrealistic for histidine for two reasons. First, the pK_a of His-159 in papain which participates in the catalytic activity is reported to be 8.4, and the high pK_a has been ascribed to ion pair formation between His-159 and Cys-25.[22] Second, Harmsen *et al.*[23] and Labro and Janssen[24] have reported that imidazole groups which show an abnormally high pK_a value (8.0 or 8.3) are involved in the N-B transition, forming salt bridges in bovine serum albumin (BSA) or HSA. Nevertheless, the reason for the exceptional pK_a value estimated in this study remains unclear. It is also possible that carbethoxylation of histidine, trinitrophenylation of lysine and diisopropylphosphorylation of tyrosine might induce a conformational change in HSA, leading to steric hindrance for MUGB binding. Although differential ultraviolet (UV) spectroscopy of the native and modified HSA did not reveal any gross conformational change induced by DEP or DFP modification, further studies may have to be extended to estimate the helical content.

As previously reported,[3] GM, which is also a cationic substrate carrying a guanidino group, was degraded in a similar manner to MUGB, and an active site of the degradation with a pK_a value about 6 was identified from the log k_2–pH profile. Tsou's analysis demonstrated that only one histidine residue was involved in the degradation, which is identical with MUGB, *i.e.* $p=i=1$. However, $i=1$ does not always mean that the same histidine residue contributes to the degradation of both drugs. In other words, it is possible that different histidine residues on HSA may participate in the degradation of MUGB and GM, respectively.

To examine this point further, the effects of site-specific ligands on the HSA activity for both MUGB and GM were investigated. Warfarin and phenylbutazone can be classified as a U-site binding drug and clofibric acid a R-site binding drug.[1b] As shown in Fig. 5, the U-site drugs caused no inhibition of the activity for MUGB degradation up to a 2 : 1 molar ratio to the protein, whereas they caused inhibition for GM degradation. These results suggest that the active site for MUGB degradation is located far from the U-site and is different from the active site for GM degradation, but both the active sites exist within the range susceptible to the influences of R-site binding.

When MUGB is employed as a contraceptive agent, the HSA esterase-like activity should be observed if the drug is administered as a vaginal cream.[25] BSA can also degrade MUGB with 3-fold less activity than HSA (data not shown), and attention should be paid to the use of albumin solution as a medium in fertilization experiments with aryl 4-guanidinobenzoates.[7]

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