Esterase-like Activity of Human Serum Albumin. IV. 1) Reactions with Substituted Aspirins and 5-Nitrosalicyl Esters

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To elucidate the reactivity of the lysine-199 residue of human serum albumin (HSA) with ester-type drugs and to characterize the region near the lysine, the reactions of substituted aspirins and 5-nitrosalicyl esters with HSA were investigated kinetically at 25℃. The Michaelis constant (K_{m} in m) and the catalytic rate constant (k_{c} in s^{-1}) were determined, and the relationship between these constants and the structure of the substrates was explored. For the reactions with substituted aspirins at pH 9.9, the logarithm of k_{c} is correlated with the pK_{a} value of the hydroxyl group in the parent salicylic acid. The regression line is as follows: log k_{c} = -0.843pK_{a} + 6.75 (r = -0.988). The K_{m} value is influenced by the number of substituents on the phenyl ring rather than the nature of the substituents. For the reactions with 5-nitrosalicyl esters at pH 7.4, the k_{c}/K_{m} values were correlated with Hansch's hydrophobic substituent constant π and Taft's steric constant E_{s} as follows: log(k_{c}/K_{m}) = 0.423π + 0.386E_{s} + 1.35 (r = 0.908).

Keywords—human serum albumin; esterase-like activity; quantitative structure-activity relationship; kinetics; aspirin; 5-nitrosalicyl ester; amino acid sequence

In the previous papers 1−3) it was reported that human serum albumin (HSA) has esterase-like activity toward phenyl acetates, p-nitrophenyl esters, and cinnamoylimidazoles. The quantitative structure-activity relationships for the reactions with the ester substrates were examined in order to characterize the reactive site of HSA. 2) The reactive site toward these substrates was found to be located near the tyrosine-411 (Tyr-411) residue of the HSA amino acid sequence, 4) and was named the R site. 5) However, it was found that the reactive site towards some substituted aspirins (which are phenyl acetates with a carboxyl group at the ortho position) is located near the lysine-199 (Lys-199) residue, away from the R site. 6) The site near the Lys-199 residue, referred to the U site, is one of the important drug-binding sites. 5−7) It is of importance not only to characterize the U site but also to elucidate the reactivity with substituted aspirins, since HSA may affect the cleavage of these ester-type drugs in vivo.

In this study the reactions of substituted aspirins and 5-nitrosalicyl esters with the U site of HSA were investigated kinetically in order to characterize the U site and to elucidate the reactivity of HSA with ester-type drugs. The Michaelis constants and the catalytic rate constants for the reactions were determined, and the relationships between these constants and the structures of the substrates were explored.

Experimental

Materials—HSA (Fraction V, lots 47C-04421, 37F-02271, and 100F-02061, Sigma Chem. Co.) was used after purification by Chen's procedure. 5) The molecular weight of HSA was assumed to be 69000 and the concentration was determined based on an extinction coefficient E_{1%}^{1cm} of 0.331 at 278 nm. 5) According to the method of Zaugg et al., 10) substituted aspirins and 5-nitrosalicyl esters were synthesized from the corresponding salicylic acid and acid
Table I. Substrates Used and Their Melting Points

<table>
<thead>
<tr>
<th>Substrates</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>mp (°C)</th>
<th>Literature values, mp (°C)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
<td>132—134</td>
<td>135&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>NO₂</td>
<td>H</td>
<td>CH₃</td>
<td>163—165</td>
<td>164—165&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>153.5—154.5&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>NO₂</td>
<td>NO₂</td>
<td>CH₃</td>
<td>93—94</td>
<td>92.5—94.0&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Br</td>
<td>Br</td>
<td>CH₃</td>
<td>153—154</td>
<td>155—157&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>Cl</td>
<td>Cl</td>
<td>CH₃</td>
<td>138—141</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NO₂</td>
<td>H</td>
<td>CH₂CH₃</td>
<td>104—105</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>NO₂</td>
<td>H</td>
<td>CH₂(CH₃)₂</td>
<td>95—97</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>NO₂</td>
<td>H</td>
<td>CH₂(CH₂)₂CH₃</td>
<td>74—76</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>NO₂</td>
<td>H</td>
<td>CH₂(CH₂)₂CH₃</td>
<td>86—88</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>NO₂</td>
<td>H</td>
<td>CH(CH₃)₂</td>
<td>119—124</td>
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<tr>
<td>11</td>
<td>NO₂</td>
<td>H</td>
<td>CH₂CH(CH₃)₂</td>
<td>91—93</td>
<td></td>
</tr>
</tbody>
</table>

anhydride in the presence of a small amount of sulfuric acid. The esters were recrystallized from ether or benzene. The esters used and their melting points are listed in Table I. The results of elemental analyses of the synthesized esters are not listed, because the analytical values obtained for carbon, hydrogen, and nitrogen were all within the range of the calculated value ± 0.3%. The salicylic acids, acid anhydrides, aspirin (1), and all other chemicals used were obtained commercially. The numbers in Table I are those used in this paper to refer to the substrates.

**Kinetic Runs**—The reaction rate of the substrate with HSA was followed spectrophotometrically at an appropriate wavelength for monitoring the appearance of the corresponding salicylic acid. A Hitachi spectrophotometer (UV-124) and a Union Giken stopped-flow spectrophotometer (RA-401) were used for the measurements of the rates. The reaction was carried out in the presence of excess HSA over the substrate. The concentrations of the substrates ranged from 2.0 x 10⁻⁶ to 2.0 x 10⁻⁵ M, depending on the molar absorptivities of the salicylic acids and also on the binding affinities of the substrates to HSA. The pseudo-first order rate constant (k<sub>obs</sub>) was determined from a plot of log (A<sub>e</sub>—A) against time, where A<sub>e</sub> and A are the absorbances at completion of the reaction and at time t, respectively. The reaction solution always contained 0.5% (v/v) acetonitrile for experimental convenience, and the temperature was 25°C. The buffer solutions used were pH 7.4, 1/15 M phosphate (μ=0.2, adjusted with sodium chloride) and pH 9.9, 2/5 M carbonate for the reactions with 5-nitrosalicylic esters and with substituted aspirins, respectively.

**Determination of Kinetic Parameters for the Reaction of Substrates with HSA**—The reaction of a substrate (S) with HSA can be expressed as shown in Chart 1. <sup>2,6</sup> Abbreviations in Chart 1 are: S·HSA, the Michaelis–Menten type complex between S and HSA; P, the corresponding salicylic acid released; Acyl-HSA, HSA acetylated with S; K<sub>S</sub>, dissociation constant of S·HSA; k<sub>2</sub>, catalytic rate constant; k<sub>0</sub>, hydrolysis rate constant of S. According to Chart 1, the k<sub>obs</sub> value determined experimentally can be represented by equation (1).<sup>1—3,6</sup>

\[
k_{\text{obs}} = \frac{k_0K_S + k_2[HSA]_0}{K_S + [HSA]_0}
\]  

(1)

where [HSA]₀ is the initial concentration of HSA. The K<sub>S</sub> and k<sub>2</sub> values can be calculated from the slope and intercept of the double-reciprocal plot based on equation (2).
\[ \frac{1}{k_{\text{obs}} - k_0} = \frac{K_3}{(k_2 - k_0) [\text{HSA}]]_0} + \frac{1}{k_2 - k_0} \]  

(2)

**Determination of pK_3 for Salicylic Acids** — The pK_3 value of the hydroxyl group in salicylic acids was determined spectrophotometrically based on equation (3). \(^{15}\)

\[ \log \frac{A - A_p}{A_p - A} = pK_3 - pH \]  

(3)

In equation (3), \(A_p\), \(A_p\), and \(A\) are the absorbances at an appropriate wavelength for the free form, ionic form, and their mixture, respectively.

**Results and Discussion**

**Substituted Aspirins**

Table II lists the kinetic parameters for the reactions of substituted aspirins with HSA, and also the pK_3 values of the hydroxyl group in the parent salicylic acid molecules. Although the number of substituted aspirins used is small (\(n = 5\)), the logarithm of \(k_2\) was correlated well with the pK_3 value of the hydroxyl group. The regression line is expressed by equation (4).

\[ \log k_2 = -0.843 \pm 0.246 \text{ pK}_3 + 6.75 \pm 2.59 \]  

(4)

\[ n=5, s=0.313, r=-0.988 \]

In equation (4), the values in parentheses are the 95\% confidence intervals; \(n\), the number of aspirin derivatives used; \(s\), the standard deviation; \(r\), the correlation coefficient. In the previous study, \(^6\) it was found that the catalytic group of HSA for the reactions with 1 and 2 is the lysine-199 (Lys-199) residue. The negative slope (−0.843) hence suggests that the reaction of the substrate with HSA occurs through the nucleophilic attack of the free ε-amino group of Lys-199 at the carbonyl carbon atom of the substrate. It is of interest to compare the correlation for the aspirin derivatives with that for phenyl acetates obtained previously. \(^2\)

The relationship for phenyl acetates excluding aspirin was expressed by equation (5).

\[ \log k_2 = -0.751 \pm 0.169 \text{ pK}_3 + 5.55 \pm 1.51 \]  

(5)

\[ n=5, s=0.122, r=-0.993 \]

Although many differences exist between the two reactions, e.g., in the catalytic group of HSA, *ortho* substituent effect, and charge of the substrates, the differences in the slope and intercept between equations (4) and (5) are small. This phenomenon may be explained as follows. The nucleophilicity of a catalyst is related to pK_3 of the catalyst. \(^{17,18}\) The pK_3 values of both Lys-199 and Tyr-411 of HSA were found previously \(^2,6\) to be about 9.5, and thus the nucleophilicities of both groups are presumed to be identical. \(^{17,18}\)

Moreover, the *ortho* effect

**Table II. Rates and Dissociation Constants for Reactions of Substituted Aspirins with HSA\(^a\)**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>(k_2) (s(^{-1}))</th>
<th>(K_3) (M)</th>
<th>(k_0) (s(^{-1}))</th>
<th>pK_3 (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(4.0 \times 10^{-4})</td>
<td>(2.2 \times 10^{-3})</td>
<td>(2.1 \times 10^{-5})</td>
<td>12.4 (^c)</td>
</tr>
<tr>
<td>2</td>
<td>(4.2 \times 10^{-2})</td>
<td>(1.6 \times 10^{-4})</td>
<td>(6.4 \times 10^{-4})</td>
<td>9.9</td>
</tr>
<tr>
<td>3</td>
<td>6.1</td>
<td>(7.3 \times 10^{-5})</td>
<td>(1.9 \times 10^{-3})</td>
<td>7.1</td>
</tr>
<tr>
<td>4</td>
<td>(9.8 \times 10^{-4})</td>
<td>(3.6 \times 10^{-5})</td>
<td>(1.3 \times 10^{-5})</td>
<td>11.2</td>
</tr>
<tr>
<td>5</td>
<td>(1.2 \times 10^{-3})</td>
<td>(7.3 \times 10^{-5})</td>
<td>(2.5 \times 10^{-5})</td>
<td>11.2</td>
</tr>
</tbody>
</table>

\(a\) pH 9.9, 2/5 M carbonate buffer containing 0.5% (v/v) acetonitrile and 25°C.

\(b\) pK_3 of the hydroxyl group of the corresponding salicylic acid.

\(c\) Literature value. \(^{10}\)
Table III. Rates and Dissociation Constants for Reactions of 5-Nitrosalicyl Esters with HSA

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$K_S$ (M)</th>
<th>$k_0$ (s$^{-1}$)</th>
<th>$k_2/K_2$ (M$^{-1}$ s$^{-1}$)</th>
<th>$\pi$</th>
<th>$E_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$8.4 \times 10^{-4}$</td>
<td>$2.6 \times 10^{-5}$</td>
<td>$7.6 \times 10^{-6}$</td>
<td>$3.2 \times 10^{-1}$</td>
<td>0.50</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>$4.4 \times 10^{-4}$</td>
<td>$8.5 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-5}$</td>
<td>$5.2 \times 10^{-1}$</td>
<td>1.00</td>
<td>-0.07</td>
</tr>
<tr>
<td>7</td>
<td>$1.2 \times 10^{-3}$</td>
<td>$1.5 \times 10^{-5}$</td>
<td>$5.9 \times 10^{-6}$</td>
<td>$8.0 \times 10^{-1}$</td>
<td>1.50</td>
<td>-0.36</td>
</tr>
<tr>
<td>8</td>
<td>$9.3 \times 10^{-4}$</td>
<td>$9.1 \times 10^{-6}$</td>
<td>$5.2 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-2}$</td>
<td>2.00</td>
<td>-0.39</td>
</tr>
<tr>
<td>9</td>
<td>$1.8 \times 10^{-4}$</td>
<td>-</td>
<td>$6.0 \times 10^{-6}$</td>
<td>-</td>
<td>2.50</td>
<td>-0.40</td>
</tr>
<tr>
<td>10</td>
<td>$2.5 \times 10^{-3}$</td>
<td>$3.7 \times 10^{-5}$</td>
<td>$8.3 \times 10^{-6}$</td>
<td>$6.8 \times 10^{-1}$</td>
<td>1.30</td>
<td>-0.47</td>
</tr>
<tr>
<td>11</td>
<td>$6.2 \times 10^{-4}$</td>
<td>$1.3 \times 10^{-5}$</td>
<td>$1.8 \times 10^{-6}$</td>
<td>$4.8 \times 10^{-1}$</td>
<td>1.80</td>
<td>-0.93</td>
</tr>
</tbody>
</table>

a) pH 7.4, 1/15 M phosphate buffer ($\mu=0.2$) containing 0.5% (v/v) acetonitrile and 25°C.
b) Hansch's hydrophobic substituent constant.$^{20}$
c) Taft's steric substituent constant.$^{21}$
d) Could not be determined accurately.

and the charge difference of the substrates seem to be reflected in the individual p$K_a$ values of the hydroxy groups in salicylic acids and phenols. The correlations expressed by equations (4) and (5) were obtained using such p$K_a$ values. Hence, the similarity of the two correlations, obtained for substituted aspirins and phenyl acetates, may be reasonable.

The binding affinity of the substrate to HSA, which is reflected by $K_S$, is affected in a complicated manner by the substituents. The $K_S$ values are influenced by the number of the substituents rather than the nature of the substituents, since the values of $K_S$ decrease in the order $1 > 2 > 3$ and are identical for 3 and 5.

Since the kinetics and mechanism of the hydrolysis of substituted aspirins have been reported in the literature,$^{13,14,19}$ the $k_0$ values given in Table II will not be discussed here.

5-Nitrosalicyl Esters

Table III lists the kinetic parameters for the reactions of 5-nitrosalicyl esters with HSA, and also gives $k_0$ for comparison. The $K_S$ value for 9 was too small to be determined accurately under the experimental conditions employed. For the same carbon number in the acyl group, the $K_S$ value of a compound with a branched chain is larger than that of the compound with a normal chain ($K_S$ for 7 < $K_S$ for 10 and $K_S$ for 8 < $K_S$ for 11). As regards $k_2$ and $k_0$, we could not find any clear correlations.

For the reactions of p-nitrophenyl esters with a-chymotrypsin, Milstien and Fife$^{22}$ found that a plot of log ($k_2/K_S$) against Taft's steric substituent constant $E_S$ gives a fair correlation. Moreover, Hansch and Coats$^{20}$ reported that the addition of the hydrophobic substituent constant $\pi$ to the above correlation results in a much better correlation. Hence, similar regression analyses were tried for the $k_2/K_S$ values obtained for the reactions of 5-nitrosalicyl esters with HSA. The plot of log ($k_2/K_S$) versus $\pi$ is shown in Fig. 1 ($\bullet$). With the exception of 11, a good relationship was obtained, as expressed by equation (6).

$$\log (k_2/K_S) = 0.335 (\pm 0.109) \pi + 1.37 (\pm 0.148)$$

(6)

$$n = 6, s = 0.0384, r = 0.985$$

Since 11 has a relatively bulky side chain, the steric factor $E_S$ was added for the analysis for all compounds including 11. The result was as follows:

$$\log (k_2/K_S) = 0.423 (\pm 0.374) \pi + 0.386 (\pm 0.616) E_S$$

$$+ 1.35 (\pm 0.388)$$

(7)

$$n = 6, s = 0.0955, r = 0.908$$
Although the effect of addition of the $E_s$ term is not statistically significant at the 0.95 level of significance, the point for 11 appears to be better fitted than in the case of the log($k_2/K_3$) versus $\pi$ plot, as shown in Fig. 1 (□).

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**References**