Physicochemical Property Modification Strategies Based on Enzyme Substrate Specificities II: α-Chymotrypsin Hydrolysis of Aspirin Derivatives

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Abstract □ Three aspirin derivatives, aspirin phenylalanine ethyl ester, aspirin phenylalanine amide, and aspirin phenyllactic ethyl ester, were investigated with respect to their hydrolysis by α-chymotrypsin. Of the three compounds, aspirin phenylalanine ethyl ester was the best substrate, with $k_{cat} = 25\ sec^{-1}$ and $K_m = 1.3 \times 10^{-6}\ M$ at pH 8.0. The results for all substrates were in the range of expectation based on kinetic data for other substrates. The apparent latitude in the nature of the acylamide substituent of α-chymotrypsin substrates makes this enzyme a good potential reconversion site for many drug derivatives.

Keyphrases □ Aspirin—derivatives, hydrolysis by α-chymotrypsin □ Derivatives—aspirin, synthesis by α-chymotrypsin hydrolysis □ α-chymotrypsin—hydrolysis of aspirin derivatives □ Physicochemistry—modification strategies based on enzyme substrate specificities, α-Chymotrypsin hydrolysis of aspirin derivatives

Previously (1), a rationale for selecting amino acid derivatives of aspirin was described and the syntheses of aspirin phenylalanine ethyl ester (I), aspirin phenylalanine amide (II), and aspirin phenyllactic ethyl ester (III) were reported. These derivatives were so designed that after an initial cleavage of the terminal ethyl ester or amide linkage, carboxypeptidase A regenerates aspirin *in vivo*. This paper reports kinetic studies of these derivatives in the presence of α-chymotrypsin.

EXPERIMENTAL

Materials—α-Chymotrypsin was obtained as a dialyzed, salt-free, lyophilized-powder[1]. The operational normality of α-chymotrypsin solutions was determined by a direct spectrophotometric titration as described previously (2).

Kinetic Measurements—The hydrolysis of the prodrugs was followed by automatic titration[2] at a constant pH.

All solvents and chemicals were reagent grade. Triple-distilled water was used in preparing solutions, and the reaction vessel was thermostated at 25.00 ± 0.02°. Dry nitrogen gas was gently blown into the reaction vessel to exclude atmospheric carbon dioxide from the system.

The titrating mechanism was set at the desired pH, and sufficient volume of the prodrug solution containing 0.1 *M* potassium chloride was pipetted into the reaction vessel and allowed to equilibrate for 10 min during magnet stirring. The solution was then brought to the desired pH; when a stable baseline was obtained, 10 μl of a suitable concentration of enzyme was added. The automatic titrating mechanism maintained the set pH during the hydrolytic reaction by adding increments of base as small as 0.1 μl and continuously recording the amount of base consumed *versus* time.

In runs with III, substrate stock solutions were made in acetonitrile. An aliquot of the stock solution was added to the reaction vessel containing a predetermined volume of 0.1 *M* potassium chloride solution, and then the titration was performed in the same manner as described.

The pH-stat results were fit by regression analysis to a polynomial of the form $y = a_0 + a_1 t + a_2 t^2$, where y is the moles of alkali consumed, t is time, and a_1 is the initial rate of hydrolysis.

[1] Aldrich Chemical Co.

[2] Radiometer TTI60 titrator, SBR3 titrigraph, ABU12 autoburet, and PHM61 pH meter.

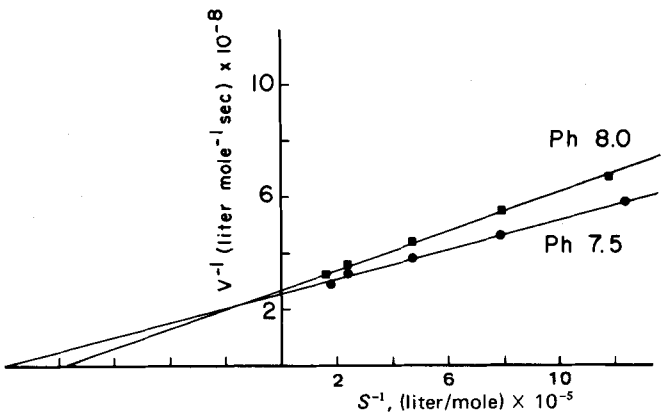

Figure 1—*Lineweaver–Burk plot for the hydrolysis of I by 1.79×10^{-9} M α-chymotrypsin at pH 7.5 and by 2×10^{-10} M α-chymotrypsin at pH 8.0.*

RESULTS AND DISCUSSION

α-Chymotrypsin-catalyzed hydrolysis follows the general kinetic scheme shown in Scheme I.

$$E + S \overset{K_S}{\rightleftharpoons} E \cdots S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2$$
$$+$$
$$P_1$$

Scheme I

The initial rate is given by the Michaelis–Menten equation:

$$V_0 = \frac{k_{cat} E_0 S_0}{K_m + S_0} \qquad \text{(Eq. 1a)}$$

where S_0 and E_0 are the initial amounts of substrate and enzyme ($S_0 \gg E_0$), or by the Lineweaver–Burk equation:

$$\frac{1}{V_0} = \frac{1}{k_{cat} E_0} + \frac{K_m}{k_{cat} E_0} \frac{1}{S_0} \qquad \text{(Eq. 1b)}$$

Plots of $1/V_0$ *versus* $1/S_0$ for I and II at pH 7.5 and 8.0 are shown in Figs. 1 and 2. Straight lines were obtained in all cases, and k_{cat} and K_m were calculated from the slopes and intercepts. These results are summarized in Table I.

It was shown (3) that k_{cat}/K_m is the most meaningful kinetic parameter for comparing different substrates. Good substrates will have a large k_{cat} and low K_m (tight binding) value and, hence, a large k_{cat}/K_m value. At low substrate concentrations ($S \ll K_m$), the enzymatic reaction is first order (with E_0 constant), with the rate constant equal to $(k_{cat}/K_m)E_0$. Hence, at a given enzyme level, E_0, k_{cat}/K_m determines the reaction rate at low substrate.

Table I shows that for I, k_{cat}/K_m is smaller by a factor of six at pH 7.5 than at pH 8.0 but that K_m does not change significantly. The k_{cat} value was eight times smaller at pH 7.5 than at pH 8.0. For *N*-acetyl phenylalanine ethyl ester, k_{cat} is three times smaller at pH 7 than at pH 7.8 (4). Both K_m and k_{cat} values for I were smaller than those for the *N*-acetyl derivatives. However, the ratio of k_{cat}/K_m was of comparable magnitude to that for the *N*-acetyl derivative. A similar change in k_{cat}/K_m was also noticed for I hydrolysis. The change in structure of the prodrug from ethyl ester to an amide caused a reduction in the hydrolysis rate by a factor of 10^5. This finding was consistent with the results reported (4) for *N*-acetyl-L-phenylalanine derivatives.

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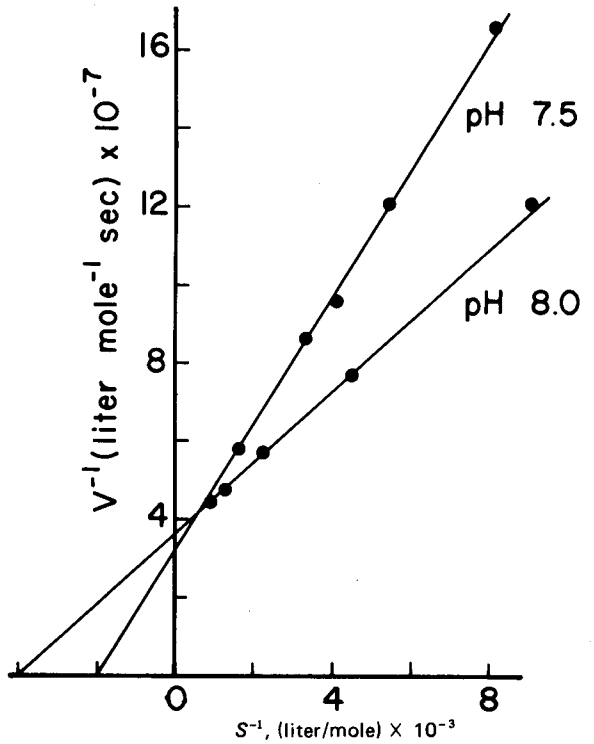

Figure 2—*Lineweaver–Burk plot for the hydrolysis of II by 6.26 × 10^{-6} M α-chymotrypsin at pH 7.5 and by 4.4 × 10^{-6} M α-chymotrypsin at pH 8.0.*

Correlation between hydroxide-ion-catalyzed hydrolysis rate constants and the first-order enzymatic hydrolysis rate constant, k_{cat}/K_m, was shown (3). For aspirin phenylalanine derivatives, the change in reactivity may be attributed to the electronic effect of the substituent rather than to any change in binding with the active site. Table I also shows that *N*-acetyl and *N*-aspirin derivatives have k_{cat}/K_m values within an order of magnitude. Therefore, for these substrates, the chymotrypsin-catalyzed hydrolysis is reasonably independent of the drug molecule, a requirement for general application of this approach. The hydrolysis rates obtained were within the expected range.

Experiments with III had to be carried out under conditions of approximately equal substrate and enzyme concentrations. This approach violates one basic assumption of the derivation of the Michaelis–Menten equation ($S_0 \gg E_0$). Dixon (5) described an elegant and simple direct plot for determining K_m when a substantial fraction of the substrate is bound.

Table I—Kinetic Parameters for α-Chymotrypsin-Catalyzed Hydrolysis

Substrate	K_m, mole/liter	k_{cat}, sec^{-1}	$\frac{k_{cat}}{K_m}$, moles sec/liter	pH
Aspirin phenylalanine ethyl ester (I)	1×10^{-6}	2.91	2.91×10^6	7.5
	1.3×10^{-6}	25	1.92×10^7	8.0
N-Acetyl phenylalanine[a] ethyl ester	1.2×10^{-3} [a]	160[a]	1.33×10^5 [a]	7.8
	0.88×10^{-3} [a]	63[a]	7.16×10^4 [a]	6.99
Aspirin phenyllactic ethyl ester (III)	2.02×10^{-5}	7.93×10^{-3}	3.96×10^2	8.0
	2.5×10^{-2}	3.7×10^{-3}	1.48×10^2	7.5
O-Acetyl phenyllactic[b] ethyl ester	2.3×10^{-2}	0.6	26.1[b]	8.0
Aspirin phenylalanine amide (II)	5.1×10^{-4}	5×10^{-3}	9.8	7.5
	2.4×10^{-4}	6.2×10^{-3}	24.7	8.0
N-Acetyl phenylalanine[a] amide	3×10^{-2} [a]	4.6×10^{-2} [a]	1.53[a]	7.9

[a] Taken from Ref. 4. [b] Taken from Ref. 12.

The velocity at any $[S]$ is given by $v = k_{cat}[ES]$. At some given point on the velocity curve, one can write:

$$v = V_{max}\left(\frac{n-1}{n}\right) \quad \text{(Eq. 2)}$$

where n is a whole number. Therefore, at the given point on the velocity curve:

$$k_{cat}[ES] = V_{max}\left(\frac{n-1}{n}\right) \quad \text{(Eq. 3)}$$

$$K_m = \frac{[E][S]}{[ES]} = \frac{([E]_0 - [ES])([S]_0 - [E])}{[ES]} \quad \text{(Eq. 4)}$$

After making appropriate substitutions, one obtains:

$$K_m = \frac{[S]_t}{n-1} - \frac{[E]_t}{n} \quad \text{(Eq. 5)}$$

A series of lines are drawn from the origin through points on the velocity curve where $v = V_{max}[(n-1)/n]$. Since n is a whole number, the points correspond to $1/2V_{max}$, $2/3V_{max}$, $3/4V_{max}$, *etc.* Each line intersects a horizontal line of height V_{max} at different $[S]_n$ values, called $[S]_2$ (for the line through $1/2V_{max}$), $[S]_3$ (for the line through $2/3V_{max}$), $[S]_3$ (for the line through $2/3V_{max}$), and so on. The value of each $[S]_n$ is given by:

$$[S]_n = \left(\frac{n}{n-1}\right)[S]_t \quad \text{(Eq. 6)}$$

where $[S]_t$ is the total substrate concentration required for a given n. Consequently:

$$[S]_t = \left(\frac{n-1}{n}\right)[S]_n \quad \text{(Eq. 7)}$$

and from Eq. 5:

$$K_m = \frac{[S]_n}{n} - \frac{[E]_t}{n} \quad \text{(Eq. 8)}$$

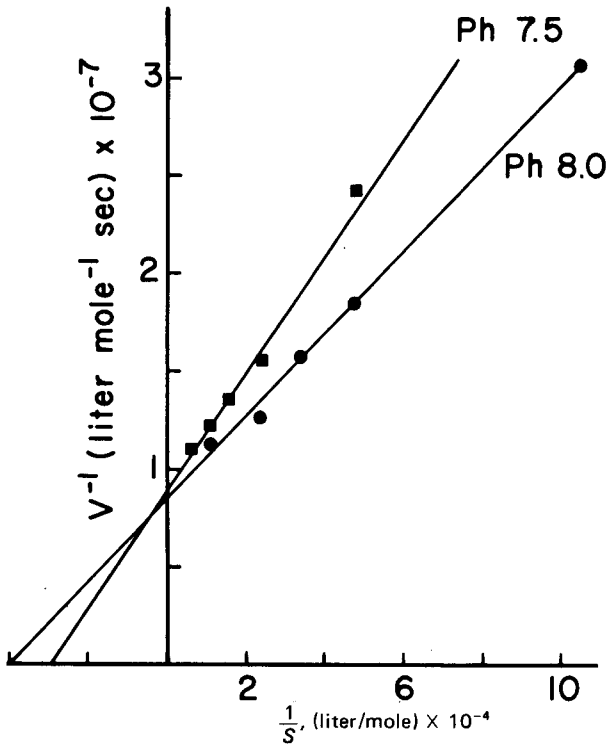

Figure 3—*Lineweaver–Burk plot for the hydrolysis of III by 3.02 × 10^{-5} M α-chymotrypsin at pH 7.5 and by 1.5 × 10^{-5} M α-chymotrypsin at pH 8.0.*

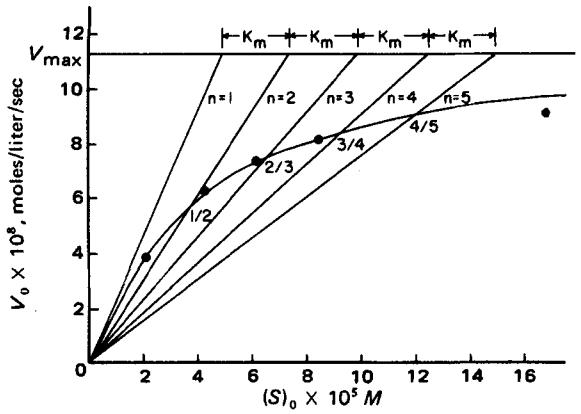

Figure 4—*Dixon plot for determining* K_m *for the hydrolysis of III by* 3.02×10^{-5} M *α-chymotrypsin at pH 7.5.*

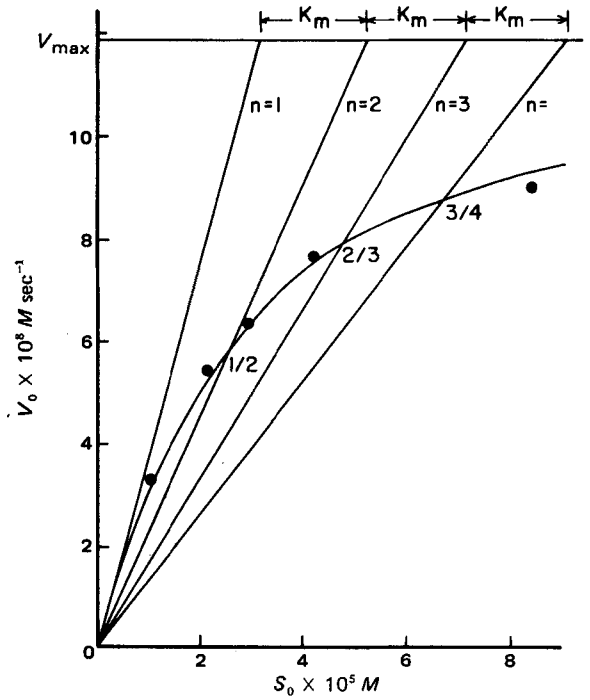

Figure 5—*Dixon plot for determining* K_m *for the hydrolysis of III by* 1.5×10^{-5} M *α-chymotrypsin at pH 8.0.*

or:

$$[S]_n = nK_m + [E]_t \qquad \text{(Eq. 9)}$$

The intercepts on the V_{max} line occur at increments of K_m, *i.e.*:

$$[S]_n - [S]_{n-1} = K_m \qquad \text{(Eq. 10)}$$

This procedure requires a knowledge of V_{max}. It was shown (6) that if the V_{max} value chosen is too low, the distances between the intercepts (*i.e.*, K_m) decrease toward the right. If the V_{max} value chosen is too high, the intervals increase toward the right. Thus, the method gives a check on the assumed value of V_{max}.

The V_{max} values used in the Dixon plots were obtained from the Lineweaver–Burk plots (Fig. 3) of the data. The Dixon plots are shown in Figs. 4 and 5; the intercepts were at constant intervals on the V_{max} axis, indicating the validity of the V_{max} values used. The K_m values obtained by the Dixon method (Table I) were only slightly lower than those found by the Lineweaver–Burk method.

Table I shows that the k_{cat}/K_m ratio was much lower for III than for its phenylalanine analog. This finding may be explained by the specificity of α-chymotrypsin in terms of sites on the enzyme complementary to the four groups oriented tetrahedrally about the α-carbon atom of the substrates (6–8). The substrate groups may fit into the corresponding sites or associate with them with varying effects. The α-acylamido group fits into its site and associates by hydrogen bonding (9, 10). For the phenyllactic acid derivative, the acylamido hydrogen capable of hydrogen bonding is lacking. This condition probably leads to less favorable orientation and a concomitant reduction in the reaction rate (k_{cat}).

It was shown (11) that all esters of the same acyl group give the same steady-state hydrolysis rate, indicating that deacylation is the rate-limiting step. For the amides, the rate depends on the amide group involved (*i.e.*, acylation is rate limiting). Therefore, for ester hydrolysis with α-chymotrypsin, $k_{cat} \approx k_3$; for amides, $k_{cat} \approx k_2$. It may be shown from the enzyme kinetic scheme (Scheme I) that K_m and the equilibrium constant K_s are related as follows:

$$K_m = \frac{k_3}{k_2 + k_3} K_s \qquad \text{(Eq. 11)}$$

If $k_2 \gg k_3$:

$$K_m \approx \frac{k_3}{k_2} K_s \qquad \text{(Eq. 12)}$$

If $k_3 \gg k_2$:

$$K_m \approx K_s \qquad \text{(Eq. 13)}$$

Bender and coworkers (4, 11) outlined some consequences of Scheme I for two types of substrates, esters and amides. They showed that the leaving groups (the ester or amide moieties) contribute very little, if anything, to the binding of substrates to α-chymotrypsin. Thus, K_s values for amides and esters of the same acylamino acids should be similar. Because $K_s = K_m$ for the amide substrate, this experimentally determined value may be used as K_s for the ester. In this way, all rate constants for various substrates can be evaluated. At pH 8.0, the values obtained for I are $K_s = 2.4 \times 10^{-4}$ *M*, $k_2 = 9.8 \times 10^3$ sec^{-1}, and $k_3 = 25$ sec^{-1}. The values for II are $K_s = 2.4 \times 10^{-4}$ *M*, $k_2 = 6.1 \times 10^{-3}$ sec^{-1}, and $k_3 = 25$ sec^{-1}.

Therefore, of the three synthesized prodrugs of aspirin, I showed the highest hydrolysis rate in the presence of α-chymotrypsin. The kinetic parameters obtained were within the range of expectation based on prior knowledge of the properties of α-chymotrypsin.

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