Physicochemical Property Modification Strategies Based on Enzyme Substrate Specificities III: Carboxypeptidase A Hydrolysis of Aspirin Derivatives

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Abstract □ The aspirin derivatives aspirin phenylalanine and aspirin phenyllactic acid were studied as substrates for carboxypeptidase A. The phenyllactic acid derivative (an ester) was the best substrate but showed considerable product inhibition. The kinetic parameters for both substrates were in the range expected on the basis of other known substrates. The results indicate that the acylamide substituent (drug) has only a small effect on the enzyme kinetic parameters. Consequently, carboxypeptidase A may serve as a reconversion site for many drug derivatives.

Keyphrases □ Aspirin—derivatives, hydrolysis by carboxypeptidase A □ Derivatives—aspirin, hydrolysis by carboxypeptidase A □ Carboxypeptidase A—hydrolysis of aspirin derivatives □ Physicochemistry—modification strategies based on enzyme substrate specificities, carboxypeptidase A hydrolysis of aspirin derivatives

A rationale for making amino acid derivatives of aspirin and their synthesis was reported previously (1). The kinetics of hydrolysis of the derivatives were studied in the presence of α-chymotrypsin, and aspirin phenylalanine ethyl ester was the most rapidly hydrolyzed substrate (2). The amino acid derivatives of aspirin were so designed that after the initial cleavage of the terminal ester or amide linkage by α-chymotrypsin, carboxypeptidase A would hydrolyze the remaining ester or amide linkage to regenerate aspirin *in vivo*. This paper reports the results of the kinetic studies of aspirin phenylalanine (I) and aspirin phenyllactic acid (II) in the presence of carboxypeptidase A.

EXPERIMENTAL

Materials—Compounds I and II were prepared by the methods already described (1). Carboxypeptidase A was obtained as a suspension of crystals in toluene–water[1]. Before use, enzyme crystals were washed twice with distilled water and dissolved in 3.0 *M* sodium chloride solution. The solutions were centrifuged and stored at 4° in concentrations of ~30 mg/ml. Such solutions were reported (3) to have exhibited no change in activity over several months. Working solutions were prepared daily by diluting the stock solutions to the desired concentrations with 1.0 *M* sodium chloride. Enzyme concentrations were measured by absorbance at 278 nm and using a molar absorptivity of 6.49×10^4 liters/mole cm (4).

For measuring enzyme activity, stock enzyme was diluted to a 10–25 μg/ml concentration in 10% lithium chloride immediately before use. The substrate solution was freshly prepared and contained 0.001 *M* hippuryl-L-phenylalanine, 0.025 *M* tromethamine, and 0.5 *M* sodium chloride. The spectrophotometer was set at 254 nm. In the control cell were placed 2.9 ml of substrate solution and 0.1 ml of 10% lithium chloride; in the test cell, 0.1 ml of enzyme solution was added at zero time to 2.9 ml of substrate solution. The increase in absorbance was recorded. Enzyme activity was calculated using the following:

$$\text{units/mg of protein} = \frac{\Delta A_{254}/\text{min}}{0.36 \times \dfrac{\text{mg of enzyme}}{\text{ml of reaction mixture}}} \quad \text{(Eq. 1)}$$

where a unit of activity is defined to be equal to 1 μmole of substrate hydrolyzed/min under the specified conditions and 0.36 is the molar absorbance index of hippuric acid that is formed stoichiometrically.

Kinetic Measurements—The hydrolyses of I and II were followed by automatic titration at constant pH.[2] The method was previously described (2).

RESULTS AND DISCUSSION

Aspirin Phenylalanine (I)—The products of carboxypeptidase A-catalyzed hydrolysis of I are aspirin and phenylalanine. The pKa of the α-amino group of phenylalanine is 9.24; at pH 7.5, it is present almost completely as NH_3^+. Thus, at pH 7.5, the dissociable proton is consumed by the product amino acid, thereby not requiring any external alkali to maintain the pH. However, at pH 8.5, 18.19% of the phenylalanine is present in the nonionized form. Experiments were performed at pH 8.5 rather than at pH 7.5, accounting for this factor.

In these studies, the Michaelis–Menten assumption of $S_0 \gg E_0$ was

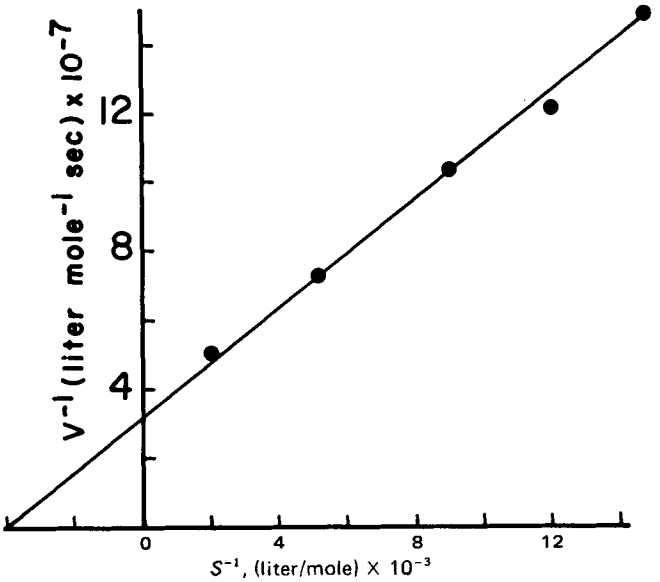
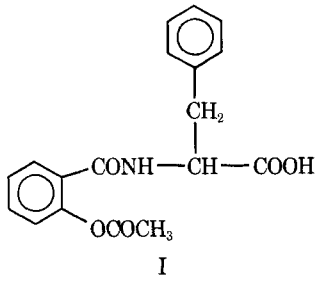
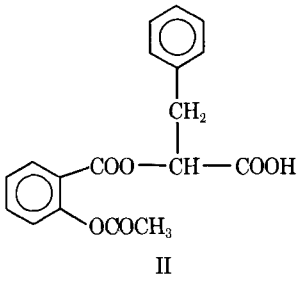


Figure 1—*Lineweaver–Burk plot for the hydrolysis of I catalyzed by 1.0×10^{-5} M carboxypeptidase A at pH 8.5.*



I



II

[1] Worthington Biochemicals Inc.

[2] Using a Radiometer TTI-60 titrator in conjunction with type SBR-3 titrigraph.

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Table I—Kinetic Parameters for Carboxypeptidase Hydrolysis at pH 7.5

Substrate	K_m, mole/liter	k_{cat}, sec^{-1}	K_i, mole/liter	$\frac{k_{cat}}{K_m}$, moles/sec/liter
Cinnamoyl phenylalanine[a]	6×10^{-4}	2.1×10^{-2}	—	35
Indoleacryloylphenylalanine[a]	5.84×10^{-4}	1.4×10^{-3}	—	2.39
Aspirin phenylalanine[b] (I)	1.3×10^{-4}	8.47×10^{-2}	—	445
Cinnamoyl phenyllactic acid	1.87×10^{-4}	67	5.78×10^{-5}	3.58×10^{5}
Furylacryloyl phenyllactic acid	1.32×10^{-4}	47	—	3.56×10^{5}
Aspirin phenyllactic acid (II)	1×10^{-4}	25	4×10^{-6}	2.5×10^{5}

[a] Taken from Ref. 3. [b] Performed at pH 8.5.

not adhered to. A Lineweaver–Burk plot (Fig. 1) was used to obtain V_{max}, which was then used in the Dixon plot (Fig. 2) to obtain K_m. The K_m value obtained from the Dixon plot was slightly lower than that obtained from the Lineweaver–Burk plot. The K_m value for I shown in Table I was obtained from the Dixon plot.

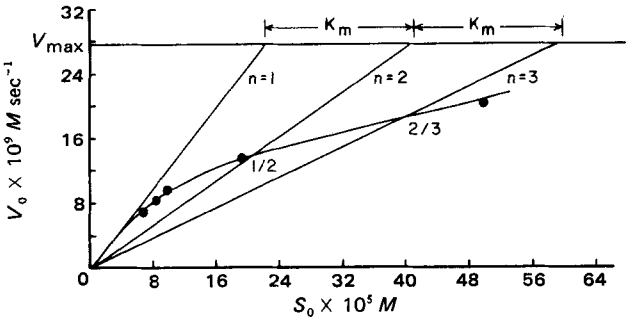

Figure 2—*Dixon plot for determining* K_m *for the hydrolysis of I by 1.0* $\times 10^{-5}$ M *carboxypeptidase A at pH 8.5.*

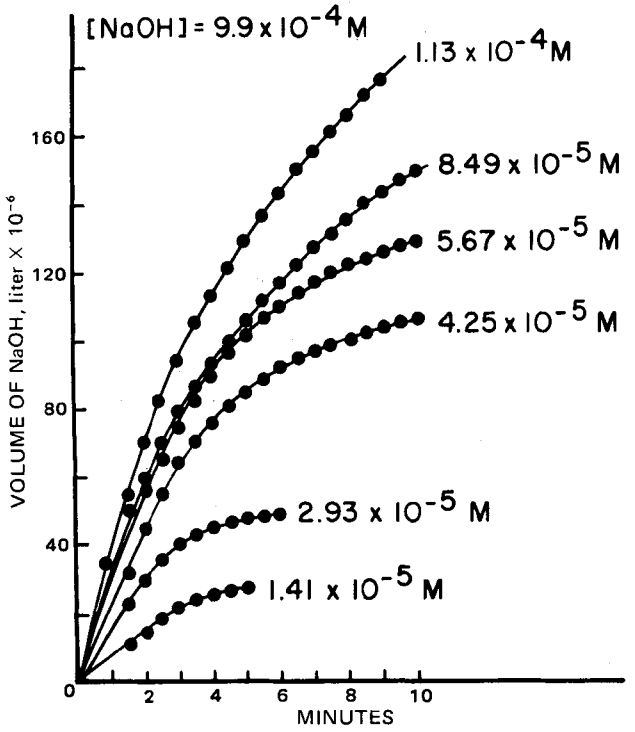

Figure 3—*Plot of volume of sodium hydroxide consumed* versus *time for the hydrolysis of II by 2.45* $\times 10^{-10}$ M *carboxypeptidase A at pH 7.5.*

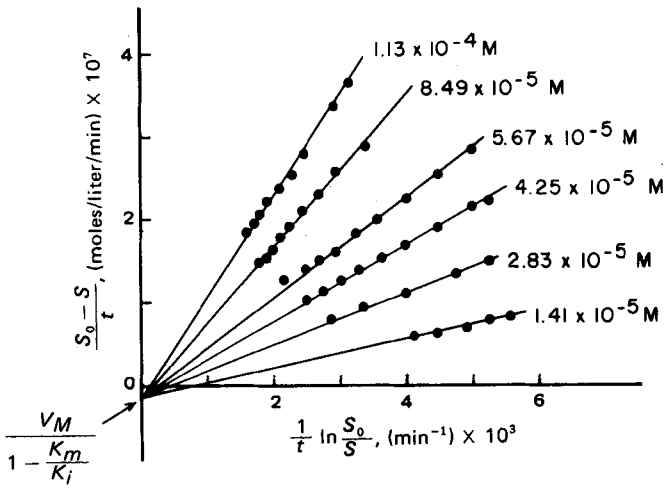

Figure 4—*Plot of integrated Michaelis–Menten equation for competitive product inhibition for the hydrolysis of II by 2.45* $\times 10^{-10}$ M *carboxypeptidase A at pH 7.5.*

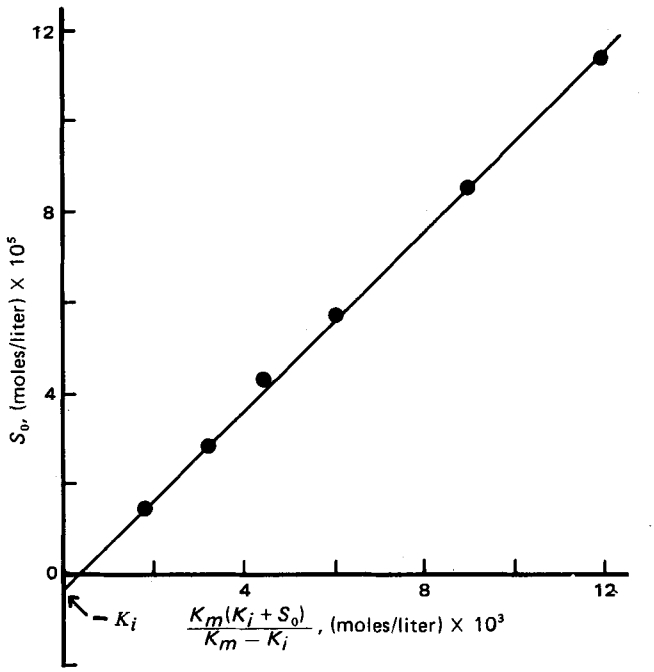

Figure 5—*Plot to obtain* K_m *and* K_i *from integrated Michaelis–Menten equation.*

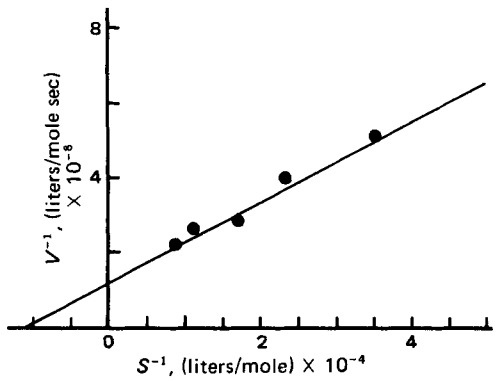

Figure 6—*Lineweaver–Burk plot for the hydrolysis of II by 2.45 × 10^{-10} M carboxypeptidase A at pH 7.5.*

Aspirin Phenyllactic Acid (II)—The results of six runs measuring the hydrolysis rates of II at six different concentrations catalyzed by carboxypeptidase A at pH 7.5 are illustrated in Fig. 3. The points shown were taken from the continuous record of the titrator for calculation purposes. The curves of this plot do not seem to follow a simple kinetic order, and the sharp decrease of hydrolysis rates with time suggests that product inhibition of the enzyme plays an important role. Significant product inhibition of carboxypeptidase A was reported (5, 6).

The scheme that fits the kinetics of the carboxypeptidase A-catalyzed hydrolysis of II is shown in Scheme I (E is carboxypeptidase A, S is II, P_1 is aspirin, and P_2 is L-phenyllactic acid). The L-phenyllactic acid (P_2) produced during hydrolysis acts as a competitive inhibitor.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \overset{k_{cat}}{\rightleftarrows} E + P_1 + P_2$$

$$E + P_2 \underset{k_{-i}}{\overset{k_i}{\rightleftarrows}} EP_2$$

Scheme I

The corresponding integrated Michaelis–Menten equation is:

$$\frac{[S]_0 - [S]}{t} = \frac{K_m(K_i + [S]_0)}{(K_m - K_i)t} \ln([S]_0/[S]) + \frac{V_{max}}{1 - K_m/K_i} = \frac{a}{t} \ln \frac{[S]_0}{[S]} + \frac{V_{max}}{1 - K_m/K_i} \quad \text{(Eq. 2)}$$

where:

$$K_m = \frac{k_{-1} + k_2}{k_1}$$

$$K_i = \frac{k_{-i}}{k_i}$$

$$a = \frac{K_m(K_i + [S]_0)}{K_m - K_i}$$

Table II—Kinetic Parameters for Carboxypeptidase A Hydrolysis of II

Kinetic Parameter	Calculated by Lineweaver–Burk Method	Calculated by Integrated Michaelis–Menten Equation
K_m, mole/liter	8.74×10^{-5}	1×10^{-4}
K_i, mole/liter	—	4×10^{-6}
V_{max}, mole/liter	8.55×10^{-9}	6×10^{-9}
k_{cat}, sec^{-1}	35	25

The integrated Michaelis–Menten equation for competitive product inhibition applies satisfactorily to the kinetic data obtained at pH 7.5 for II. This finding is illustrated by Fig. 4 in which $([S]_0 - [S])/t$ is plotted *versus* $1/t \ln([S]_0/[S])$. The linearity of the plots obtained is consistent with the proposed competitive product inhibition since the lines would curve for other types of product inhibition.

The intercept of the ordinate of Fig. 4 is a function of V_{max}, K_m, and K_i, and the slopes of the straight lines, a, are related to K_m, K_i, and $[S]_0$ by:

$$[S]_0 = a\left(1 - \frac{K_i}{K_m}\right) - K_i \quad \text{(Eq. 3)}$$

Thus, to separate the various kinetic parameters, Fig. 5 plots the values of $[S]_0$ *versus* the respective slopes, a, from Fig. 4. The intercept along the $[S]_0$ axis is $-K_i$; hence, K_m and V_{max} can be calculated. The K_i value was considerably smaller than K_m, indicating strong competitive inhibition by the product, L-phenyllactic acid.

As a check on the applicability of the integrated Michaelis–Menten equation and the computed kinetic constants, K_m and V_{max} also were determined by the Lineweaver–Burk procedure. Figure 6 shows that the double reciprocal plot of the kinetic results is linear. The agreement between kinetic parameters calculated by the two procedures at pH 7.5 is good (Table II).

An overall summary of the carboxypeptidase A-catalyzed hydrolysis is given in Table I along with some results obtained from the literature for comparison purposes. Compound II was a better substrate than the corresponding phenylalanine derivative. The values of k_{cat}/K_m obtained were comparable to those of the substrates reported previously, indicating that the reconversion rate was in the expected range and that the acylamide drug moiety had only a small effect on enzyme kinetics.

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