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# SUBSTRATE INHIBITION IN THE HYDROLYSIS OF *N*-ACYLGLYCINE ESTERS BY CARBOXYPEPTIDASE A

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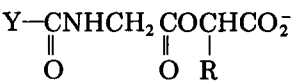
## Summary

The rates of hydrolysis of a series of 21 *N*-acylglycine esters ($YCONHCH_2CO_2CH(CH_2CH_3)CO_2H$ (2)) by bovine pancreatic carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2) have been studied over the substrate concentration range $10^{-4}$—$10^{-1}$ M at pH 7.5, 25°C, ionic strength 0.5. All substrates display substrate inhibition except $Y = CH_3$, $CH_3CH_2$ and $(CH_3)_3C$ for which normal Michaelis-Menten kinetics are observed. In all cases substrate inhibition is consistent with the formation of an $ES_2$ complex and parameters for the second-degree rate equation $v/E = (k_2^{app}S + k_3^{app}S^2/K_{SS}^{app})/(K_S^{app} + S + S^2/K_{SS}^{app})$ have been evaluated. For a series of eight aliphatic groups varying in size between $Y = CH_3$ and Y = cyclo-$C_6H_{11}$ the following linear correlations were observed: $-\log K_S^{app} = 0.82\pi + 1.32$ and $\log k_2^{app}/K_S^{app} = 0.71\pi + 5.81$ ($\pi$ is Hansch's hydrophobicity parameter). Aryl and aralkyl Y moieties deviate from these correlation lines. $K_{SS}^{app}$ also depends on the hydrophobicity of Y but no quantitative correlation is obvious. Thus the Y unit of 2 is involved in a hydrophobic interaction with the enzyme when 2 binds at both the catalytically productive and inhibitory sites. Parameters for the enzymic hydrolysis of the esters $YCONHCH_2CO_2CH(CH_2CH(CH_3)_2)CO_2H$ (3) ($Y = C_6H_5(CH_2)_n$ ($n = 0$, 1, 2)) are also presented. Pronounced nonproductive 1 : 1 enzyme · substrate complex formation is observed for each of 2: $Y = C_6H_5(CH_2)_n$ ($n = 2$, 3) and 3: $Y = C_6H_5(CH_2)_2$. Hippurate anion is shown to be an uncompetitive inhibitor ($K_i = 12$ mM) for the hydrolysis of 2: $Y = (CH_3)_3C$. Data are now available which can only be interpreted in terms of at least three enzymic sites being available for hydrophobic interactions with ester substrate molecules.

## Introduction

It is now well established [1—3] that the substrate inhibition which complicates the kinetics of the hydrolysis of some esters by bovine pancreatic

carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2) is due to the formation of a 1 : 2 enzyme · substrate complex ($ES_2$) which is catalytically less active than the simple 1 : 1 Michaelis complex (ES). This phenomenon is particularly obvious in the hydrolysis of esters of hippuric acid (1: Y = $C_6H_5$) by this enzyme.



(1)

2: R = $CH_3CH_2$

3: R = $(CH_3)_2CHCH_2$

In a study [4] in which systematic modifications were made to the structure of the hippuric acid moiety of the ester 2: Y = $C_6H_5$, it was established that the integrity of the amide unit (CONH) in 1 is an essential requirement for the occurrence of substrate inhibition in the enzymic hydrolysis of such esters. In addition, it was observed that substrate inhibition occurs in the enzymic hydrolysis of the esters 2: Y = $C_6H_5$ and $C_6H_5CH_2$, but simple Michaelis-Menten kinetics are observed if Y = $CH_3$ or $C_6H_5CONHCH_2$. In order to further elucidate the specificity of the enzyme for the Y group in 1 in binding ester substrates at both the catalytically productive site and the inhibitory binding site, we have now synthesized a series of 21 esters 2, in which Y is either an alkyl, aryl or aralkyl group, and have studied the steady-state kinetics of the hydrolysis of these esters by bovine pancreatic carboxypeptidase A at pH 7.5. Analogous data for the hydrolysis of the esters 3: Y = $C_6H_5(CH_2)_n$ ($n$ = 0, 1, 2) are also presented.

## Materials and Methods

*Synthesis of substrates.* Most of the *N*-acylglycines were prepared from glycine and the appropriate acid chloride by the method of Ingersoll and Babcock [5]. Solid acid chlorides were first dissolved in a minimum volume of tetrahydrofuran. *N*-Acylglycines bearing simple alkyl Y substituents (4: Y = $CH_3(CH_2)_n$, $n$ = 1—4) were made by the following general route:

Sodium (0.2 mol) was weighed in petroleum, washed with ether, dried and dissolved in anhydrous methanol (85 ml). Glycine (0.1 mol) was added, and the mixture was boiled under reflux for a few minutes to obtain a clear solution. Acid chloride (0.1 mol) was then added to the cooled solution, and the reaction mixture was refluxed for 3 days. The ether was removed, and the residue was dissolved in 5% aqueous $NaHCO_3$ solution. After ether extraction, the aqueous solution was acidified to pH 2. After removal of the water, the crude *N*-acylglycine was recrystallized several times.

Racemic esters (2) were prepared from the appropriate *N*-acylglycine and either benzyl 2-bromobutanoate or *tert*-butyl 2-bromobutanoate by the general method previously described [4]. Racemic esters (3) were similarly prepared using *tert*-butyl 2-bromo-4-methylpentanoate. Repeated recrystallization gave the pure esters in most cases, although conversion to the sodium or potassium

TABLE I

CHARACTERIZATION OF *N*-ACYLGLYCINE ESTERS

| Ester | Y | m.p. (°C) | PMR ($CF_3CO_2H$) |
|---|---|---|---|
| 2 | $CH_3CH_2$ | 142—143 * | δ1.30(6H,m), 2.18(2H,m), 2.66(2H,m), 4.43(2H,s), 5.27(1H,t,J = 6 Hz), 8.03(1H, broad) |
| 2 | $CH_3(CH_2)_2$ | 163—164 ** | δ1.10(6H,m), 1.93(4H,m), 2.65(2H,t,J = 2 Hz), 4.28(1H,t,J = 5 Hz), 4.47(2H,d,J = 4 Hz), 8.10(1H, broad) |
| 2 | $CH_3(CH_2)_3$ | 73— 74 | δ0.8—2.3(12H,m), 2.6(2H,t,J = 7 Hz), 4.4(2H,d,J = 4 Hz), 5.25(1H,t,J = 6 Hz), 7.9(1H, broad) |
| 2 | $CH_3(CH_2)_4$ | 71— 72 | δ0.9—2.3(14H,m), 2.6(2H,t,J = 7 Hz), 4.45(2H,d,J = 4 Hz), 5.25(1H,t,J = 6 Hz), 7.9(1H, broad) |
| 2 | $(CH_3)_3C$ | 87— 88 | δ1.15(3H,t,J = 7 Hz), 1.33(9H,s), 2.0(2H,m), 4.35(2H, broad s), 5.25(1H,t,J = 6 Hz), 7.4(1H, broad) |
| 2 | cyclo-$C_5H_9$ | 112—113 | δ1.1(3H,t,J = 7 Hz), 1.65—2.2(10H, m), 2.75(1H, broad), 4.35(2H, broad s), 5.2(1H,t,J = 6 Hz), 7.2(1H, broad) |
| 2 | cyclo-$C_6H_{11}$ | 107—108 | δ1.07(3H,t,J = 7 Hz), 1.20—2.20(13H,m), 4.30(2H,d,J = 4 Hz), 5.25(1H,t,J = 6 Hz), 7.50(1H, broad) |
| 2 | cyclo-$C_6H_{11}CH_2$ | 118—119 | δ1.1(3H,t,J = 7 Hz), 1.3—2.5(15H,m), 4.4(2H, broad s), 5.2(1H,t,J = 6 Hz), 7.35(1H, broad) |
| 2 | cyclo-$C_6H_{11}(CH_2)_2$ | 61— 62 | δ1.1(3H,t,J = 7 Hz), 1.3—2.7(17H,m), 4.4(2H, broad s), 5.3(1H,t,J = 6 Hz), 7.6(1H, broad) |
| 2 | 4-$CH_3C_6H_4$ | 124—125 | δ1.12(3H,t,J = 7 Hz), 2.10(2H,m), 2.37(3H,s), 4.60(2H,s), 5.30(1H,t,J = 6 Hz), 7.5—7.9(5H,m) |
| 2 | 4-$ClC_6H_4$ | 129—130 | δ1.07(3H,t,J = 7 Hz), 2.09(2H,m), 4.52(2H,d,J = 6 Hz), 5.24(1H,t,J = 6 Hz), 7.3—7.8(5H,m) |
| 2 | 4-$NO_2C_6H_4$ | 154—155 | δ1.17(3H,t,J = 7 Hz), 2.14(2H,m), 4.63(2H,d,J = 6 Hz), 5.34(1H,t,J = 6 Hz), 7.9—8.5(5H,m) |
| 2 | 4-$(CH_3)_3CC_6H_4$ | 102—103 | δ1.1(3H,t,J = 7 Hz), 1.4(9H,s), 2.1(2H,m), 4.55(2H, broad s), 5.25(1H,t,J = 6 Hz), 7.5—7.8(5H,m) |
| 2 | 4-$C_6H_5C_6H_4$ | 157—159 | δ1.05(3H,t,J = 7 Hz), 2.0(2H,m), 4.5(2H, broad s), 5.2(1H,t,J = 6 Hz), 7.25—7.80(10H,m) |
| 2 | $C_6H_5(CH_2)_2$ | 115—116 | δ1.07(2H,t,J = 7 Hz), 2.00(2H,m), 2.90(4H,m), 4.30(2H,d,J = 5 Hz), 5.17(1H,t,J = 6 Hz), 7.27(5H,s), 7.43(1H, broad) |
| 2 | $C_6H_5(CH_2)_3$ | 99—100 | δ1.1(3H,t,J = 7 Hz), 1.8—2.8(8H,m), 4.2(2H,d,J = 4 Hz), 5.15(1H,t,J = 6 Hz), 7.2(6H, broad s) |
| 2 | 1-Naphthyl | 99—101 | δ1.1(3H,t,J = 6 Hz), 2.2(2H,m), 4.6(2H,d,J = 5 Hz), 5.15(1H,t,J = 6 Hz), 7.4—8.2(8H,m) |
| 2 | 2-Naphthyl | 148—150 | δ1.05(3H,t,J = 6 Hz), 2.1(2H,m), 4.6(2H, broad s), 5.3(1H,t,J = 6 Hz), 7.45—7.95(7H,m), 8.3(1H,s) |
| 3 | $C_6H_5CH_2$ | 109—110 | δ1.0(6H,m), 1.8(3H,m), 3.9(2H,s), 4.35(2H,d,J = 6 Hz), 5.35(1H,t,J = 6 Hz), 7.35(6H, broad s) |
| 3 | $C_6H_5(CH_2)_2$ | 110—111 *** | δ1.05(6H,m), 1.9(3H,m), 3.0(4H,t,J = 4 Hz), 4.3(2H, broad), 5.3(1H, broad), 7.2(5H,s), 7.7(1H,-broad) |

* Sodium salt, monohydrate.

** Sodium salt.

*** Potassium salt.

TABLE II

KINETIC PARAMETERS FOR THE ENZYMIC HYDROLYSIS OF 2

Parameters for Eqn. 1; pH 7.5, 25°C, ionic strength 0.5.

| No. | Y | $K_S^{app}$ (mM) * | $K_{SS}^{app}$ (mM) | $k_2^{app}$ ($10^3$ $min^{-1}$) | $k_3^{app}$ ($min^{-1}$) | $k_2^{app}/K_S^{app}$ ($10^7$ $M^{-1} \cdot min^{-1}$) | $k_2^{app}/k_3^{app}$ |
|---|---|---|---|---|---|---|---|
| 1 | $CH_3$ ** | 15 | — | 24 | — | 0.16 | — |
| 2 | $CH_3CH_2$ | 5.9 ± 0.4 | — | 19.0 ± 0.4 | — | 0.32 | — |
| 3 | $CH_3(CH_2)_2$ | 3.3 ± 0.3 | 14 ± 2 | 33 ± 2 | 4000 ± 1000 | 1.0 | 8.3 |
| 4 | $CH_3(CH_2)_3$ | 1.4 ± 0.1 | 5.5 ± 0.2 | 26.0 ± 0.3 | 1700 ± 200 | 1.9 | 15 |
| 5 | $CH_3(CH_2)_4$ | 0.47 ± 0.07 | 9.0 ± 0.2 | 17.7 ± 0.4 | 1900 ± 200 | 3.8 | 9.3 |
| 6 | $(CH_3)_3C$ | 3.9 ± 0.2 | — | 20.2 ± 0.4 | — | 0.52 | — |
| 7 | cyclo-$C_5H_9$ | 1.07 ± 0.05 | 3.1 ± 0.3 | 25.0 ± 0.2 | 1900 ± 400 | 2.3 | 13 |
| 8 | cyclo-$C_6H_{11}$ | 0.26 ± 0.04 | 3.0 ± 0.2 | 9.3 ± 0.6 | 280 ± 170 | 3.6 | 33 |
| 9 | cyclo-$C_6H_{11}CH_2$ | 0.91 ± 0.04 | 3.5 ± 0.3 | 27.1 ± 0.3 | 680 ± 240 | 3.0 | 40 |
| 10 | cyclo-$C_6H_{11}(CH_2)_2$ | 0.061 ± 0.013 | 1.2 ± 0.1 | 6.9 ± 0.4 | 320 ± 60 | 11 | 22 |
| 11 | $C_6H_5$ ** | 0.31 | 1.2 | 6.4 | 200 | 2.1 | 32 |
| 12 | 4-$CH_3C_6H_4$ | 0.18 ± 0.03 | 1.1 ± 0.1 | 4.8 ± 0.3 | 340 ± 110 | 2.7 | 14 |
| 13 | 4-$ClC_6H_4$ | 0.28 ± 0.05 | 1.2 ± 0.1 | 6.2 ± 0.6 | 260 ± 80 | 2.2 | 24 |
| 14 | 4-$NO_2C_6H_4$ | 0.26 ± 0.05 | 3.9 ± 0.3 | 7.1 ± 0.3 | 1700 ± 100 | 2.7 | 4.2 |
| 15 | 4-$(CH_3)_3CC_6H_4$ | 0.52 ± 0.06 | 0.090 ± 0.004 | 14.9 ± 0.5 | 110 ± 30 | 2.9 | 135 |
| 16 | 4-$C_6H_5C_6H_4$ | 0.075 ± 0.005 | 0.049 ± 0.001 | 3.6 ± 0.1 | 58 ± 2 | 4.8 | 62 |
| 17 | $C_6H_5CH_2$ ** | 2.9 | 4.9 | 51 | 2000 | 1.8 | 26 |
| 18 | $C_6H_5(CH_2)_2$ | <0.1 | 42 ± 2 | 1.33 ± 0.05 | 380 ± 20 | >1.3 | 3.5 |
| 19 | $C_6H_5(CH_2)_3$ | <0.05 | 11 ± 1 | 2.4 ± 0.1 | 370 ± 40 | >4.8 | 6.5 |
| 20 | 1-Naphthyl | 0.18 ± 0.02 | 1.1 ± 0.1 | 8.6 ± 0.1 | 650 ± 50 | 4.8 | 13 |
| 21 | 2-Naphthyl | 0.14 ± 0.02 | 0.050 ± 0.02 | 6.6 ± 0.4 | 115 ± 15 | 4.7 | 57 |

* $K_S^{app} = K_m$; $k_2^{app} = k_{cat}$.

** Data from ref. 4.

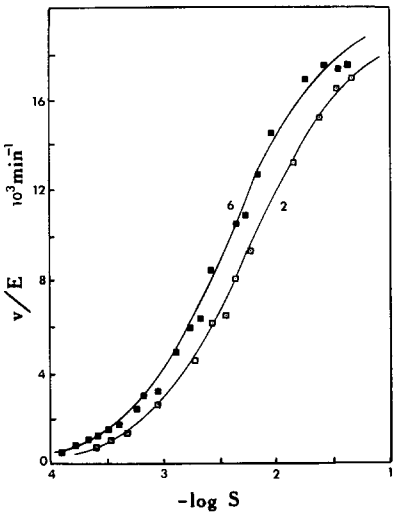



Fig. 1. Dependence of initial velocity of enzymic hydrolysis on substrate concentration for esters Nos. 2 and 6 (Table II) (pH 7.5, 25°C, ionic strength 0.5). Curves are calculated using Eqn. 1 and the parameters in Table II.

salt [4] and recrystallization was occasionally necessary. Detailed melting points and proton magnetic resonance (PMR) spectral data for each new ester are given in Table I.

*Kinetic studies.* Preparation of enzyme and substrate stock solutions and all kinetic details are as previously described [2—4]. All kinetic data are for pH 7.5, 25°C and ionic strength 0.5.

## Results

The initial velocity ($v/E$) of the enzymic hydrolysis of each of the esters 2 in Table II was measured at pH 7.5, 25°C, ionic strength 0.5 at a number of substrate concentrations ($S$) * in the range of $10^{-4}$—$10^{-1}$ M in the racemic ester. In several cases this range was extended to concentrations as low as $2 \cdot 10^{-5}$ M and as high as 0.5 M. In all cases, only 50% of the racemic ester was enzymically hydrolyzed at equilibrium. This is consistent with the general observation [2,6—9] that only the L-isomers of such esters are substrates for bovine pancreatic carboxypeptidase A.

The dependence of $v/E$ on log $S$ for the esters 2 is presented in Figs. 1—3. These three figures represent the three different types of substrate concentration dependence of the initial velocity that were observed for the esters 2 in the current study. Lineweaver-Burk plots of the data for each ester in Fig. 1 (2: Y = $CH_3CH_2$ and $(CH_3)_3C$) are quite linear over the whole substrate concentration range that was investigated, and clearly indicate that normal Michaelis-Menten kinetics are applicable to these two substrates. Derived values of the parameters $k_{cat}$ and $K_m$ are given in Table II.

* Throughout this paper $S$ refers to the concentration of the L-isomer only, unless otherwise indicated.

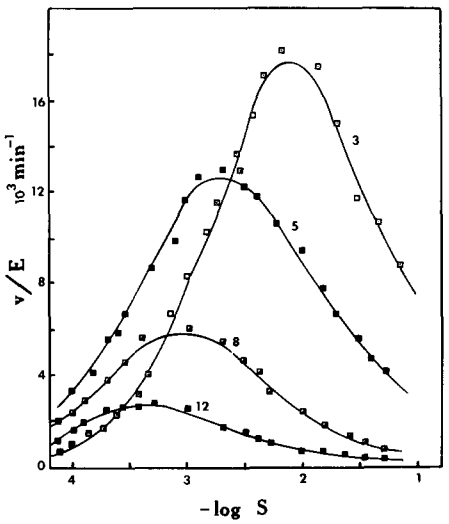



Fig. 2. Dependence of initial velocity of enzymic hydrolysis on substrate concentration for esters Nos. 3, 5, 8 and 12 (Table II) (pH 7.5, 25°C, ionic strength 0.5). Curves are calculated using Eqn. 1 and the parameters in Table II. Similar dependences of $v/E$ on $S$ are observed for the esters Nos. 4, 7, 9, 10, 13–16, 20, 21.

For most of the esters 2 that were investigated in the present study, the initial velocity of enzymic hydrolysis increases with increasing substrate concentration at low substrate concentrations. However, $v/E$ reaches a maximum value, and at higher substrate concentrations substrate inhibition becomes apparent as $v/E$ decreases once again (Fig. 2). This phenomeon is similar to that previously examined and analyzed in considerable detail for hippuric acid esters (1: Y = $C_6H_5$) [1,2]. For hippurate esters, the dependence of $v/E$ on $S$ can be fitted by the four parameter equation

$$\frac{v}{E} = \frac{k_2^{app}S + k_3^{app}S^2/K_{SS}^{app}}{K_S^{app} + S + S^2/K_{SS}^{app}} \quad (1)$$

The data for each of the esters in Fig. 2 were fitted to Eqn. 1 by the iterative technique that has been described previously [10].

In all cases acceptable fits were obtained with average differences between calculated and experimental initial velocities of around 5%. This difference is similar to the experimental error in the measured velocities. Values of the four parameters evaluated in this way for each ester are given in Table II.

A third type of dependence of $v/E$ on $S$ was observed for the esters 2: Y = $C_6H_5(CH_2)_n$ ($n$ = 2, 3), and is illustrated in Fig. 3. In these cases, $v/E$ is independent of $S$ at the lowest substrate concentrations that are experimentally accessible, while at high substrate concentrations substrate inhibition is observed. These data can be considered to be modified forms of the curves in Fig. 2, with $K_S^{app}$ being much smaller than the lowest value of $S$ that is experimentally accessible. Using this assumption, Eqn. 1 may be modified to Eqn. 2,

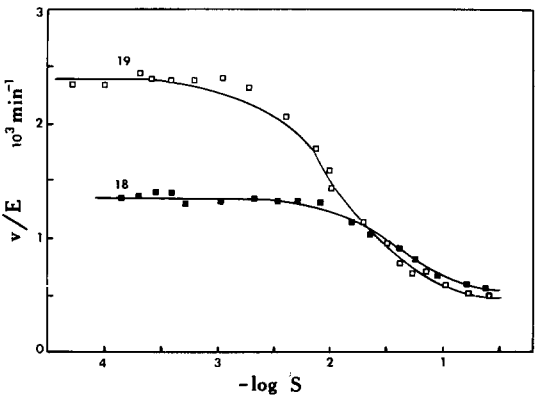



Fig. 3. Dependence of initial velocity of enzymic hydrolysis on substrate concentration for esters Nos. 18 and 19 (Table II) (pH 7.5, 25°C, ionic strength 0.5). Curves are calculated using Eqn. 1 and the parameters in Table II.

which can be rearranged to the form shown in Eqn. 3.

$$\frac{v}{E} = \frac{k_2^{app} + k_3^{app} S/K_{SS}^{app}}{1 + S/K_{SS}^{app}} \tag{2}$$

$$\frac{v}{E} = K_{SS}^{app} \frac{(k_2^{app} - v/E)}{S} + k_3^{app} \tag{3}$$

Now, $k_2^{app}$ is the value of $v/E$ in the plateau region of Fig. 3. Plots of $v/E$ against $(k_2^{app} - v/E)/S$ for the high substrate concentration data in Fig. 3 are linear and the parameters $k_2^{app}$, $k_3^{app}$ and $K_{SS}^{app}$ have been evaluated and are included in Table II.

We have also measured the initial velocity of the enzymic hydrolysis of the esters 3: Y = $C_6H_5(CH_2)_n$ ($n$ = 1, 2) as a function of substrate concentration at

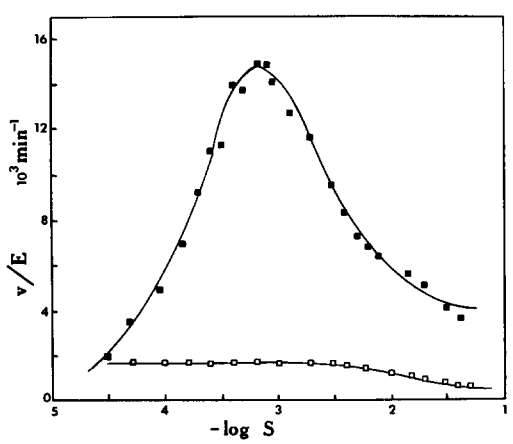



Fig. 4. Dependence of initial velocity of enzymic hydrolysis on substrate concentration for 3: Y = $C_6H_5CH_2$ (■) and 3: Y = $C_6H_5(CH_2)_2$ (□) (pH 7.5, 25°C, ionic strength 0.5). Curves are calculated using Eqn. 1 and the parameters in Table III.

TABLE III

KINETIC PARAMETERS FOR THE ENZYMIC HYDROLYSIS OF 3

Parameters for Eqn. 1; pH 7.5, 25°C, ionic strength 0.5.

| Y | $K_S^{app}$ (mM) | $K_{SS}^{app}$ (mM) | $k_2^{app}$ ($10^3$ $min^{-1}$) | $k_3^{app}$ ($min^{-1}$) | $k_2^{app}/K_S^{app}$ ($10^7$ $M^{-1} \cdot min^{-1}$) | $k_2^{app}/k_3^{app}$ |
|---|---|---|---|---|---|---|
| $C_6H_5$ * | 0.021 | 0.78 | 4.4 | 160 | 21 | 28 |
| $C_6H_5CH_2$ | 0.63 ± 0.06 | 0.62 ± 0.02 | 41 ± 2 | 3600 ± 200 | 6.5 | 11 |
| $C_6H_5(CH_2)_2$ | <0.05 | ≈30 ** | 1.65 ± 0.05 | <600 ** | >3.3 | >3 |

* Data from ref. 2.

** Data is not available at sufficiently high substrate concentrations to allow an accurate estimate of $k_3^{app}$ and $K_{SS}^{app}$.

pH 7.5. The data for these two esters are given in Fig. 4. Parameters for Eqn. 1 for these two esters were evaluated as described above, and are collected in Table III, and compared with data for 3: Y = $C_6H_5$ which was studied earlier [2].

The reversible inhibition of the enzymic hydrolysis of 2: Y = $(CH_3)_3C$ by the hippurate anion ($C_6H_5CONHCH_2CO_2^-$) has been investigated and compared with the previously reported [11] inhibition of the hydrolysis of 1: Y = $C_6H_5$, R = $C_6H_5CH_2$ by the same anion. Lineweaver-Burk plots for the hydrolysis of 2: Y = $(CH_3)_3C$ in the presence and absence of hippurate anion are parallel. Such parallel double-reciprocal plots are typical of uncompetitive inhibition in which the inhibitor only binds to the ES complex and not to the free enzyme (Scheme I). Scheme I generates a Michaelis equation

$$E + S \underset{K_S}{\rightleftharpoons} ES \overset{+I}{\underset{K_i}{\rightleftharpoons}} ESI$$
$$\downarrow k_2$$
$$E + P_1 + P_2$$

Scheme I

having $k_{cat}^{app} = k_2/(1 + I/K_i)$ and $K_m^{app} = K_S/(1 + I/K_i)$ at inhibitor concentration $I$. Calculation of $K_i$ from $k_{cat}^{app}$ derived from the current data gives $K_i = 1.2 \cdot 10^{-2}$ M at pH 7.5, 25°C, ionic strength 0.2, which is in agreement with $K_i \approx 1.0 \cdot 10^{-2}$ M observed for this anion as an uncompetitive inhibitor of 1: Y = $C_6H_5$, R = $C_6H_5CH_2$ under the same conditions [11].

## Discussion

We have previously investigated [2] a series of hippurate esters, including 2: Y = $C_6H_5$ and 3: Y = $C_6H_5$, that display substrate inhibition of their enzymic hydrolysis of the same form as that shown in Fig. 2. For hippurate esters it was established that Eqn. 1 is consistent with the kinetic mechanism shown in Scheme II, where $S_D$ and $S_L$ represent the D and L isomers of the ester,

respectively, and $K_n$ ($n$ = 1—7) are dissociation constants. Detailed

$$\begin{array}{ccc} S_L E & \overset{S_L}{\rightleftharpoons} & S_L E S_L \overset{k_3}{\rightarrow} E + S_L + \text{products} \\ {\scriptstyle S_L}\uparrow\downarrow {\scriptstyle K_2\ S_L} & & \uparrow\downarrow {\scriptstyle K_3} \\ E & \overset{S_L}{\rightleftharpoons} & E S_L \overset{k_2}{\rightarrow} E + \text{products} \\ {\scriptstyle S_D}\uparrow\downarrow {\scriptstyle K_5\ S_D} & & \uparrow\downarrow {\scriptstyle K_6} \\ S_D E & \overset{S_L}{\rightleftharpoons} & S_D E S_L \overset{k_4}{\rightarrow} E + S_D + \text{products} \end{array}$$

Scheme II

investigation of the influence of the D-isomer of the ester indicated that: (a) it was not enzymically hydrolyzed; (b) it competed with the L-isomer for binding at a catalytically non-productive site (i.e. $S_LE$ and $S_DE$ in Scheme II); (c) $K_3 = K_6$; $K_2$, $K_5 >> K_1$, $K_6$ and $k_3 \approx k_4 < k_2$; and (d) the formation of $S_LES_L$ and $S_DES_L$ closely approximates ordered binding in all cases. Under these conditions it can be shown that for hydrolysis of the racemic ester, the parameters of Eqn. 1 are defined by

$$K_S^{app} = K_1 \tag{4}$$

$$k_2^{app} = k_2 \tag{5}$$

$$K_{SS}^{app} = K_3/2 = K_6/2 \tag{6}$$

$$k_3^{app} = k_3 \tag{7}$$

There is a general similarity of the values for the parameters for the esters 2 in Table II and the parameters for the hippurate ester 2: Y = $C_6H_5$. It therefore seems reasonable to assume that the above general features a—d also apply to the other esters 2. Exceptions to this general conclusion may be necessary for 2: Y = $C_6H_5(CH_2)_n$ ($n$ = 2, 3) which display the unusual profiles of Fig. 3. Thus for the majority of the data in Table II, $K_S^{app}$, $k_2^{app}$ and $k_3^{app}$ seem to represent real equilibrium and rate constants, while $K_{SS}^{app}$ bears a very simple relation-

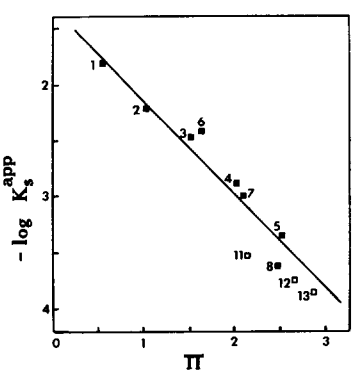



Fig. 5. Dependence of log $K_S^{app}$ for 2 on Hansch's $\pi$-parameter. Esters are indicated by the numbers in Table II. Esters Nos. 11—13 are not included in the correlation line.

ship to the dissociation constant for L- or D-ester molecules bound at the inhibitory site.

For related ester substrates which do not display substrate inhibition, there is independent evidence that the kinetic parameter $K_m$ does represent the true dissociation constant of the catalytically productive Michaelis complex [12]. Our general conclusion that $K_S^{app}$ ($=K_m$) is equal to $K_1$ in Scheme II is also consistent with this result, and is even further strengthened by the linear relationship in Fig. 5 between log $K_S^{app}$ for 2 and Hansch's $\pi$-parameter which is a quantitative measure of the hydrophobicity of Y [13,14]. This linear correlation covers all Y up to and including Y = cyclohexyl, and can be represented by the least-squares correlation line (corr. coeff. = 0.968):

$$-\log K_S^{app} = 0.82\,\pi + 1.32 \tag{8}$$

While the data for Y = 4-$CH_3C_6H_4$ and 4-$ClC_6H_4$ are in the vicinity of this correlation line, the hippurate ester (Y = $C_6H_5$) shows a significant deviation. In general, log $K_S^{app}$ shows little dependence on structure for aryl Y moieties.

A linear correlation also exists between log $k_2^{app}/K_S^{app}$ (= log $k_{cat}/K_m$) for the same Y substituents as were considered in Fig. 5. The least-squares correlation line in this case is given by Eqn. 9 (corr. coeff. = 0.978)

$$\log(k_2^{app}/K_S^{app}) = 0.71\,\pi + 5.81 \tag{9}$$

Eqn. 8 indicates that the Y-substituent of an ester substrate 2 is bound in a hydrophobic region of the enzyme active site when 2 is bound in a catalytically productive manner. The deviations from Eqn. 8 which are observed for large Y substituents presumably indicate the limited size of this hydrophobic region. Increases in $K_S^{app}$ between Y = $C_6H_5$ and $C_6H_5CH_2$ for both 2 and 3 (Tables II and III) and Y = $C_6H_{11}$ and $C_6H_{11}CH_2$ for 2 seem to indicate that this hydrophobic region is not sufficiently large to adequately accommodate the larger groups, although it should be noted that this increase in $K_S^{app}$ is accompanied by an increase in $k_2^{app}$ of approximately the same magnitude. The unexpected decrease in $K_S^{app}$ upon further homologation of Y in both the phenyl and cyclohexyl series will be considered separately below.

The fact that $k_2^{app}$ and $K_S^{app}$ for 2 show quite different dependences on Y, while $K_S^{app}$ and $k_2^{app}/K_S^{app}$ show similar Y dependences, further supports our interpretation of kinetic parameters in terms of Eqns. 4–7. For the most general solution to the rate Eqn. 1 according to Scheme II, the ratio $k_2^{app}/K_S^{app}$ is equal to the true specificity constant $k_2/K_1$. However, $k_2^{app}$ and $K_S^{app}$ are independently given by Eqns. 10 and 11 for racemic ester substrates [2].

$$k_2^{app} = \frac{k_2}{1 + (K_1/K_2) + (K_1/K_5)} \tag{10}$$

$$K_S^{app} = \frac{K_1}{1 + (K_1/K_2) + (K_1/K_5)} \tag{11}$$

Clearly, the relationships observed in this work for the Y dependence of $k_2^{app}$, $K_S^{app}$ and $k_2^{app}/K_S^{app}$ are most simply reconciled with $K_2$, $K_5 >> K_1$ as has been

previously established [2] for hippurate esters.

For the esters 2: Y = $C_6H_5(CH_2)_2$ and $C_6H_5(CH_2)_3$ and 3: Y = $C_6H_5(CH_2)_2$, $K_S^{app}$ becomes so small (Figs. 3 and 4) that it is not directly measureable by the current techniques and only an upper limit, equal to the lowest substrate concentration investigated, can be set. Such parallel decreases in both $K_S^{app}$ and $k_2^{app}$ are often indicative of the formation of non-productive 1 : 1 enzyme · substrate complexes. Significantly, the values of the ratio $k_2^{app}/K_S^{app}$ for these three esters seem to be about the same size as for their lower homologs, and the values for $k_3^{app}$ appear to be "normal".

The data for $K_{SS}^{app}$ as a function of Y in Table II indicate that there is a requirement for a hydrophobic Y substituent to allow substrate binding at the inhibitory site. Whereas 2: Y = $CH_3$, $CH_3CH_2$ and $(CH_3)_3C$ do not display substrate inhibition, this phenomenon is apparent for the larger aliphatic Y units that were examined. There does not appear to be any simple quantitative relationship between $K_{SS}^{app}$ and the size or hydrophobicity of Y, although substrate binding at the inhibitory site does appear to display a preference for cyclic Y groups rather than acyclic Y groups.

$YCONHCH_2CO_2^-$ (4)

For substituted phenyl groups there is a reasonable correlation (Table IV) between $K_3$ ($=2K_{SS}^{app}$) for 1 and $K_i$ for the corresponding *N*-acylglycine anions (4), which have been established previously [11] to be uncompetitive inhibitors of the enzymic hydrolysis of *O*-hipuryl-L-3-phenyllactic acid (1: Y = $C_6H_5$, R = $C_6H_5CH_2$). Thus the conclusion [11] that 4 bind to the same enzymic site as inhibitory substrate molecules seems secure. The interpretation of uncompetitive inhibition by 4 as ordered binding of substrate followed by inhibitor, reinforces the conclusion that substrate molecules bind in an ordered manner to the catalytically productive and inhibitory sites on the enzyme; i.e. $K_2$, $K_5 >> K_1$, which is consistent with the assumptions in the derivation of Eqns. 4—7.

The value of $K_3/K_i$ for Y = $C_6H_5(CH_2)_2$ in Table IV is significantly different from the other values for this ratio in this table. The reason for this difference can be traced to the unusually large value for $K_{SS}^{app}$ for 2: Y = $C_6H_5(CH_2)_2$ in

TABLE IV

INHIBITION OF THE HYDROLYSIS OF 1 BY UNCOMPETITIVE INHIBITORS (4) AND SUBSTRATES (2)

| Y | $K_i$ (mM) * | $K_3$ (mM) ** | $K_3/K_i$ |
|---|---|---|---|
| $C_6H_5$ | 10 | 2.4 | 0.24 |
| 4-$ClC_6H_4$ | 8.2 | 2.4 | 0.29 |
| 4-$NO_2C_6H_4$ | 63 | 7.8 | 0.12 |
| $C_6H_5CH_2$ | 19 | 9.8 | 0.52 |
| $C_6H_5(CH_2)_2$ | 4.3 | 42 | 9.8 |

* Data from ref. 11.

** From Table II, $K_3 = 2K_{SS}^{app}$.

Table II. As pointed out above, this ester seems to be particularly prone to forming non-productive 1 : 1 enzyme · substrate complexes.

It has been noted previously [2] that for hippurate esters (1: Y = $C_6H_5$) the ratio $k_2^{app}/k_3^{app} \approx 25$ and is essentially independent of the side-chain R of the alcohol moiety. Perusal of the $k_2^{app}/k_3^{app}$ ratios in Table II shows that this ratio does display some dependence on the nature of Y in 2. While there appears to be a tendency for this ratio to vary inversely with $K_{SS}^{app}$, no overall correlation with this or any other parameter is apparent. When significant non-productive binding of substrate occurs (2: Y = $C_6H_5(CH_2)_n$, ($n$ = 2, 3)), the ratios $k_2^{app}/k_3^{app}$ will not be directly comparable with the other values in Table II, since such non-productive binding artificially reduces the value of $k_2^{app}$.

It is clear from Eqns. 8 and 9 that the productive 1 : 1 enzyme · substrate complex for the esters 2 is the same, irrespective of whether or not the ester displays substrate inhibition at high substrate concentrations. As a further check on this point we investigated the reversible inhibition of the enzymic hydrolysis of 2: Y = $(CH_3)_3C$ by hippurate anion (4: Y = $C_6H_5$). This inhibitor displayed uncompetitive inhibition of the enzymic hydrolysis of the ester 2: Y = $(CH_3)_3C$, which does not display substrate inhibition, just as it does for hippurate ester substrates which are subject to substrate inhibition. The $K_i$ values for this inhibitor with both types of substrate are quite similar, and so there can be little doubt that, irrespective of the substrate present, hippurate anion is binding to the same site of the productive ES complex.

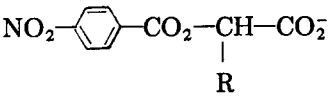
It has been established previously [11] that it is likely that a conformational change in the enzyme upon formation of the productive ES complex produces a binding site that is accessible to the uncompetitive inhibitors 4 and also to inhibitory substrate molecules. The integrity of the amide functional group of the ester substrates 1 was established [4,11] as a prerequisite for this conformational change. The current data indicate that the nature of Y has little, if any effect on this conformational change and on the resulting accessibility of the inhibitory binding site. Taken in conjunction with the pronounced dependence of $K_{SS}^{app}$ on Y, this gives a clear indication that the interactions with the enzyme of the Y moieties of 1 are quite independent for substrate molecules bound at the productive and inhibitory sites.

## General consideration of substrate binding

There is now strong evidence that at least three hydrophobic regions in each carboxypeptidase A molecule are available for the accommodation of hydrophobic groups of *N*-acylglycine ester substrates. The importance of hydrophobic interactions between the R side-chain of the alcohol moiety of 1 and the enzyme for the formation of the productive ES complex has been clearly demonstrated previously [2]. This interaction will be referred to below as involving site D of the enzyme. The current work shows that the Y substituent of 1 is also involved in a hydrophobic interaction with the enzyme (site E) in this same productive ES complex (Eqns. 8 and 9). Another hydrophobic interaction between Y and the enzyme (at site F) seems to be predominantly responsible for substrate binding at the inhibitory site to produce the catalytically less active $ES_2$ complex. Sites D and E must clearly be hydrophobic areas

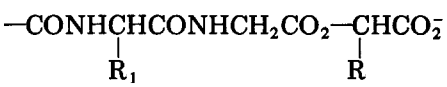
of the same general active site region of the enzyme. Site F may possibly be relatively remote from the active site, since it appears that a conformational change in the enzyme is necessary in order to make site F accessible for binding Y of the inhibitory substrate molecules.

Interaction with site D would be expected to be common to all esters that are productively bound to carboxypeptidase A. We have recently established [15] that the R side-chains of ester substrates as diverse as hippurate (1: Y = $C_6H_5$) and *p*-nitrobenzoate (5) esters are bound in the same hydrophobic region of the enzyme active site (i.e. site D).



(5)

Interaction with site E would be expected to be specific for *N*-acyl amino acid ester substrates. This site may also be important for binding larger depsipeptide ester substrates 6 bearing hydrophobic $R_1$ substituents. The only ester substrates of this



(6)

type for which data are currently available appear to be *N*-acylglycylglycine derivatives (6: $R_1$ = H) [12], so that it is not possible to test this hypothesis on the basis of current literature data.

There is some evidence that a hydrophobic interaction at site F is alone responsible for binding uncompetitive inhibitors or inhibitory substrate molecules. The D- and L-isomers of the ester substrates 1 bind equally well at this site [2] and structural modifications in inhibitors related to hippurate anions have little effect on $K_i$ for uncompetitive inhibition [11].

The identification of sites D, E and F with specific regions of the enzyme molecule does not seem to be possible at the present time. The relationship of each of these sites to binding sites for peptide substrates is also unclear in view of the recent conclusions in favour of quite different productive binding sites for ester and peptide substrate molecules [9,11,12,15,16]. Attempts have been made [17,18] to systematically study structural effects in peptide substrates on substrate binding and rate of enzymic hydrolysis. However, no simple relationships are apparent between substituent effects in peptide substrates and the substituent effects reported for ester substrates in the present study.

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