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Kinetic Investigation of the α-Chymotrypsin-Catalyzed Hydrolysis of Peptide-Ester Substrates

The Relationship between the Structure of the Peptide Moiety and Reactivity

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A number of peptide-ester substrates of the general structure Ac-L_{xn}-. . .-L_{x2}-L_{x1}-OMe have been synthesized and their α-chymotrypsin-catalyzed hydrolysis studied. The kinetic analysis involved varying the concentration of substrate and methanol product, and measuring rates along the entire progression curve.

For the dipeptide esters Ac-L_{x2}-L_{x1}-OMe and the amino-acid derivatives Ac-L_{x1}-OMe the following constants could be determined: the dissociation constant of the enzyme-substrate complex, K_{EA}, both rate constants of the acylation step, k_{23} and k_{32}, and the forward rate constant of the deacylation step, k_{31}. For the tripeptide ester Ac-Ala-Ala-Tyr-OMe it appears that the rate constant for the dissociation of the enzyme-substrate complex, k_{21}, is smaller than the rate constant for acylation, k_{23}. Thus, for this substrate only the association and dissociation rate constants k_{12} and k_{21} could be determined and the values of k_{23}, k_{32} and k_{31} only indirectly estimated. The influence of structural changes in the peptide moiety of the substrates on reactivity has been established by comparing the rate constants of appropriate pairs of substrates. It was found that the substrate reactivity, as measured by k_{23}/K_{EA}, increases with the number and strength of the secondary interactions in a manner consistent with the binding scheme which has been proposed on the basis of crystallographic studies. The effect of a particular interaction on k_{23} and on K_{EA} is dependent on the nature of the other interactions. However, the effect on k_{23}/K_{EA} appears to be independent of the presence of the other interactions and therefore characteristic of that particular interaction. The results for these substrates are compared with those found previously for a series of peptide substrates of the structure Ac-L_{xn}-. . .-L_{x2}-. . .-L_{x1}-Gly-NH_2 which have the same acyl moiety as the peptide esters studied in this work.

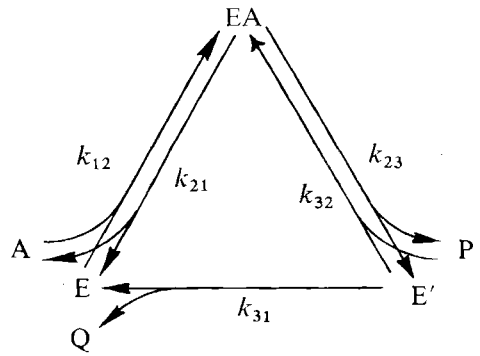
The interactions of peptide substrates with the active site of α-chymotrypsin are known to involve several amino-acid residues on both sides of the bond to be cleaved. The most important interactions for determining the reactivity of a substrate are due to the residue containing the carbonyl group of the cleaved bond (primary interactions), but recent evidence indicates that interactions involving adjacent residues (secondary interactions) also have a significant influence on reactivity. Structural information about secondary interactions between the enzyme and the residues on the acyl side of peptide substrates has been provided by a crystallographic study of γ-chymotrypsin inhibited by peptidyl chloromethyl ketones [1]. It was shown that the peptide moiety of the inhibitor forms an antiparallel β-structure with the residues Ser-214, Trp-215 and Gly-216 of the enzyme. On the basis of this work an interaction scheme had been proposed whose general validity for substrates [2,3] and non-covalently bound inhibitors [4] was confirmed by subsequent kinetic investigations with α-chymotrypsin.

Moreover, kinetic studies of substrate hydrolysis allow the analysis of the relative influence of the secondary interactions on the stability of the enzyme-substrate complex and on the rates of the catalytic steps. In the case of the peptides Ac-L_{xn}-. . .-L_{x1}-Gly-NH_2 it was found that these interactions mainly influence the rate of the acylation step [3]. In a further approach to the study of the relationship between structure and reactivity a similar analysis can be done using peptide-ester substrates Ac-L_{xn}-. . .-L_{x1}-OMe, which have the same acyl moiety. From a comparison of the influence that structural changes in the acyl moiety of the substrates have on the reactivity of peptide and ester bonds it is expected that interesting information about the factors determining reactivity can be obtained.

Abbreviations. IUPAC/IUB rules for peptides are followed, see *Eur. J. Biochem.* (1972) *27*, 201–207.

Enzyme. α-Chymotrypsin (EC 3.4.21.1).

A detailed investigation of these relationships requires the accurate determination of the rate constants of the single reaction steps, which for the α-chymotrypsin-catalyzed hydrolysis are defined by the following three-centre model:



where EA represents the enzyme-substrate complex and E′ the acyl-enzyme intermediate. In the case of peptide hydrolysis, the steady-state constants k_{cat} and K_m reduce to the acylation rate constant k_{23} and the dissociation constant of the enzyme-substrate complex, K_{EA}, respectively. The values of k_{23} and K_{EA} can, therefore, be determined by the simple analysis of the dependence of the hydrolysis rate on the substrate concentration. In the case of peptide-ester hydrolysis, however, this simplification can no longer be made and the determination of k_{23} and K_{EA} requires a more complex kinetic analysis involving the study of the dependence of the hydrolysis rates on both substrate and alcohol-product concentrations [5]. In addition this more complex analysis allows the determination of the deacylation rate constant k_{31} and the rate constant of the methanolysis of the acyl enzyme, k_{32}.

The work presented in this paper is concerned with the hydrolysis of the tripeptide ester Ac-Ala-Ala-Tyr-OMe, the dipeptide esters Ac-L_{x2}-L_{x1}-OMe, where L_{x1} is tyrosine or phenylalanine and L_{x2} is glycine, alanine, valine, or proline, and the amino-acid derivative Ac-Tyr-OMe.

EXPERIMENTAL PROCEDURE

SUBSTRATES

General Remarks

Melting points were measured in open capillary tubes and are uncorrected. Optical rotations were measured with a Zeiss photoelectric polarimeter in a 10-cm cell and are estimated to be correct to ± 1 °. Thin-layer chromatograms were run on silica gel HF_{254} (Merck, Darmstadt, Germany) with chloroform–methanol (9:1, v/v) (A), (4:1, v/v) (B), and (2:1, v/v) (C). Nuclear magnetic resonance spectra were recorded using a Varian A-60 or HA-100 spectrometer and showed the expected signals in all cases; this data is available on request. Samples for elemental analysis were dried at room temperature and 0.001 Torr (0.133 Pa) for 24–48 h. The substrate Ac-Tyr-OMe, m.p. 133–134 °C, was purchased from Schwarz/Mann (New York). The protected dipeptide Z-Val-Tyr-OMe and most amino-acid derivatives and reagents were purchased from Fluka AG, Buchs (Switzerland).

The standard procedures for coupling with *N*-hydroxysuccinimide esters of *tert*-butyloxycarbonyl amino acids, removal of *tert*-butyloxycarbonyl amino-protecting group and *N*-acetylation with *N*-acetoxysuccinimide have been described previously [3].

Synthesis of Ac-Gly-Tyr-OMe (1)

H-Gly-Tyr-OMe · HCl (8). H-Tyr-OMe · HCl (3.66 g, 15.8 mmol) was coupled with Boc-Gly-ONSu (4.25 g, 15.6 mmol) in the presence of triethylamine (1.59 g, 15.7 mmol) under standard conditions. The chromatographically homogeneous protected dipeptide obtained, R_F 0.53 (B), was then treated with hydrogen-chloride-saturated 1,2-dimethoxyethane. The crystalline crude product was twice recrystallized from methanol–2-propanol to give 3.30 g (73%) of pure material, m.p. 226.5–227.5 °C ([6] 223 °C), R_F 0.35 (C), $[\alpha]_D^{25}$ = + 23 ° (*c* = 1.0, methanol).

$C_{12}H_{17}ClN_2O_4$: calcd C 49.92, H 5.93, N 9.70, Cl 12.28%; found C 49.88, H. 5.94, N 9.65, Cl 12.20%.

Ac-Gly-Tyr-OMe (1). The dipeptide methyl ester hydrochloride (8) (2.13 g, 7.39 mmol) was neutralized with triethylamine (746 mg, 7.38 mmol) and acetylated with *N*-acetoxysuccinimide (1.16 g, 7.38 mmol). The crude product was twice crystallized from ethyl acetate; yield, 1.61 g (74%), m.p. 141.5–142 °C, R_F 0.46 (B), $[\alpha]_D^{25}$ = + 21.5° (*c* = 1.0, 96% ethanol).

$C_{14}H_{18}N_2O_5$: calcd C 57.13, H 6.17, N 9.52%; found C 57.08, H 6.30, N 9.38%.

Synthesis of Ac-Ala-Tyr-OMe (2)

H-Tyr-OMe · HCl (4.10 g, 17.7 mmol) was coupled with Boc-Ala-ONSu (5.00 g, 17.5 mmol) in the presence of *N*-methylmorpholine (1.74 g, 17.6 mmol) under standard conditions. The chromatographically homogeneous protected dipeptide obtained, R_F 0.57 (B), was treated with hydrogen-chloride-saturated 1,2-dimethoxyethane to give the dipeptide methyl ester hydrochloride. This was acetylated with *N*-acetoxysuccinimide (2.75 g, 17.5 mmol) in the presence of *N*-methylmorpholine (1.73 g, 17.5 mmol). The crude product was crystallized from ethyl acetate; yield, 3.83 g (71%), m.p. 123–124 °C, R_F 0.50 (B), $[\alpha]_D^{22}$ = −25.5 ° (*c* = 1.0, 96% ethanol).

$C_{15}H_{20}N_2O_5$: calcd C 58.43, H 6.54, N 9.09%; found C 58.45, H 6.50, N 9.16%.

Synthesis of Ac-Val-Tyr-OMe (3)

Z-Val-Tyr-OMe (2.61 g, 6.15 mmol) was dissolved in anhydrous methanol (50 ml) containing dry hydrogen chloride (15 mmol) and hydrogenated at normal pressure over palladium on charcoal (10% Pd, 1.56 g) for 4 h. The catalyst was removed by filtration and the filtrate evaporated to dryness. The residue was freed of hydrogen chloride by drying at 0.001 Torr (0.133 Pa) over potassium hydroxide. The dipeptide methyl ester hydrochloride obtained was neutralized with triethylamine (610 mg, 6.02 mmol) and acetylated with *N*-acetoxysuccinimide (941 mg, 5.98 mmol). The crude product was twice crystallized from ethylacetate; yield, 870 mg (43%), m.p. 193–194 °C, R_F 0.61 (B), $[\alpha]_D^{24} = -31°$ ($c = 1.0$, 96% ethanol).

$C_{17}H_{24}N_2O_5$: calcd C 60.70, H 7.19, N 8.33%; found C 60.78, H 7.15, N 8.21%.

Synthesis of Ac-Pro-Tyr-OMe (4)

H-Tyr-OMe · HCl (2.36 g, 10.2 mmol) was coupled with Boc-Pro-ONSu (3.12 g, 10.0 mmol) in the presence of *N*-methylmorpholine (1.02 g, 10.1 mmol). The protected dipeptide obtained was treated with hydrogen-chloride-saturated 1,2-dimethoxyethane to give the dipeptide methyl ester hydrochloride. This was suspended in anhydrous *tert*-butanol (25 ml) and neutralized with *N*-methylmorpholine (911 mg, 9.00 mmol). The solution thus obtained was added to a solution of acetic acid (522 mg, 8.69 mmol) and carbonyldiimidazole (1.38 g, 8.51 mmol) in anhydrous 1,2-dimethoxyethane (25 ml). The reaction mixture was allowed to stand at room temperature for 16 h, reduced to one-half volume, diluted with 2 N hydrochloric acid (20 ml) and extracted several times with ethyl acetate. The combined ethyl acetate phases were washed with 1 M sodium hydrogen carbonate and water, dried over potassium sulphate, and evaporated to dryness. The residue (1.65 g), which could not be crystallized, was chromatographed twice on silica gel columns. By eluting with methanol–acetone–ethyl acetate 1:5:4 (by vol.) and methanol-ethyl acetate 1:9 (v/v), 870 mg (30%) of chromatographically homogenous, hygroscopic white powder was obtained: R_F 0.37 (A), 0.33 (methanol–benzene 1:4 v/v), $[\alpha]_D^{24} = -36°$ ($c = 1.0$, 96% ethanol).

$C_{17}H_{22}N_2O_5$: calcd C 61.06, H 6.63, N 8.38%; found C 60.75, H 6.66, N 8.32%.

Synthesis of Ac-Ala-Phe-OMe (5)

Boc-Ala-Phe-OMe (9). H-Phe-OMe · HCl (2.16 g, 10.0 mmol) was coupled with Boc-Ala-ONSu (2.85 g, 10.0 mmol) in the presence of *N*-methylmorpholine (1.01 g, 10.0 mmol). The crystalline crude product was recrystallized from carbon tetrachloride–cyclohexane to give 3.11 g (89%) of pure material, m.p. 80–83 °C, R_F 0.63 (A), $[\alpha]_D^{22} = -15.0$ C ($c = 1.0$, 96% ethanol).

$C_{18}H_{26}N_2O_5$: calcd C 61.70, H 7.48, N 8.00%; found C 61.59, H 7.50, N 7.98%.

The literature [7] records a m.p. 188–189 C for a product crystallized from ethyl acetate–petroleum ether.

Ac-Ala-Phe-OMe (5). The protected dipeptide (9) (2.85 g, 8.16 mmol) was treated with hydrogen-chloride-saturated 1,2-dimethoxyethane. The dipeptide methyl ester hydrochloride obtained was neutralized with *N*-methylmorpholine (821 mg, 8.12 mmol) and acetylated with *N*-acetoxy succinimide (1.27 g, 8.11 mmol) under standard conditions. The crystalline residue (2.26 g) was recrystallized from water; yield, 1.66 g (70%), m.p. 133.5–134 °C, R_F 0.61 (B), $[\alpha]_D^{22} = -36.5°$ ($c = 1.0$, 96% ethanol).

$C_{15}H_{20}N_2O_4$: calcd C 61.63, H 6.90, N 9.58%; found C 61.60, H 6.93, N 9.70%.

Synthesis of Ac-Pro-Phe-OMe (6)

H-Phe-OMe · HCl (2.46 g, 11.4 mmol) was coupled with Boc-Pro-ONSu (3.57 g, 11.4 mmol) in the presence of *N*-methylmorpholine (1.15 g, 11.4 mmol). The protected dipeptide obtained was treated with hydrogen-chloride-saturated 1,2-dimethoxyethane to give the dipeptide methyl ester hydrochloride. This was then dissolved in formamide (16 ml) and neutralized with *N*-methylmorpholine (1.01 g, 10.0 mmol). The solution was diluted with 1,2-dimethoxyethane (50 ml), and 1-acetoxybenzotriazole (1.77 g, 10.0 mmol) was added. The reaction mixture was allowed to stand at room temperature for 4 h, reduced to one-half volume, diluted with water (25 ml), and extracted with ethyl acetate in the usual way. The crude product was purified chromatographically on a silica gel column by eluting with chloroform–methanol 9:1 (v/v). The purified product was crystallized from benzene–cyclohexane and from ethyl acetate–diisopropyl ether to give 1.65 g (52%) of pure material, m.p. 106.5–108 °C, R_F 0.44 (A), $[\alpha]_D^{25} = -45°$ ($c = 1.0$, 96% ethanol).

$C_{17}H_{22}N_2O_4$: calcd C 64.13, H 6.97, N 8.80%; found C 64.04, H 6.97, N 8.78%.

Synthesis of Ac-Ala-Ala-Tyr-OMe (7)

H-Tyr-OMe · HCl (1.46 g, 6.30 mmol) was coupled with Boc-Ala-ONSu (1.80 g, 6.28 mmol) in the presence of *N*-methylmorpholine (638 mg, 6.30 mmol). The protected dipeptide obtained was treated with hydrogen-chloride-saturated 1,2-dimethoxyethane to give the dipeptide methyl ester hydrochloride. This was neutralized with *N*-methylmorpholine (630 mg, 6.22 mmol) and coupled with Boc-Ala-ONSu (1.71 g,

5.98 mmol). The chromatographically homogeneous protected tripeptide obtained, R_F 0.56 (B), was dissolved in cold trifluoroacetic acid (12 ml) and the solution allowed to stand for 1 h at 0 °C. The tripeptide methyl ester trifluoroacetate obtained on evaporating the reagent was dried at 0.001 Torr (0.133 Pa) over potassium hydroxide, dissolved in 20 ml of 1,2-dimethoxyethane–water 9:1 (v/v), and freed of trifluoroacetic acid by stirring for 1 h with an excess of solid sodium hydrogen carbonate. The remaining solid was removed by filtration, *N*-methylmorpholine (588 mg, 5.81 mmol) and *N*-acetoxysuccinimide (863 mg, 5.49 mmol) were added to the filtrate, and the solution was allowed to stand at room temperature for 4 h. The reaction mixture was worked up according to standard procedure to yield 1.62 g of crude product, which was crystallized from methanol–chloroform–diisopropyl ether and from water to give 896 mg (43%) of pure material, m.p. 208–210 °C, R_F 0.48 (B), $[\alpha]_D^{23} = -43°$ (c = 1.0, 96% ethanol).

$C_{18}H_{25}N_3O_6$: calcd C 56.98, H 6.64, N 11.08%; found C 57.15, H 6.60, N 10.85%.

KINETIC ANALYSIS

The hydrolysis of the substrates was followed by measuring with a pH-stat the amount of carboxylic acid product Q formed during the reaction. The dependence of the rates of product formation, v, on the concentrations of substrate A and alcohol product P was analyzed on the basis of Eqn (1) derived from the three-centre model:

$$\frac{v}{[E]} = \frac{V_A \frac{[A]}{K_A}}{1 + \frac{[A]}{K_A} + \frac{[P]}{K_P} + \frac{[A][P]}{K_{AP}}} \quad (1)$$

The composite kinetic constants of this equation are related to the rate constants of the single steps by the following equations:

$$V_A = \frac{k_{23}\, k'_{31}}{k_{23} + k'_{31}} \quad (2)$$

$$K_A = \frac{k'_{31}(k_{21} + k_{23})}{k_{12}(k_{23} + k'_{31})} \quad (3)$$

$$K_P = \frac{k'_{31}(k_{21} + k_{23})}{k_{21}\, k_{32}} \quad (4)$$

$$K_{AP} = \frac{k'_{31}(k_{21} + k_{23})}{k_{12}\, k_{32}} \quad (5)$$

where

$$k'_{31} = k_{31}\,[H_2O].$$

The constants V_A and K_A are identical with the Michaelis-Menten parameters k_{cat} and K_m.

The rates v were calculated at different points (reference points) along the entire progression curve according to the general-rates method described in a previous publication [5]. Since the initial substrate concentration was at least three times the value of K_A, and the reaction was followed to completion, all effective substrate concentrations become available in a single experiment. The substrate concentration at time t was determined from the difference between the initial amount of substrate and the amount of product Q formed at time t. In order to verify that the amount of base added did not systematically deviate from the amount of product formed, the total amount of base added was compared with the initial amount of substrate. If a deviation by more than 0.5% was observed the progression curve was not used for evaluation.

It was found that the product P (methanol for all substrates) formed during the reaction has no measureable influence on the rates; thus product P had to be added at different concentrations in order to be able to measure its effect on the rates. The plots of v *versus* $v/[A]$ at various product concentrations appear as series of straight lines, each line being characterized by a $k_{cat(P)}$ and a $K_{m(P)}$ value which are related to the initial product concentration $[P]_0$ by the equations

$$k_{cat(P)} = \frac{V_A}{1 + \frac{K_A[P]_0}{K_{AP}}} \quad (6)$$

$$K_{m(P)} = \frac{1 + \frac{[P]_0}{K_P}}{\frac{1}{K_A} + \frac{[P]_0}{K_{AP}}} \quad (7)$$

Plots of $1/k_{cat(P)}$ and $K_{m(P)}/k_{cat(P)}$ *versus* $[P]_0$ should therefore be linear. Preliminary experiments showed that this is the case when $[P]_0$ does not exceed 1.5 M. Higher $[P]_0$ values created unwanted side-effects and must be avoided. It has also been shown that the carboxylic acid product formed during the reaction does not affect the rates, as required by Eqn (1). The kinetic data was processed using the numerical procedure described previously (5). For the substrates reported here, the general rates were determined at 15–25 reference points along the progression curves by fitting second-order polynominals to 8–15 data points (values of the amount of Q) spaced at 3–9-s intervals. The parameters V_A, K_A, K_P and K_{AP} of Eqn (1) and their standard deviations were calcu-

Table 1. *Concentrations of the reactants*
The number of initial methanol concentrations within the range indicated is given in parentheses

Substrate	Enzyme concn	Initial substrate concn	Range of initial methanol concns
	nM	mM	M
Ac-Tyr-OMe	39.5	2.44	0–0.80 (10)
Ac-Gly-Tyr-OMe	33.2	4.62	0–1.24 (11)
Ac-Ala-Tyr-OMe	35.0	4.74	0–1.21 (11)
Ac-Val-Tyr-OMe	18.1	0.593	0–1.27 (8)
Ac-Pro-Tyr-OMe	18.6	1.74	0–1.28 (8)
Ac-Phe-OMe	77.0	3.52	0–1.23 (11)
Ac-Ala-Phe-OMe	95.6	4.74	0–1.07 (10)
Ac-Pro-Phe-OMe	54.2	2.12	0–1.30 (8)
Ac-Ala-Ala-Tyr-OMe	12.3	0.423	0–1.30 (8)

lated by iterative regression using first estimates of the parameters determined by linear regression on the reciprocal form of this equation [5].

MEASUREMENTS

The concentration of α-chymotrypsin (three-times crystallized, salt-free preparation from Sigma, lot 50 C-2550) was determined by the *N*-cinnamoylimidazole method [8]. All reagents were of analytical grade, and the water was doubly distilled (quartz apparatus).

Ester hydrolysis was carried out in a measuring cell containing 1 ml 10 mM potassium phosphate buffer pH 7.9, 1 ml 2 M NaCl solution, 1–5 ml substrate solution, 0–1.25 ml 10 M methanol solution, and water to a final volume of 9.5 ml. All solutions were pipetted using calibrated Hamilton syringes. The reaction mixture and samples of the enzyme solution (stock solution stored at 4 °C) were thermostatted in a water-bath at 25 °C. They were then placed in the titration apparatus and their pH adjusted manually to 7.90 by the addition of small amounts of base. The measurements at 25 °C were started by the addition of 0.5 ml of enzyme solution. In all cases the reaction was followed to completion. The titrating base solution was 0.04–0.1 M NaOH. The initial concentrations of substrate, product, and enzyme are summarized in Table 1. Two or three experiments were done at each set of initial concentrations.

RESULTS AND INTERPRETATION OF THE KINETIC PARAMETERS

The composite kinetic constants V_A, K_A, K_P and K_{AP} determined from the general rates of hydrolysis on the basis of Eqn (1) are summarized in Table 2.

Table 2. *Composite kinetic constants for the ester substrates measured in 0.2 M sodium chloride at pH 7.90 and 25 °C*

Substrate	V_A	K_A	K_P	K_{AP}
	s^{-1}	μM	mM	mM^2
Ac-Tyr-OMe	222 ±4	382 ± 5	425± 6	5250±500
Ac-Gly-Tyr-OMe	248 ±4	287 ± 2	435± 5	2220± 70
Ac-Ala-Tyr-OMe	141 ±2	182 ± 2	626± 7	5650±510
Ac-Val-Tyr-OMe[a]	86 ±3	41 ± 1	422± 10	–
Ac-Pro-Tyr-OMe	172 ±4	76 ± 1	471± 9	2260±270
Ac-Phe-OMe[b]	109 ±2	700 ±20	452± 11	2680±200
Ac-Ala-Phe-OMe	62.7±0.8	231 ± 5	476± 7	6760±640
Ac-Pro-Phe-OMe	72.0±1.6	79 ± 2	370± 10	3700±700
Ac-Ala-Ala-Tyr-OMe	128 ±5	13.4± 0.3	3590±360	421± 42

[a] The constant K_{AP} for this substrate could not be determined, since the dependence on $[P]_0$ of $k_{cat([P]=0)}/k_{cat[P]} = 1 + (K_A/K_{AP})[P]_0$ (see Kinetic Analysis) was too small to be measured.
[b] Reported in a previous publication [5].

From the ratio K_{AP}/K_P the values for the dissociation constants of the enzyme-substrate complexes, K_{EA}, were obtained (Table 3). The interpretation of the composite kinetic constants is facilitated by considering the following ratios:

$$\frac{V_A}{K_A} = \frac{k_{12}\,k_{23}}{k_{21} + k_{23}} = \alpha$$

$$\frac{V_A\,K_{AP}}{K_A\,K_P} = \frac{k_{21}\,k_{23}}{k_{21} + k_{23}} = \beta\,.$$

When the free enzyme and the enzyme-substrate complex are in rapid equilibrium, *i.e.* $k_{21} > k_{23}$, α reduces to k_{23}/K_{EA} and the acylation rate constant k_{23} can be determined. Conversely, when k_{21} is smaller than k_{23}, α approximates to k_{12}, as has been found in the case of acetyl-L-tryptophan *p*-nitrophenyl ester [9, 10]. Thus by comparing α with a reliable estimate of k_{12} it would be possible to determine which condition applies to the substrates investigated. The rate constant k_{12} has been shown to be little dependent on the structure of the substrate or inhibitor. The values reported for a number of ligands of widely different structure [11–13] are within a factor of ten, ranging from 6×10^6 to $6\times10^7\ M^{-1}\,s^{-1}$. It can be seen from Table 3 that the α values for the amino-acid derivatives Ac-L_{x1}-OMe and the dipeptide substrates Ac-L_{x2}-L_{x1}-OMe are below this range. Thus for these substrates the condition $k_{21} > k_{23}$ can be considered to apply. The α value of the tripeptide ester Ac-Ala-Ala-Tyr-OMe, however, is well within the range indicated for k_{12} and it can be expected that $k_{21} \leqslant k_{23}$. The following considerations seem to indicate that for this substrate $k_{21} < k_{23}$.

Table 3. *Dissociation constants of the enzyme-substrate complexes and rate constants calculated from the composite kinetic constants given in Table 2*
β approximates to k_{23} except for Ac-Ala-Ala-Tyr-OMe where it approximates to k_{21}

Substrate	K_{EA}	$10^{-3} \times \alpha$	β	k_{32}	k'_{31}
	mM	$M^{-1} s^{-1}$	s^{-1}	$M^{-1} s^{-1}$	s^{-1}
Ac-Tyr-OMe	12 ± 2	581 ± 18	7000 ± 1000	539 ± 19	229 ± 5
Ac-Gly-Tyr-OMe	5.1 ± 0.2	864 ± 20	4410 ± 270	604 ± 19	263 ± 5
Ac-Ala-Tyr-OMe	9.0 ± 0.9	775 ± 20	7000 ± 700	230 ± 8	144 ± 3
Ac-Pro-Tyr-OMe	4.8 ± 0.7	2260 ± 80	10800 ± 1800	372 ± 18	175 ± 5
Ac-Phe-OMe	5.9 ± 0.5	156 ± 7	920 ± 90	274 ± 20	124 ± 5
Ac-Ala-Phe-OMe	14 ± 2	271 ± 9	3800 ± 600	134 ± 7	64 ± 2
Ac-Pro-Phe-OMe	10 ± 3	911 ± 43	9100 ± 2500	195 ± 11	72 ± 2
Ac-Ala-Ala-Tyr-OMe	0.12 ± 0.03	9550 ± 580	1100 ± 250	–	–

Table 4. *Comparison of the V_A/K_A values of ester and peptide [3] substrates*

Substrate	Y = OMe	Y = Gly-NH_2	$10^{-4} \times \alpha/(k_{23}/K_{EA})$
	$\frac{V_A}{K_A} = \alpha$	$\frac{V_A}{K_A} = \frac{k_{23}}{K_{EA}}$	
	$mM^{-1} s^{-1}$	$M^{-1} s^{-1}$	
Ac-Tyr-Y	581	27.5	2.11
Ac-Gly-Tyr-Y	864	61.5	1.40
Ac-Ala-Tyr-Y	775	48.4	1.60
Ac-Val-Tyr-Y	2100	190	1.11
Ac-Pro-Tyr-Y	2260	140	1.81
Ac-Phe-Y	156	9.6	1.62
Ac-Ala-Phe-Y	271	15.3	1.77
Ac-Pro-Phe-Y	911	51.3	1.78
Ac-Ala-Ala-Tyr-Y	9550	5500	0.174

a) The value of β approximates to the acylation rate constant k_{23} when $k_{21} > k_{23}$. For the amino-acid derivatives and dipeptide esters, to which this condition has been shown to apply, β increases with the ability of the substrate to form secondary interactions with the enzyme and reaches an upper limit of about 10^4 s^{-1}. For the tripeptide ester, which is expected to form even more secondary interactions, β is only 1.1×10^3 s^{-1} (Table 3). This can be taken as evidence that for this substrate β does not represent the acylation rate constant k_{23}.

b) When $k_{21} > k_{23}$ the composite kinetic constant

$$K_P = \frac{k'_{31}\,(k_{21} + k_{23})}{k_{32}\,k_{21}}$$

approximates to k'_{31}/k_{32} and thus is a measure of the partitioning of the acyl enzyme between the attacking nucleophiles. This ratio is expected to remain practically constant for the series of the esters investigated, since it is unlikely that substrates giving rise to differences only in the secondary interactions would influence the partitioning of the acyl enzyme between such small and closely related nucleophiles as water and methanol. For the amino-acid derivatives and dipeptide esters similar values of K_P with an average of 460 ± 70 mM are observed, whereas the K_P of the tripeptide ester is ten times larger (Table 2). This suggests that for the latter substrate K_P does not reduce to k'_{31}/k_{32}.

c) In Table 4 the α values of the peptide esters E_x-. . .-L_{x1}-OMe can be compared with the k_{23}/K_{EA} values of the corresponding peptides E_x-. . .-L_{x1}-Gly-NH_2 reported in a previous paper [3]. When α reduces to k_{23}/K_{EA} the ratio $\alpha_{ester}/(k_{23}/K_{EA\ peptide})$ is expected to reflect the intrinsic difference in rates of enzyme acylation by peptide-ester and peptide substrates and therefore to be virtually constant. This is again found to be true for the amino-acid derivatives and dipeptide esters, the average value of the ratio being (16 ± 1) $\times 10^3$. The ratio pertaining to the tripeptide ester is ten times smaller, indicating that for this substrate α does not approximate to k_{23}/K_{EA}.

The apparently anomalous values of α, β and K_P found for this substrate, all deviating by a factor of ten from those expected, can be explained by assuming that in this case $k_{21} < k_{23}$. Under this condition α and

Table 5. *Rate constants for the hydrolysis of Ac-Ala-Ala-Tyr-OMe*

Constant	Value	Unit
k_{23}	10500	s^{-1}
k_{32}	350	$M^{-1}\,s^{-1}$
k'_{31}	130	s^{-1}
k'_{31}/k_{32}	370	mM

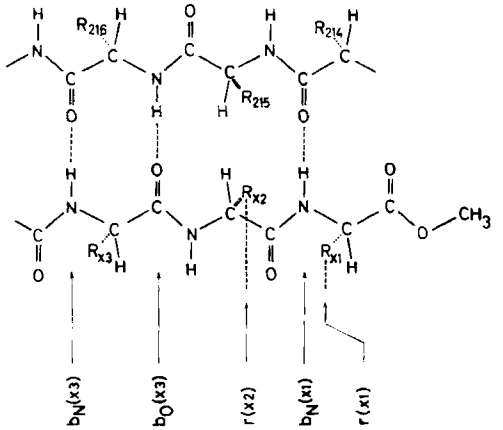


Fig. 1. *Interaction scheme.* The upper part represents the peptide chain of the enzyme active site and the lower part the peptide chain of the substrate. Two types of interactions are shown: the b(*xn*) interactions which occur between the backbones and the r(*xn*) interactions which occur between the side-chains. The positions at which these interactions are assumed to occur [1] are indicated by arrows

β approximate to k_{12} and k_{21} respectively and K_P becomes $k'_{31}k_{23}/k_{32}k_{21}$. From the average value of the ratio $(k_{23}/K_{EA})_{ester}/(k_{23}/K_{EA})_{peptide}$, 1.6×10^4, and from k_{23}/K_{EA} for Ac-Ala-Ala-Tyr-Gly-NH_2, 5.5×10^3 $M^{-1}\,s^{-1}$ [3], k_{23}/K_{EA} for Ac-Ala-Ala-Tyr-OMe is estimated to be 9×10^7 $M^{-1}\,s^{-1}$. On the basis of this estimate the rate constants k_{23}, k_{32} and k'_{31} for the tripeptide ester were calculated (Table 5). It is worth noting that the values obtained for k_{23} and for k'_{31}/k_{32} are consistent with those found for the other substrates.

DISCUSSION

As can be seen from Tables 3 and 5, structural changes involving the residues L_{x1} and L_{x2} result in small changes in the pseudo-first-order rate constant of acyl enzyme hydrolysis, k'_{31}. The absence of a large effect on k'_{31} is not surprising, since the acyl moiety of the substrate is firmly bound to the enzyme *via* a covalent bond. The introduction of secondary interactions, which are relatively weak bonds, would therefore have little influence on the spatial arrangement of the reacting atoms of the acyl enzyme and hence on its rate of hydrolysis. The influence of secondary interactions on the rate constant of acyl enzyme methanolysis, k_{32}, is similar to that on k'_{31} and can be explained accordingly. Consequently the ratio k'_{31}/k_{32}, which measures the water-methanol partitioning of the acyl enzyme, is similar for all substrates.

The relationship between the structure of the acyl part of peptide-ester substrates and their reactivity can best be discussed on the basis of the acylation rate constant k_{23}, the dissociation constant of the enzyme-substrate complex, K_{EA}, and their ratio k_{23}/K_{EA}, which represents the overall reactivity with respect to enzyme acylation. The effects of changes in the substrate structure on these constants are determined by comparing pairs of substrates which differ in a single structural feature. They are then related to the number and type of interactions which may occur between this structural feature and the active site, according to the binding scheme derived from crystallographic studies [1] (Fig. 1). The replacement of phenylalanine by tyrosine at position *x*1, as in comparison 1 of Table 6, results in an approximately 3-fold increase in k_{23}/K_{EA} for the three substrate pairs considered. It appears that a hydrogen bond is formed between the hydroxyl group of the tyrosine residue and a group of the active site which strengthens the r(*x*1) interaction (the symbols are defined in Fig. 1). The introduction of a glycine residue in *x*2 (comparison 2) leads to a 1.5-fold increase of k_{23}/K_{EA}. Substituting this glycine by an alanine (comparison 3) has little effect on the ratio. However, the replacing of this alanine by residues with larger hydrophobic side-chains, such as valine or proline (comparisons 5 and 6) leads to an approximately 3-fold increase in k_{23}/K_{EA}. On the basis of the interaction scheme these observations would appear to be consistent with the formation of a favorable interaction between the side-chain of the residue at *x*2 and a hydrophobic region of the enzyme surface. Consideration of the 3-dimensional structure of the enzyme, as visualized by a skeletal model constructed on the basis of the coordinates published by Birktoft *et al.* [14], indicates that this hydrophobic region consists of the side-chains of His-57 and Ile-99, and the α and β-carbons of Trp-215. Extending the peptide chain of Ac-Ala-Tyr-OMe by an alanine residue results in an increase in k_{23}/K_{EA} of 116-fold. This large effect can be attributed to the hydrogen bonds $b_O(x3)$ [1] and $b_N(x3)$ (Fig. 1) and possibly to interactions of the methyl side chain of L_{x3} with the backbone section of the enzyme near Ser-218.

As can be seen in Table 6 all these effects are markedly similar to those observed with the corresponding peptide substrates, indicating that they are independent of the type of bond being cleaved.

[1] From the investigation of the peptide substrates [3] it was concluded that this hydrogen bond is not formed when the acyl moiety of the peptide is Ac-L_{x2}-L_{x1}, even when the interaction scheme would predict such an interaction to occur.

Table 6. *Relationship between the structural changes of the substrates, the presence or absence of a particular interaction, and the changes in the steady-state kinetic constants*
The symbols for the interactions are defined in Fig. 1. The occurence of b(*xn*) interactions is denoted by + and that of r(*xn*) interactions by a letter representative of the side-chain involved (a, v, p, f, and y for alanine, valine, proline, phenylalanine and tyrosine, respectively). For each of the substrate pairs considered the values given are the ratios of the constant for the lower substrate to the constant for the upper substrate. The values without brakets are for Y = OMe and those in brakets for Y = Gly-HN_2

Substrates	Interactions proposed on the basis of the interaction scheme					Ratios resulting from comparisons 1 – 7 of																									
						k_{23}/k_{EA}							k_{23}							K_{EA}												
	b_N (*x*3)	b_O (*x*3)	r (*x*2)	b_N (*x*1)	r (*x*1)	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7						
Ac-Phe-Y				+	f	3.7 (2.9)			1.7 (4.6)				7.6 (4.6)			4.1 (2.5)				2.0 (1.6)			2.4 (1.6)								
Ac-Tyr-Y				+	y		1.5 (2.2)							0.6 (0.8)							0.4 (0.3)										
Ac-Gly-Tyr-Y		+		+	y			0.9 (0.8)							1.6 (2.9)							1.8 (3.7)									
Ac-Ala-Phe-Y		+	a	+	f	2.9 (3.3)			1.3 (1.8)		3.4 (3.3)		1.8 (4.0)			1.5 (3.1)		2.4 (2.1)		0.6 (1.3)			0.8 (1.2)		0.7 (0.6)						
Ac-Ala-Tyr-Y		+	a	+	y					2.7 (3.9)		116 (114)							1.5 (7.3)							0.013 (0.06)					
Ac-Val-Tyr-Y		+	v	+	y																										
Ac-Pro-Phe-Y		+	p	+	f	2.5 (2.7)					2.9 (2.9)		1.2 (5.8)					1.5 (3.1)		0.5 (2.1)					0.5 (1.1)						
Ac-Pro-Tyr-Y		+	p	+	y																										
Ac-Ala-Ala-Tyr-Y	+	+	a	+	y																										

It should also be noted that the same structural change in different substrate pairs (comparisons 1, 4 and 6) leads to similar increases in k_{23}/K_{EA}, suggesting that the influence of a particular structural change on this ratio is very little dependent on the nature of the remainder of the substrate. The effects on k_{23}/K_{EA} can, therefore, be considered a good measure for the total free-energy change associated with a particular interaction, whereas the effects on k_{23} and K_{EA} will indicate how this overall effect is partitioned into a rate effect (change in the activation energy) and an affinity effect (change in the stability of the enzyme-substrate complex).

The similarity of the effects on k_{23}/K_{EA} observed for the peptide-ester and peptide substrates as well as the regularities found within each of the two series is no longer apparent when k_{23} and k_{EA} are considered separately. In comparison 1, for example, where the k_{23}/K_{EA} ratios are constant, the k_{23} ratios vary from 1.2–7.6. Similarily, in comparison 4 the k_{23}/K_{EA} ratios are both approximately 1.5 while the k_{23} ratios are 1.5 and 4.1. On the basis of these observations it would appear that the effect of a particular structural change on the acylation rate constant k_{23}, unlike the overall rate constant k_{23}/K_{EA}, are dependent on the nature of the remainder of the substrate, *i.e.* the partitioning of the overall effect into a rate and an affinity effect is dependent on the nature of the remainder of the substrate. This conclusion is more clearly demonstrated by a comparison of the phenylalanine esters Ac-Phe-OMe, Ac-Ala-Phe-OMe, and Ac-Pro-Phe-OMe with the corresponding tyrosine esters Ac-Tyr-OMe, Ac-Ala-Tyr-OMe, and Ac-Ala-Pro-Tyr-OMe. In the case of the phenylalanine esters the introduction of an alanine at the $x2$ position and the replacement of this alanine by a proline lead to a 4.1-fold and 2.4-fold increase in k_{23} respectively. For the tyrosine esters, however, the introduction of the same secondary interactions results in no increase and a 1.5-fold increase in k_{23} respectively. In contrast, when the k_{23}/K_{EA} values are considered, the same sequence of increases is observed for both series of esters.

The differences in kinetic behaviour between the tyrosine and phenylalanine peptide esters can be rationalized on the basis of the principle that the rate constant for enzyme acylation reflects the spatial arrangement of the reacting atoms within the active site and that this arrangement is determined by the interactions which occur between the enzyme and the substrate. In the case of the tyrosine esters, it appears that the optimal arrangement of the reacting atoms is attained by the interactions of the L_{x1} residue alone. The introduction of additional residues on the x-side has therefore little influence on the spatial arrangement, and k_{23} remains unchanged. The interactions arising from these additional residues do, however, contribute to the stability of the enzyme substrate complex. This is clearly exemplified by comparison 7, where the peptide chain of Ac-Ala-Tyr-OMe is extended by an alanine residue. The estimated value of k_{23} for Ac-Ala-Ala-Tyr-OMe is 10^4 s^{-1} and is close to that for Ac-Ala-Tyr-OMe, while the K_{EA} value for the tripeptide ester is a factor of 75 smaller than that of the dipeptide ester. The value 10^4 s^{-1} for k_{23} appears to be near the maximum value which can be achieved with peptide methyl ester substrates and probably reflects an optimal arrangement for the reacting atoms of these substrates. It is interesting to note that Ac-Gly-Tyr-OMe is the only substrate in the tyrosine series which has a value significantly lower than 10^4 s^{-1}. A rather low value of k_{23} when Gly is at the $x2$ position is also observed for the corresponding peptide substrate and has been attributed to the formation of non-specific interactions, which produces a less favourable spatial arrangement of the reacting atoms [3].

For the phenylalanine-peptide esters the spatial arrangement induced by the formation of the interactions involving L_{x1} seems to be less favourable than for the tyrosine peptide esters. This difference in behaviour probably results from the absence of an interaction involving the phenolic hydroxyl group, so that the attainment of an optimal arrangement of the reacting atoms for the phenylalanine esters requires more interactions on the x-side than those provided by L_{x1} alone. The introduction of additional interactions should, therefore, result in a favourable reorientation of the substrate within the active site and a corresponding increase in k_{23}, while the value of K_{EA} should remain virtually unchanged. It can be seen from Table 3 that this kinetic behaviour is observed for the phenylalanine esters with the exception of the somewhat lower value of K_{EA} for Ac-Phe-OMe.

Further insight into the relationship between structure and reactivity is gained by comparing the kinetic behaviour of the tyrosine esters Ac-L_{xn}-. . .-Tyr-OMe and that of the corresponding tyrosine peptides Ac-L_{xn}-. . .-Tyr-Gly-NH_2 (Table 6). An increase in number and strength of the interactions on the x-side produces mainly a decrease in K_{EA} in the former series of substrates and mainly an increase in k_{23} in the latter; these effects can respectively be defined as an affinity and a rate effect. Considering the structural differences between peptide-ester and peptide substrates, it seems reasonable to propose that the difference in kinetic behaviour is related to the absence or presence of a glycine-amide moiety on the y-side of the substrate. It is known that such a moiety interacts with the enzyme [15, 16]; in particular a hydrogen bond between the NH of its terminal amide and the carbonyl oxygen of Phe-41 is likely to be formed, as suggested by an analogous interaction occurring between chymotrypsin and pancreatic trypsin inhibitor [17].

The finding that for the tyrosine peptides the introduction of secondary interactions on the *x*-side does not lead to a stabilization of the enzyme-substrate complex seems to indicate that interactions on the *y*-side interfere with the formation of interactions on the *x*-side, particularly those involving the residue in *x*2. The rate effect, which is observed with these substrates, can be explained by assuming that the introduction of further *x*-side interactions modifies the balance between the *x* and the *y*-side interactions and that the associated reorientation of the substrate results in a better spatial arrangement of the reacting atoms. For the tyrosine esters, where the *y*-side interactions are practically missing, no restraints prevent the formation of the *x*-side interactions in the enzyme-substrate complex. Thus in the case of these substrates the introduction at the *x*2 position of residues with increasing side-chain size and at the *x*3 position of an alanine residue does stabilize the complex. Since these additional residues can be accomodated in the active site without a reorientation of the substrate part containing the reacting atoms (see above) no rate effect is observed.

It can, therefore, be concluded that the orientational factor which had been proposed earlier to explain the behaviour of peptide substrates [3] also seems to provide a good basis for the interpretation of the results obtained in this work. The view that the precise orientation of the reacting atoms is the critical factor in determining the acylation rate is also supported by recent studies, which revealed a strongly preferred orientation for the nucleophilic attack of a carbonyl in general [18–21] as well as in the special case of the chymotrypsin-catalyzed reactions [22].

However, the aforementioned interference between the *x* and *y*-side interactions in the case of the tyrosine peptides suggests that an additional factor may be responsible for the rate effect: the steric restrictions which impede the simultaneous formation of optimal interactions on both sides of the substrate in the enzyme-substrate complex may become weaker when the reaction proceeds towards the transition state. This could occur as a consequence of the change in the geometry of the reacting group (tetrahedralisation of the carbonyl carbon). Additional groups on the *x*-side then can only lead to optimal interactions when the transition state is reached, thereby providing a net transition-state stabilization. Similar arguments have been proposed by other authors to explain the leaving-group specificity in the α-chymotrypsin-catalyzed hydrolysis of peptides [16]. Probably both, this and the orientational factor, contribute to the catalytic efficiency of the enzyme but it is obviously not yet possible to determine their relative importance.

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