Biochimica et Biophysica Acta, 660 (1981) 65–72
Elsevier/North-Holland Biomedical Press

BBA 69306

THE KINETICS OF HYDROLYSIS OF SOME EXTENDED *N*-AMINOACYL-L-PHENYLALANINE METHYL ESTERS BY BOVINE CHYMOTRYPSIN A_α

EVIDENCE FOR ENZYME SUBSITE S_5

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(Received February 5th, 1981)

Key words: Chymotrypsin kinetics; Peptide methyl ester hydrolysis; Activation constant; Subsite S_5; (Bovine)

A series of *N*-acetylated peptide methyl esters of general formula *N*-acetyl-(glycyl)$_n$-L-phenylalanine methyl ester (n = 0–3) has been synthesized to study the effect of varying aminoacyl chain length on the efficiency of chymotrypsin A_α (EC 3.4.21.1) catalysed ester hydrolysis. Values of k_{cat} and K_m for each substrate have been obtained from kinetic measurements at pH 8.00 and 25.0°C. It has been found that for the first three members of the series (n = 0–2) there is an increase in k_{cat} value as the aminoacyl chain length is increased. However, the kinetic constants (k_{cat} and K_m) for the third (n = 2) and fourth (n = 3) members of the series were found to be very similar. These results are shown to be consistent with a substrate binding scheme proposed for the isomeric enzyme chymotrypsin A_γ. The enzyme-catalysed reactions were also investigated over a range of temperature (15–35°C). In each case the Arrhenius law was obeyed, within experimental error, and evaluation of meaningful values for the thermodynamic functions of activation ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) was possible with certain assumptions. In contrast to the similarity of kinetic constants found for the third and fourth members of the substrate series, the corresponding values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ were markedly different. These results, together with those for the first two members of the series are interpreted in terms of a model binding system which is consistent with the existence of further enzyme subsites in the S_4–S_5 region.

Introduction

In early studies on proteolytic enzymes, the specificity of a particular enzyme was described in terms of the nature of the side chain of an amino acid residue which participates in the peptide bond broken by the enzyme-catalysed process. For example, 'trypsin-like' enzymes are specific for basic amino acids, viz. arginine and lysine. This type of specificity is now termed the 'primary specificity'. In recent years, X-ray crystallographic studies on enzyme-inhibitor complexes and a number of kinetic studies have shown that additional interactions between the enzyme surface and portions of the substrate molecule both adjacent to and remote from the primary specificity residue have a considerable effect on the stability of enzyme-substrate complexes and the overall efficiency of catalysis. Such effects contribute to what is now termed the 'secondary specificity' of the enzyme.

Chymotrypsin preferentially hydrolyses peptide bonds C-terminal to aromatic amino acid residues [1]. A number of kinetic investigations have been undertaken to define the secondary specificities of this enzyme. The effect of extending the aminoacyl chain of specific substrates on the amidase [2,3] and peptidase [4] activities of chymotrypsin A_α has shown that the enzyme has as least two subsites, termed S_2 and S_3 in the notation of Schechter and Berger [5], which bind substrate residues N-terminal

to the primary specificity residue (P_1).

An important contribution to an understanding of the intimate interaction between peptide substrates and the enzyme surface was made by Segal et al. [6]. In this work an X-ray crystallographic study of the complex formed between chymotrypsin A_γ and a peptide chloromethyl ketone inhibitor indicated that the backbone of the inhibitor was bound in an antiparallel β-type configuration with three extended residues of the main chain of the enzyme. In a parallel kinetic investigation of the esterase activity of chymotrypsin A_γ on a series of aminoacyl-L-phenylalanine methyl esters, Segal [7] demonstrated that the peptide binding scheme deduced from the X-ray crystallographic study was probably closely related to that occurring during enzymic hydrolysis. However, it is not clear from these studies whether potential enzyme subsites exist beyond S_3.

The aim of this study is to investigate the effect of secondary interactons in the enzyme subsite S_2 to S_5 region upon the esterase activity of chymotrypsin A_α. A series of extended specific substrates of general formula Ac-$(Gly)_n$-Phe-OMe, where $n = 0-3$, has been synthesized and the results of the kinetic work on the chymotrypsin A_α-catalysed hydrolysis of these substrates are reported here. In the design of this series of peptide substrates, the justifications of the choice of glycine as the aminoacyl chain extender and the acyl group as the N-terminal blocking group have been given by Green and Tomalin [8] for the design of a similar series of specific substrates for bovine α- and β-trypsins. The choice of L-phenylalanine as the primary specificity residue (P_1) was dictated primarily by synthetic considerations.

The kinetics of the enzymic catalysis have also been investigated over a range of temperature (15–35°C in 5°C intervals). Provided that the Arrhenius law is obeyed for all four substrates and that the rate of reaction is limited by a single chemical step (in this case enzyme deacylation) it is possible to evaluate the thermodynamic functions of activation ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$). These quantities are interpreted in terms of substrate binding to the secondary specificity sites in the region S_2-S_5.

Materials and Methods

Substrates

General. 4-Methylumbelliferyl-*p*-(*N,N,N*-trimethylammonium)-cinnamate hydrochloride was synthesized by the method of Jameson et al. [9]. Ac-Phe-OMe was obtained from Pfaltz and Bauer, Inc., Stamford, CT, U.S.A. *N*-Acetoxysuccinimide was prepared from *N*-hydroxysuccinimide (Sigma London Chemical Co. Ltd.) by the method of Treadway and Schultz [10]. Melting points were measured in open capillary tubes and the values quoted are uncorrected. All substrates were homogeneous by thin layer chromatography (TLC). The optical purity of each substrate was checked by monitoring the rapid total chymotrypsin ester hydrolysis in the pH-stat at pH 8.00. It was found that the quantity of NaOH required to neutralise the acid liberated from each peptide was equivalent, within the limits of experimental error, to the calculated amount, indicating that the substrates were optically pure.

Standard preparative procedures. (A) Mixed anhydride coupling to Cbz-Gly. Cbz-Gly was dissolved in tetrahydrofuran (5 ml/mmol) and triethylamine (1 equiv.). The mixture was cooled to −15°C before isobutylchloroformate (1 equiv.) was added with stirring over a period of 20 min. A solution of the amino acid or peptide methyl ester salt (1 equiv.) and triethylamine (1 equiv.) in anhydrous tetrahydrofuran (5 ml/mmol) at −15°C was then added. The solution was stirred overnight and allowed to come to room temperature. The triethylamine salt was filtered from the mixture, the filtrate was evaporated to dryness under reduced pressure and the resulting residue was dissolved in ethyl acetate (10 ml/mmol). After filtration the solution was washed with cold 1 M HCl, 1 M $NaHCO_3$ and saturated aqueous NaCl, dried over $MgSO_4$ and evaporated under reduced pressure yielding the crude product.

(B) Removal of the benzyloxycarbonyl protecting group. The benzyloxycarbonyl peptide methyl ester was treated for 30 min with a solution of HBr in glacial acetic acid (45% HBr, 1.0 ml/mmol). The peptide methyl ester hydrobromide was then precipitated by pouring the reaction mixture into vigorously stirred anhydrous diethyl ether (50 ml/mmol). After 15 min vigorous stirring the product was collected by filtration, washed with diethyl ether and dried.

(C) *N*-Acetylation with *N*-acetoxysuccinimide. The product from B was suspended in anhydrous tetrahydrofuran (8 ml/mmol). Triethylamine (1 equiv.) and *N*-acetoxysuccinimide (1 equiv.) were added and the

solution stirred at room temperature for 4 h. The crude product was obtained after removal of the triethylamine hydrobromide by filtration and evaporation of the filtrate to dryness under reduced pressure.

Synthesis of Ac-Gly-Phe-OMe. Cbz-Gly was synthesized by the method of Bergman and Zervas [11].

Phe-OMe · HCl. Thionyl chloride (17.85 g, 150 mmol) was added, dropwise with stirring, to methanol (70 ml) cooled to 3°C. L-Phenylalanine (8.25 g, 50 mmol) was added and the mixture stirred overnight at room temperature. After removal of the solvent and other volatile material by evaporation under reduced pressure the crude product was crystallized from ethanol: m.p. 158–160°C (lit. 159–161°C [12]).

Cbz-Gly-Phe-OMe. Cbz-Gly (3.14 g, 15.0 mmol) was coupled with Phe-OMe · HCl (3.23 g, 15.0 mmol) as described in procedure A. The product obtained was an oil which could not be crystallized, as found by other workers [13].

Ac-Gly-Phe-OMe. Cbz-Gly-Phe-OMe was deprotected by removal of the benzyloxycarbonyl group (procedure B). The resulting Gly-Phe-OMe · HBr (4.40 g, 13.9 mmol) was taken up in tetrahydrofuran and acetylated with *N*-acetoxysuccinimide (procedure C). The product was recrystallized twice from ethanol/water. It was observed that on drying in vacuo at 60°C the product formed a sticky substance suggesting that the crystalline compound was a hydrate. Segal [7] observed a loss of 5.47% by weight on drying in vacuo overnight at 50°C indicating 0.89 mol of H_2O/mol Ac-Gly-Phe-OMe.

Found	: C, 57.2; H, 6.8; N, 9.5
$C_{14}H_{18}N_2O_4$, $0.89H_2O$ requires	: C, 57.1; H, 6.8; N, 9.5%

Synthesis of Ac-Gly-Gly-Phe-OMe. Cbz-Gly-Gly-Phe-OMe. Cbz-Gly (1.84 g, 8.8 mmol) was coupled with Gly-Phe-OMe · HBr (2.79 g, 8.8 mmol) as described in procedure A. The product was obtained as a waxy solid by precipitation with *n*-hexane from an ethyl acetate solution.

Ac-Gly-Gly-Phe-OMe. Cbz-Gly-Gly-Phe-OMe was deprotected as in procedure B. The resulting Gly-Gly-Phe-OMe · HBr (2.00 g, 5.3 mmol) was acetylated with *N*-acetoxysuccinimide (procedure C). The crude product was crystallized twice from ethanol: m.p. 183–185°C (lit. 174.5–176.5°C [7]).

Found	: C, 56.9; H, 6.3; N, 12.3
$C_{16}H_{21}N_3O_5$ requires	: C, 57.3; H, 6.3; N, 12.5%

Synthesis of Ac-Gly-Gly-Gly-Phe-OMe. Cbz-Gly-Gly-Gly-Phe-OMe. Cbz-Gly (0.50 g, 2.4 mmol) was coupled with Gly-Gly-Phe · HBr (0.90 g, 2.4 mmol) by procedure A. The product was obtained as a waxy solid by precipitation from ethyl acetate solution with *n*-hexane.

Ac-Gly-Gly-Gly-Phe-OMe. Gly-Gly-Gly-Phe-OMe · HBr (0.90 g, 2.1 mmol) was obtained by deprotection of Cbz-Gly-Gly-Gly-Phe-OMe (procedure B) and acetylated with *N*-acetoxysuccinimide (0.30 g, 2.1 mmol) (procedure C). The product was crystallized twice from ethanol: m.p. 218–220°C decomp.

Found	: C, 54,5; H, 6.1, N, 14.0
$C_{18}H_{25}N_4O_6$ requires	: C, 55.1; H, 6.1; N, 14.3%

Enzyme

Bovine chymotrypsin A_α was a triply crystallized and lyophilized preparation from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. Stock solutions were prepared at an approximate concentration of 20 μM in 1 mM HCl. The exact operational molarity of the stock enzyme solutions was determined by spectrofluorimetric active site titration with 4-methylumbelliferyl-*p*-(*N,N,N*-trimethylammonium)-cinnamate hydrochloride by the method of Jameson et al. [9].

Kinetic measurements

The progress of ester hydrolysis was followed on a Radiometer pH-stat apparatus consisting of a pH meter (PHM62), titration unit (TTT60c), automatic burette (ABU13), titration assembly (TTA60) and recorder (REC61/REA160). The jacketted reaction vessel was thermostatted to ±0.1°C by circulation from a Grant LE8 water bath and all measurements were carried out under an atmosphere of nitrogen saturated with water. The glass electrode was standardized against two standard buffer solutions

[14]. Alkali solutions were prepared by diluting an aliquot of carbonate-free NaOH solution [15] into previously boiled-out distilled water. The absolute molarities of titrant solutions were determined by titration against solutions of potassium hydrogen phthalate [15]. Substrate solutions were made to contain 0.1 M NaCl and 5% (v/v) dimethylformamide. Kinetic runs were carried out by thermostatting an aliquot of substrate solution (2–3 ml) in the reaction vessel. The pH was then brought to 8.00 by the addition of a measured quantity of alkali plus a further quantity to compensate accurately for the acidity of the stock enzyme solution. There was no evidence of non-enzymic hydrolysis. Stock enzyme solution (0.01 ml) was then added and the uptake of alkali recorded as a function of time. Examination of progress curves for reactions carried out under zero-order conditions showed that there was no progressive loss of enzyme activity within the time of the experiment. This was true over the whole temperature range. Initial velocities for all kinetic runs were computed by using a curve-fitting procedure involving orthogonal polynomials [16]. Values of k_{cat} and K_m were computed using the method of Wilkinson [17].

Results and Discussion

The kinetic results for the chymotrypsin A_α-catalysed hydrolysis of the four substrates are given in Table I. A substrate binding scheme which would seem appropriate to explain these results was proposed by Segal et al. [6], on the basis of an X-ray crystallographic study of the covalent complex formed between chymotrypsin A_γ and a series of aminoacyl-L-phenylalanine chloromethyl ketone inhibitors. The main feature of the proposed binding scheme is the existence of an antiparallel β-type interaction between the aminoacyl chain and an extended peptide chain at the enzyme surface adjacent to the primary specificity site. The protein residues involved in the interaction are Ser-214,Trp-215 and Gly-216. The scheme proposed by Segal et al. and extended to accommodate the four acyl-enzymes formed during the enzymic reaction is set out in terms of the Schechter and Berger notation in Fig. 1. All four acyl-enzymes have a hydrogen bond between the NH group of Phe(P_1) and the CO group of Ser-214(S_1). In accordance with the antiparallel β-configuration proposed there are no hydrogen bonds between Gly(P_2) and Trp-215(S_2), but there are two possible hydrogen-bonded interactions at Gly-216(S_3). The single interaction of the *N*-acetyl group of Ac-Gly-Phe-chymotrypsin A_α (designated P_3^a/S_3^a) is accompanied by a significant increase in k_{cat} value and decrease in K_m value compared to its predecessor. However, with Ac-Gly-Gly-Phe-chymotrypsin A_α the formation of the second of the potential hydrogen bonds (designated P_3^b/S_3^b) is also possible and this is accompained by the largest increase in k_{cat} value and the largest drop in K_m value. In this case the P_3/S_3

TABLE I

KINETIC CONSTANTS FOR CHYMOTRYPSIN A_α AND β-TRYPSIN-CATALYSED HYDROLYSIS OF SPECIFIC *N*-ACETYL PEPTIDE METHYL ESTER SUBSTRATES

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	Number of runs
Chymotrypsin A_α *	Ac-Phe-OMe	59.9 ± 1.7	1.24 ± 0.07	48	26
	Ac-Gly-Phe-OMe	72.6 ± 3.5	0.86 ± 0.10	84	26
	Ac-Gly-Gly-Phe-OMe	117.7 ± 4.3	0.26 ± 0.02	453	27
	Ac-Gly-Gly-Gly-Phe-OMe	134.2 ± 12.5	0.25 ± 0.04	537	24
β-trypsin **	Ac-Lys-OMe	127 ± 2	0.088 ± 0.005	1 400	36
	Ac-Gly-Lys-OMe	347 ± 10	0.190 ± 0.020	1 800	26
	Ac-Gly-Gly-Lys-OMe	285 ± 7	0.230 ± 0.020	1 200	26

* This work. pH 8.00, 25.0°C, I = 0.1 M, 5% (v/v) dimethylformamide.

** Data from Green and Tomalin [8]. pH 8.00, 25.0°C, I = 0.2 M.

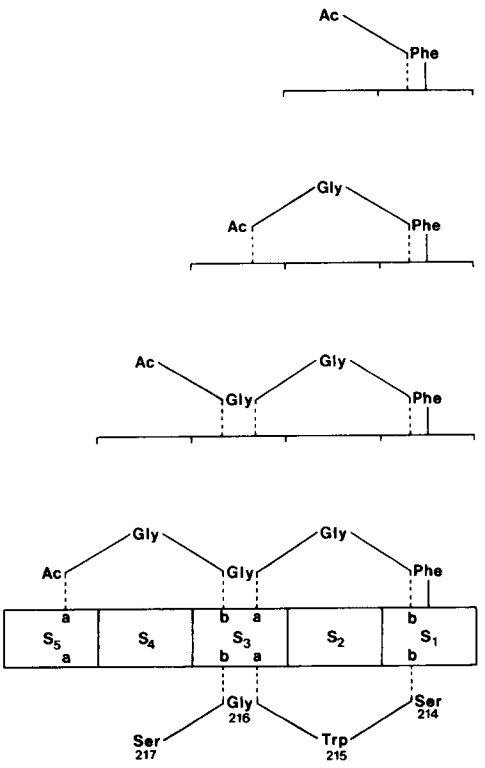

Fig. 1. Schematic diagram of the subsite binding in *N*-aminoacyl-L-phenylalanyl chymotrypsins based on the notation of Schechter and Berger [5]. The hydrogen bonds involved in the antiparallel β-configuration with residues 214–216 of the enzyme are shown.

interaction is a complete one (i.e., $P_3^a/S_3^a + P_3^b/S_3^b$) in terms of the antiparallel β-type configuration and is the cornerstone of its stability. A stable interaction of this type clearly has the greatest influence on the kinetic constants and this will be referred to later. For Ac-Gly-Gly-Gly-Phe-chymotrypsin A_α the model of Segal et al. has been extended to include subsite S_5 as shown in Fig. 1. If the β-type structure continues beyond subsite S_3 there would be no hydrogen-bonded interactions at subsite S_4 (as with S_2) but there would be the possibility of a hydrogen bond between the *N*-acetyl group of the tetrapeptide substrate and subsite S_5 (i.e., P_5^a/S_5^a). Segal et al. [6] have noted that the backbone of the protein chain bends sharply inwards between Gly-216 and Ser-217 in the crystalline inhibitor complexes, thus casting doubt on the possibility of the β-type configuration extending beyond subsite S_3. However, a significant increase in the k_{cat}/K_m value for the longest substrate indicates that there may be an interaction at subsite S_5.

The chymotrypsin A_α-catalysed hydrolysis of the *N*-aminoacyl-L-phenylalanine methyl esters was studied over the temperature range 15–35°C in 5°C intervals. The values of k_{cat} at each temperature are summarized in Table II. The Arrhenius law is obeyed, within experimental error, for each of the four substrates. In each case there was no evidence of curvature in the Arrhenius plot which is indicative that there are no gross changes in mechanism or

TABLE II

THE EFFECT OF TEMPERATURE ON THE CATALYTIC RATE CONSTANT FOR THE CHYMOTRYPSIN A_α-CATALYSED HYDROLYSIS OF AMINOACYL-L-PHENYL ALANINE METHYL ESTERS. SUMMARY OF THE VALUES OF THE THERMODYNAMIC CONSTANTS OF ACTIVATION FOR DEACYLATION OF THE ENZYME

Temperature (°C)	Catalytic rate constant k_{cat} (s^{-1}) for:			
	Ac-Phe-OMe	Ac-Gly-Phe-OMe	Ac-Gly-Gly-Phe-OMe	Ac-Gly-Gly-Gly-Phe-OMe
15.0	38.9 ± 1.4	36.4 ± 1.4	82.5 ± 7.3	79.4 ± 4.6
20.0	46.4 ± 2.6	51.8 ± 11.7	94.7 ± 3.8	97.9 ± 4.6
25.0	59.9 ± 1.7	72.6 ± 3.5	117.7 ± 4.3	134.2 ± 12.5
30.0	87.1 ± 4.1	102.0 ± 5.0	141.0 ± 4.1	173.5 ± 14.1
35.0	116.0 ± 9.0	149.4 ± 11.3	152.6 ± 5.5	234.2 ± 30.7
Activation constants				
$\Delta H_3^{\ddagger}$ ($kJ \cdot mol^{-1}$)	38.9	49.1	22.3	37.3
$\Delta S_3^{\ddagger}$ ($JK^{-1} \cdot mol^{-1}$)	−79.9	−44.3	−130.3	−77.6
$\Delta G_3^{\ddagger}$ ($kJ \cdot mol^{-1}$) (t = 25°C)	62.7	62.3	61.1	60.4

changes in the rate-determining step within this temperature range.

It is well established that the rate-determining step for the trypsin and chymotrypsin-catalysed hydrolysis of specific ester substrates is often deacylation of the acyl-enzyme intermediate (ES′) formed during the catalytic process,
i.e., $k_{cat} = k_3$ in the scheme below

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{P_1}{\overset{k_2}{\rightarrow}} ES' \overset{k_3}{\rightarrow} E + P_2 \qquad (1)$$

In order to obtain meaningful interpretation of the results it is assumed for the present that $k_{cat} \approx k_3$, $\Delta H^{\ddagger}_{obs} \approx \Delta H^{\ddagger}_3$ and $\Delta S^{\ddagger}_{obs} \approx \Delta S^{\ddagger}_3$. It can be seen that there is a slight trend to more negative values of $\Delta G^{\ddagger}_3$ corresponding to the increased k_{cat} values as the substrates are extended. The corresponding $\Delta H^{\ddagger}_3$ values, however, do not reflect this trend. Therefore any interpretation in terms of enzyme-substrate interactions must take into account the entropy term $\Delta S^{\ddagger}_3$. Furthermore, the values of both $\Delta H^{\ddagger}_3$ and $\Delta S^{\ddagger}_3$ for the tri- and tetrapeptide substrates are markedly different indicating that the similar catalytic rate constants observed for these two substrates at 25°C is fortuitous and conclusions based on this observation must be re-examined.

The simplest way the results may be interpreted is to assume that each subsite on the enzyme acts independently of its neighbours and consequently each subsite/substrate interaction contributes in a cumulative way to the activation functions. This assumption is a reasonable one for the substrate series in question since the interactions involve hydrogen bonds which will be free of steric interference or displacement by bulky side chain groups on the substrate. If it is also assumed that the contributions to the activation functions, common to all four substrates, are embodied in the values for Ac-Phe-chymotrypsin A_α then it is possible to estimate the contributions to $\Delta H^{\ddagger}_3$ and $\Delta S^{\ddagger}_3$ for each subsite interaction in the following way.

$$\Delta\Delta H^{\ddagger}_3 = \Delta H^{\ddagger}_3[\text{Ac-(Gly)}_n\text{-Phe-OMe}] - \Delta H^{\ddagger}_3[\text{Ac(Gly)}_{n-1}\text{-Phe-OMe}] \qquad (2)$$

This treatment can be similarly applied to $\Delta\Delta S^{\ddagger}_3$. The results are given in Table III. Addition of one interaction at subsite S_3 (P^a_3/S^a_3) in Ac-Gly-Phe-chymotrypsin A_α (H-bond donated from NH of Gly-216 to CO of substrate acetyl group) contributes +10.2 kJ · mol^{-1} to $\Delta H^{\ddagger}_3$ and +35.6 JK^{-1} · mol^{-1} to $\Delta S^{\ddagger}_3$. This hydrogen bond assists the catalytic process, not by lowering $\Delta H^{\ddagger}_3$, but by presumably ordering the acyl enzyme in such a way that only a small degree of further ordering is required to form the transition state for the deacylation process. Addition of a further hydrogen bond (P^b_3/S^b_3) in Ac-Gly-Gly-Phe-chymotrypsin A_α (H-bond donated from NH of the substrate Gly to CO of Gly-216) has the opposite effect. It was seen earlier that the formation of the P^b_3/S^b_3 interaction led to a relatively large increase in k_{cat} for hydrolysis of this substrate. In this case the corresponding $\Delta H^{\ddagger}_3$ value is dramatically lowered by

TABLE III

THE EFFECT OF INDIVIDUAL SUBSITE INTERACTIONS ON THE VALUES OF THE ENTHALPY AND ENTROPY OF ACTIVATION FOR THE CHYMOTRYPSIN A_α and β-TRYPSIN-CATALYSED HYDROLYSIS OF SPECIFIC *N*-AMINOACYL PEPTIDE ESTER SUBSTRATES

Enzyme	Substrate	$\Delta\Delta H^{\ddagger}_3$ (kJ · mol^{-1})	$\Delta\Delta S^{\ddagger}_3$ (JK^{-1} · mol^{-1})
chymostrypsin A_α *	Ac-Gly-Phe-OMe	+10.2	+35.6
	Ac-Gly-Gly-Phe-OMe	−26.8	−86.0
	Ac-Gly-Gly-Gly-Phe-OMe	+15.0	+52.7
β-trypsin **	Ac-Gly-Lys-OMe	+9.4	+39.4
	Ac-Gly-Gly-Lys-OMe	−2.3	−7.5

* This work.
** Green and Tomalin [8].

26.8 kJ · mol^{-1} but its effect on the catalytic rate is counteracted somewhat by a larger negative entropy requirement on forming the transition state. The net effect of the full P_3/S_3 interaction ($P_3^a/S_3^a + P_3^b/S_3^b$) is therefore to lower the enthalpy 'barrier' by 16.6 kJ · mol^{-1} and to increase the entropy 'barrier' by 23.4 JK^{-1} · mol^{-1}.

If the antiparallel β-configuration continues beyond subsite S_3 the next hydrogen bonded interactions would occur at subsite S_5. The first of the two possible interactions (P_5^a/S_5^a) would be realised with the CO group of the *N*-acetyl residue of Ac-Gly-Gly-Gly-Phe-chymotrypsin A_α. This interaction is similar to the corresponding P_3^a/S_3^a interaction and it would be reasonable to expect similar contributions to the activation functions. Table III shows this to be the case with $\Delta H_3^\ddagger$ increased by +15 kJ · mol^{-1} and $\Delta S_3^\ddagger$ increased by +52.7 JK^{-1} · mol^{-1}.

It is concluded therefore that subsite interactions may well continue up to subsite S_5 in chymotrypsin A_α. However, a possibility which cannot be excluded, is that such an interaction is only possible when subsites S_2 to S_4 are occupied by glycine residues. Although it has been noted that the antiparallel β-type configuration may not continue up to and include subsite S_5 since the main chain of the enzyme, according to Segal et al., apparently makes a sharp bend between Gly-216(S_3) and Ser-217, it is possible that the acetyl group (P_5) of the tetrapeptide substrate interacts in a similar way with either the side chain of a conveniently placed amino acid or to some other part of the enzyme backbone.

It is significant that the above results are to some extent remarkably similar to those for a previous study with β-trypsin [8], where it was definitely established that $k_{cat} = k_3$. The catalytic constants are summarized in Table I. For β-trypsin the k_{cat} value increases in going from Ac-Lys-OMe to Ac-Gly-Lys-OMe, but in contrast to chymotrypsin A_α, the value for the next substrate decreases slightly rather than increasing. Values for $\Delta H_3^\ddagger$ and $\Delta S_3^\ddagger$ are summarized in Table III for comparison. The values for the dipeptide substrates agree remarkably well indicating very similar P_3^a/S_3^a interactions. However, there is a large discrepancy in the values for the tripeptide substrates, indicating that the enzyme subsites behave very differently in the two enzymes at the P_3^b/S_3^b position. Results from X-ray crystallographic work on the porcine trypsin-soybean trypsin inhibitor (Kunitz) complex [18] indicate that the enzyme chain between residues 216 and 220 (Gly-Tyr-Gly-Cys) forms a β-bend of type I [19] in which the NH group of Cys-220 is hydrogen-bonded to the CO group of Gly-216. The CO group of Gly-216 is thus, approx. 180° from its orientation in chymotrypsin A_α. A similar situation occurs in bovine trypsin where the CO group of this residue occupies an intermediate position of 90° from its chymotrypsin A_α orientation. At subsite S_3 it appears that trypsin is able to form the P_3^a/S_3^a hydrogen bond but unlike chymotrypsin is unable to form the P_3^b/S_3^b hydrogen bond involving the CO group of Gly-216. It is therefore possible to explain the similarity of $\Delta\Delta H_3^\ddagger$ and $\Delta\Delta S_3^\ddagger$ for the two dipeptide substrates and why there is a large discrepancy for the next pair of substrates.

It may be argued that k_2 for the L-phenylalanine substrate studied here may not be sufficiently greater than k_3 to justify the assumption that $k_{cat} = k_3$ [20,21]. Consideration of the experimental evidence given and the striking parallel with the activation constants for β-trypsin indicate that the present assumption that $k_{cat} \approx k_3$ does not lead to erroneous interpretation. Using this system is is possible, in principle, with further extensive measurements to evaluate both k_2 and k_3 so that the effect of subsite interactions on acylation and deacylation may be measured.

In conclusion it appears that useful information about secondary enzyme substrate interactions may be obtained from kinetic measurements, particularly in conjunction with the results of X-ray crystallographic studies. However, it has been shown that it is possible to draw over simplistic conclusions from rate data measured at a single temperature. Unfortunately there is a considerable body of published material which draws conclusions from such data. In order to correctly understand the effect of secondary interactions more fully it is desirable to obtain values for the thermodynamic functions of activation from kinetic measurements over the largest possible temperature range. Such data are readily obtained with some enzyme systems but it must be conceded that interpretation may be more difficult in systems where there are experimental problems such as thermal denaturation of the enzyme, non-adherence to the Arrhenius law or where there is no clearly defined rate-determining step in the enzymic reaction.

Acknowledgements

The authors thank the Science Research Council for a Research Studentship (to C.R.H.), the Medical Research Council for a grant for equipment (to G.T.) and Mr. M. Hart and staff of the Microanalytical Laboratory, Department of Chemistry, University of Manchester for microanalytical work.

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