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Kinetic studies of pepsin active site model compound and porcine pepsin

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ABSTRACT: The kinetic parameters for the hydrolysis of the heptapeptide Pro–Thr–Glu–Phe-(4-NO_2)Phe–Arg–Leu by the pepsin model compound tetrabutylammonium monosalt of *m*-aminobenzoic acid diamide of fumaric acid (TBA *m*-FUM) and porcine pepsin were determined using a spectrophotometric technique. According to the $\Delta S^{\neq}$ values obtained, in the transition state the inner motion in the TBA *m*-FUM–heptapeptide complex is more restricted than that in the pepsin–heptapeptide complex. The model compound TBA *m*-FUM can cause a cleavage of the Phe—(4-NO_2)Phe bond in the substrate molecules following a mechanism similar as that suggested for pepsin, but its catalytic activity is much lower. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: pepsin model compounds; kinetics; UV spectra; peptide bond hydrolysis

INTRODUCTION

The activity of pepsin has been assayed by hydrolysis of naturally occurring proteins such as hemoglobin.[1] However, many bonds in hemoglobin molecules have been cleaved simultaneously and therefore this compound appeared unsuitable for mechanistic studies.[2] Consequently, other substrates, i.e. dipeptides and polypeptides, were used in investigations of the catalytic action of enzymes.[3–5] The kinetic data for the hydrolysis of a series of peptide substrates have revealed that the Phe—Phe and Phe—(4-NO_2)Phe bonds were hydrolysed more rapidly than the other peptide bonds,[5] while the reaction rate was markedly affected by the structure of residues on both sites of the scissile bond. The heptapeptide Pro–The–Glu–Phe–(4-NO_2)Phe–Arg–Leu was designed as one of the best substrates for detailed studies on the stereochemistry and intermolecular forces in the active site of a number of the aspartic proteinases.[6] By x-ray investigation it has been shown that an active site fissure of porcine pepsin can be occupied by a heptapeptide.[7] Such a result was previously predicted by theoretical calculations.[8] Analysis of heptapeptide hydrolysis products indicated the formation of two peptides, Pro–Thr–Glu–Phe and (4-NO_2)Phe–Arg–Leu).[6] It has been found that the kinetics of the peptide bond splitting can be observed by a spectrophotometric method involving measurement of the change in absorbance at 300 nm.[5,6,9,10]

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Recently, monosalts *of o-*, *m-* and *p*-aminobenzoic acid diamides have been synthesized as model compounds resembling the active site of pepsin.[9] These compounds include two carboxylic groups with a different distance between them. Three of the model compounds showed hydrolytic activity with respect to oxindole, while tetrabutylammonium monosalt of *m*-aminobenzoic acid diamide of fumaric acid (TBA *m*-FUM) also caused cleavage of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu.[9] The aim of this work was to study the kinetics of this reaction. For the sake of comparison, the reaction with pepsin was also investigated under comparable experimental conditions.

EXPERIMENTAL

Porcine pepsin and substrate

Pepsin was obtained from Aldrich-Chemie. The heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu and isovalerylpepstatin were purchased from Bachem. All these compounds were used as received. The activity of pepsin was determined by titration of the enzyme against a solution of isovalerylpepstatin as described by Dunn *et al.*[6]

Synthesis of tetrabutylammonium monosalt of *m*-aminobenzoic acid diamide of fumaric acid (TBA *m*-FUM)

Synthesis of *m*-FUM. *m*-FUM (Fig. 1) was prepared from fumaric acid and *m*-aminobenzoic acid. Fumaric

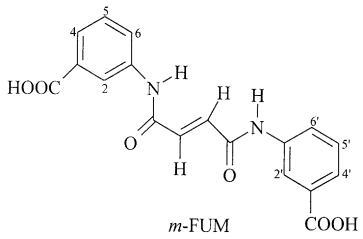

Figure 1. Structure of *m*-aminobenzoic acid diamide of fumaric acid (*m*-FUM)

acid (0.01 mol) and oxalyl chloride (0.05 mol) were stirred and refluxed for 24 h in chloroform. Fumaryl chloride was separated, diluted in 50 cm^3 of dioxane and stirred with *m*-aminobenzoic acid for 24 h. Dioxane was evaporated under reduced pressure. The *m*-FUM obtained was filtered, washed with water and dried. The purity was confirmed by elemental analysis: C 60.9 (61.0), H 4.1 (4.0), N 7.9 (7.9)%. The compound melted with carbonization at 300 °C. The reaction yield was 78%.

The ^{1}H NMR spectrum of *m*-FUM shows four aromatic signals at 7.75 (CH 6,6′), 7.51 (CH 5,5′), 7.96 (CH 4,4′) and 8.42 ppm (CH 2,2′). The signal of CH 2,2′ is shifted owing to the closeness of carboxylic and amide groups. The two acidic protons are found at 12.5 ppm. Protons, which are attached to the double bonded carbons and to the amide groups (NH), have signals at 7.2 and 10.93 ppm, respectively.

Synthesis of tetrabutylammonium monosalt of *m*-FUM (TBA *m*-FUM). A solution of 8.80 mg (2.5×10^{-5} mol) of *m*-FUM in 5 cm^3 of ethanol was added to 0.025 cm^3 of 1 m tetrabutylammonium hydroxide in methanol. The alcohol was evaporated under reduced pressure and the *m*-FUM monosalt was dissolved in 25 cm^3 of water. From this stock solution we prepared dilute solutions for further studies.

Mass spectra

Low-resolution liquid secondary ion mass spectrometry (LSIMS) of the reaction products after completed hydrolysis of the heptapetide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu with TBA *m*-FUM was performed on an AMD 604 two-sector mass spectrometer (AMD Intectra, Germany) of BE geometry. *m*-Nitrobenzyl alcohol (NBA) was used as a matrix.

Determination of kinetic parameters

Solutions of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu of concentration ranging from 5.2 to 209 μm were prepared in water (in the case of TBA *m*-FUM) or in 10^{-3} m hydrochloric acid (in the case of pepsin). The progress of the substrate hydrolysis by TBA *m*-FUM or porcine pepsin was monitored by recording the change in absorbance at 300 nm versus time (Figs 2 and 3). The kinetic runs were carried out using a stopped-flow spectrophotometer (Applied Photophysics) with the cell block termostated to ±0.1 °C.

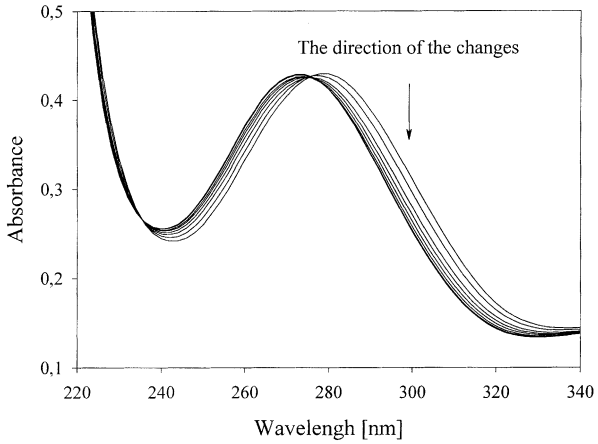

Figure 2. Change in UV absorbance of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu. Temperature, 293 K; solvent, hydrochloric acid (0.001 M), time interval, 10 s; heptapeptide concentration, 105 μM, pepsin concentration, 23 nM

The least-squares procedure was applied for computation of the initial reaction rates (V) with at least seven values of the substrate concentration ([S]) at each constant concentration of the enzyme model compound or enzyme ([E]). In all cases, a hyperbolic V vs [S] dependence was obtained according to the Michaelis–Menten equation. The maximum velocity of the heptapeptide hydrolysis (V_{MAX}) and the Michaelis–Menten constant (K_M) were derived from a non-linear regression analysis of the experimental data and a linear regression

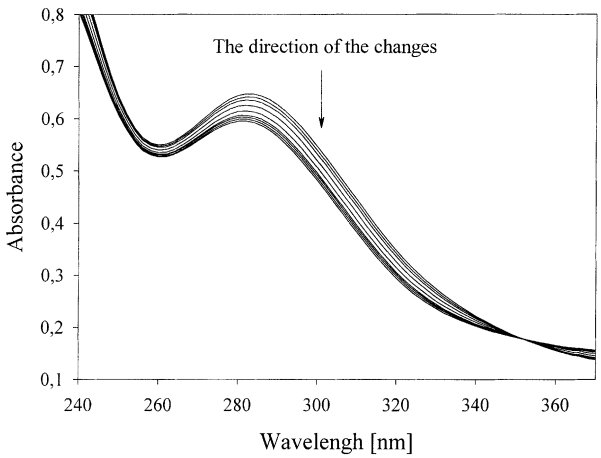

Figure 3. Change in UV absorbance of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu. Temperature, 293 K; solvent, water; time interval, 10 s; heptapeptide concentration, 105 μM; TBA *m*-FUM concentration, 99 μM

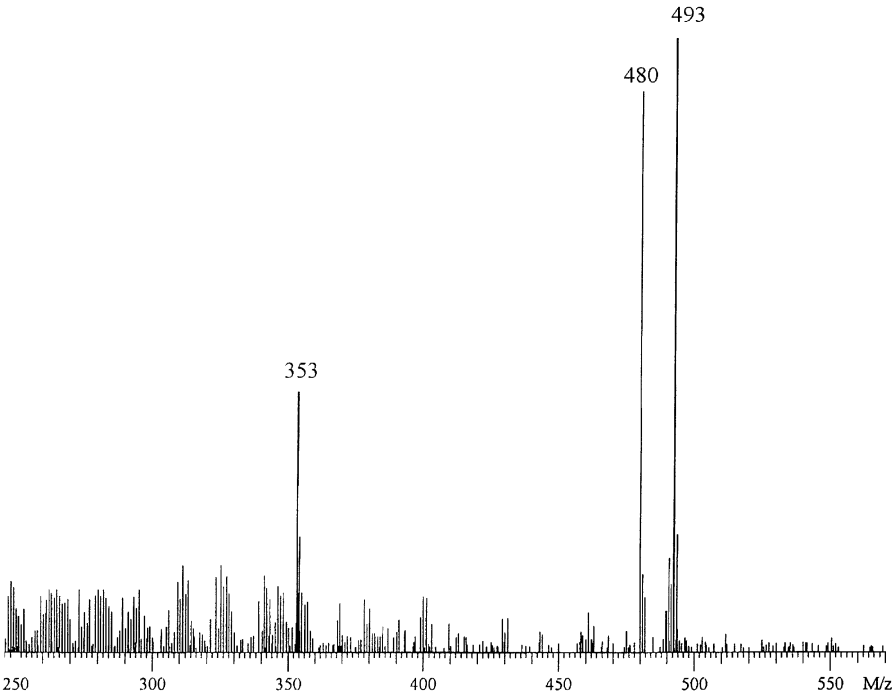

Figure 4. LSIMS of the product mixture obtained having completed hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu by TBA *m*-FUM

analysis of the Lineweaver–Burk, Hanes and Eadie–Hofstee plots.[11,12] The catalytic constants, $k_{cat} = V_{MAX}$ / [E], were calculated using the experimentally obtained V_{MAX} values and the appropriate concentration of TBA *m*-FUM or porcine pepsin. The pseudo-first-order rate constants (k_{obs}) were found from the time dependence of absorbance according to the equation $k_{obs} - (1/t)\ \ln (A_\infty - A_o)/(A_\infty - A_t)$, where A_0 initial absorbance, A_t = absorbance at time t and A_∞ = absorbance at infinite time. The activation entropy ($\Delta S^{\neq}$), enthalpy ($\Delta H^{\neq}$) and free enthalpy ($\Delta G^{\neq}$) were evaluated from the Eyring's equation by a linear least-squares fit of ln k_{obs} vs $1/T$.

RESULTS AND DISCUSSION

The hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu as a substrate by TBA *m*-FUM yields two peptides, which were identified by mass spectrometry technique. An example of the mass spectrum of the product mixture taken having completed the hydrolysis of the heptapeptide by *m*-FUM is given in Fig. 4. Examination of this mass spectrum confirms the conclusion drawn from UV and IR data that the only bond cleaved is the Phe–(4-NO_2)Phe bond.[9] The formation of the tripeptide and tertrapeptide is well evidenced by the peaks: *m/z* 480 for (4-NO_2)Phe–Arg–Leu and *m/z* 493 for Pro–Thr–Glu–Phe. The *m/z* 353 peak can be assigned to the *m*-FUM anion.

The kinetic parameters of the Michaelis–Menten equation are given in Table 1. The value of $k_{cat.}/K_M$ for pepsin is considerably higher than that for TBA *m*-FUM, whereas the affinity of TBA *m*-FUM to the substrate is twice higher. The catalytic activity (k_{cat}) of TBA *m*-FUM in the hydrolysis of the substrate molecules is about three orders of magnitude lower than that of porcine pepsin. This difference in k_{cat} values may be caused by the specific interactions existing between various parts of the heptapeptide and pepsin but not between the substrate and TBA *m*-FUM.

The pseudo-first-order rate constants for the reactions of heptapetide with porcine pepsin and TBA *m*-FUM are given in Tables 2 and 3, respectively. In the case of pepsin, with increasing temperature the $k_{obs.}$ values increase continuously, whereas in the case of TBA *m*-FUM, the $k_{obs.}$ values increase only up to 308 K. Because

Table 1. Kinetic parameters for the hydrolysis of the heptapetide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu by TBA *m*-FUM and porcine pepsin at 293 K

Substance	[E] (μM)	Average K_M (μM)	Average V_{MAX} ($\mu M s^{-1}$)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_M$ ($mM^{-1}s^{-1}$)
TBA *m*-FUM	99	61 ± 8	0.25 ± 0.01	$(2.5 \pm 0.1) \times 10^{-3}$	$(46 \pm 6) \times 10^{-3}$
Porcine pepsin	29×10^{-3}	113 ± 16	0.31 ± 0.02	10.7 ± 0.7	95 ± 8

Table 2. Pseudo-first-order rate constants $k_{obs.}$ for the hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu by porcine pepsin in 10^{-3} m hydrochloric acid, with pepsin concentrations 0.116–7.264 μm as indicated

Temperature (K)	k_{obs} (s^{-1})						
	0.116μM	0.362μM	0.580μM	1.449μM	2.899μM	5.797μM	7.264μM
288	0.0169 ± 0.0002	0.0760 ± 0.0004	0.0803 ± 0.0006	0.1555 ± 0.0010	0.5102 ± 0.0041	1.1162 ± 0.0091	1.4312 ± 0.0194
298	0.0343 ± 0.0003	0.1984 ± 0.0018	0.2126 ± 0.0018	0.520 ± 0.005	0.9170 ± 0.0109	2.4927 ± 0.0221	3.3346 ± 0.0284
308	0.0676 ± 0.0005	0.4718 ± 0.0037	0.4813 ± 0.0046	0.9996 ± 0.0090	2.457 ± 0.0214	5.7901 ± 0.0642	6.2343 ± 0.0453
318	0.11087 ± 0.0009	0.5849 ± 0.0126	0.6703 ± 0.0070	1.957 ± 0.0194	4.441 ± 0.0267	9.5746 ± 0.0936	9.0840 ± 0.1294

Table 3. Pseudo-first order rate constants k_{obs} for the hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu by TBA *m*-FUM in water, with TBA *m*-FUM concentrations of 49.71–198.87 μM as indicated

Temperature (K)	k_{obs} (s^{-1})			
	49.71μM	99.44μM	148.31μM	198.87μM
288	0.0032 ± 0.0001	0.0059 ± 0.0001	0.0094 ± 0.0001	0.0113 ± 0.0002
298	0.0055 ± 0.0001	0.0080 ± 0.0002	0.0127 ± 0.0001	0.0181 ± 0.0003
308	0.0072 ± 0.0001	0.0140 ± 0.0002	0.0260 ± 0.0002	0.0267 ± 0.0004
318	0.0048 ± 0.0001	0.0092 ± 0.0001	0.0141 ± 0.0002	0.0159 ± 0.0003

Table 4. Activation parameters for the hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu by TBA *m*-FUM and porcine pepsin

Substance	Activation enthalpy, $\Delta H^{\neq}$ (kJ mol^{-1})	Activation entropy, $\Delta S^{\neq}$ (J $mol^{-1}K^{-1}$)	Free enthalpy, $\Delta G^{\neq}$ (kJ mol^{-1})
TBA *m*-FUM	31 ± 3	-100 ± 7	61 ± 3
Porcine pepsin	53 ± 5	36 ± 2	41 ± 5

of the possible decomposition of the model compound (TBA *m*-FUM) at higher temperatures, the Michaelis–Menten kinetic parameters were obtained at 293 K (see Table 1). Changes in pH conditions of the hydrolysis reactions may be a further factor contributing to the differences in the observed kinetics.

The activation parameters are listed in Table 4. The entropy of activation ($\Delta S^{\neq}$) reflects the changes in the number and kind of the degrees of freedom if the reagents form an active complex. When the complex movement in the transition state is limited or impeded, ($\Delta S^{\neq}$) decreases. Consequently, the possibility of inner motion in the TBA *m*-FUM–heptapeptide complex should be more restricted than that of the pepsin–heptapeptide complex. The negative value of the ($\Delta S^{\neq}$) explains the higher ($\Delta G^{\neq}$) value for the reaction of heptapeptide with TBA *m*-FUM, although the activation enthalpy is clearly lower.

Previous work on the mechanism of the pepsin activity suggested that the H_2O or OH^- species were located in the active centre of pepsin [9,13–15] and, consequently, a base mechanism of hydrolysis was proposed. Recently, we have also assumed this type of mechanism for the cleavage of heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu) by TBA *m*-FUM.[9] The above-presented kinetic data for the heptapeptide hydrolysis by both pepsin and TBA *m*-FUM support this assumption.

The mechanism of the TBA *m*-FUM activity is shown in Fig. 5. The water molecule bound between the carboxylic and carboxylate groups of TBA *m*-FUM becomes distorted when approached by the heptapeptide molecule. The carbonyl group of the heptapeptide forms a hydrogen bond with the carboxylic group of TBA *m*-FUM while the negative partial charge of the O atom of water attacks the electrophilic carbon atom of the peptide bond. The formation of internal hydrogen bonds is easily visible in the FTIR spectrum.[9] The hydroxyl ion resulting from the split H_2O molecule adds to the carbon atom of the peptide bond of heptapeptide. Simultaneously, the

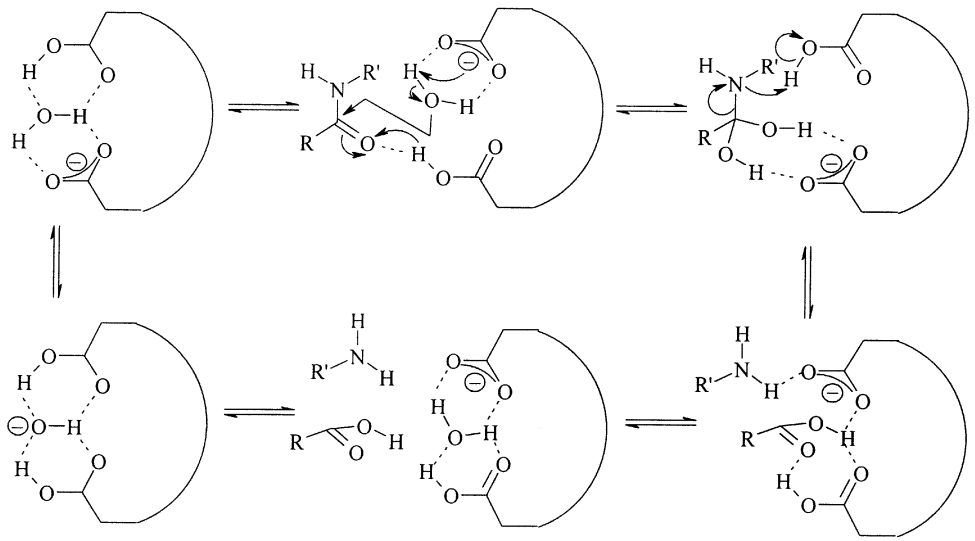

Figure 5. Suggested mechanism of the hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2) Phe–Arg–Leu by TBA *m*-FUM

amide bond is broken and the proton adds to the nitrogen atom.

Conclusions

The steric arrangement of carboxylic and carboxylate groups in the tetrabutylammonium monosalt of *m*-aminobenzoic acid diamide of fumaric acid (TBA *m*-FUM) has proved beneficial for catalytic hydrolysis of the heptapeptide Pro–Thr–Glu–Phe–(4-NO_2)Phe–Arg–Leu in water. The mechanism of cleavage of the Phe—(4-NO_2)Phe bond by TBA *m*-FUM is similar to that of pepsin. However, the catalytic activity of TBA *m*-FUM, indicated by the k_{cat} values, is much lower than that of pepsin, although the affinity of TBA *m*-FUM to the heptapeptide is higher than the affinity of pepsin. As follows from the activation parameters, the inner motion in the complex of the TBA *m*-FUM–heptapeptide in the transition state is more restricted than that in the pepsin–heptapeptide complex.

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