The kinetics of hydrolysis of some extended *N*-aminoacyl-L-arginine methyl esters by porcine pancreatic kallikrein

A comparison with human plasma kallikrein

Peter R. LEVISON and Geoffrey TOMALIN*

Department of Biochemistry, Victoria University of Manchester, Manchester M13 9PL, U.K.

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The effects of subsite interactions in the S_2 – S_4 region [Schechter & Berger (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162] of porcine pancreatic kallikrein (EC 3.4.21.8) on its catalytic efficiency have been investigated. Kinetic constants (k_{cat} , K_m) have been determined for a series of seven extended *N*-aminoacyl-L-arginine methyl esters whose sequence is based on either the C-terminal sequence of kallidin (–Pro-Phe-Arg) or (–Gly–) $_n$ Arg. With these substrates it has been found that neither acylation nor deacylation of the enzyme is rate-limiting. Values of k_{cat} range from 21.5 to 2320 s^{–1}, indicating that there are interactions with different residues in the *N*-aminoacyl chain and enzyme subsites in the S_2 – S_4 region. It is shown that possible hydrogen-bonded interactions with the enzyme in the S_3 – S_4 region have a significant effect on catalysis. The presence of L-phenylalanine at P₂ has a very large effect on both k_{cat} and K_m , giving a greatly enhanced catalytic efficiency. Substrates with L-proline at P₃ also have a marked effect, but in this case the overall effect is one of lowered catalytic efficiency. By comparison with the results of a similar study with human plasma kallikrein I (EC 3.4.21.8), it has been possible to demonstrate that there are considerable differences in kinetic behaviour between the two enzymes. These are related to relative differences in the rates of acylation and deacylation with ester substrates and also the roles of subsites S_2 and S_3 of the two enzymes.

Kallikreins are a group of enzymes from various sources that catalyse the excision of kinins from kininogens. Kallikreins from glandular sources (kidney, pancreas, salivary gland, submandibular gland) are all glycoproteins of relatively low molecular weight in the range 25 000–40 000 (Schachter, 1980). It has been found that extensive sequence homologies exist not only between the enzymes from different glands, but also between different species (Fritz *et al.*, 1977; Geiger *et al.*, 1979; Lottspeich *et al.*, 1979; Tschesche *et al.*, molecular weight in the range 25 000–40 000 kallidin (Lys-bradykinin) from both high-molecular-weight kininogen and low-molecular-weight kininogen (Carretero & Scicli, 1980). Plasma kallikreins, on the other hand, differ not only in molecular weight (85 000–95 000), but also in function, since

only bradykinin is excised from high-molecular-weight kininogen (Kerbiriou & Griffin, 1979). Plasma kallikreins also have additional functions in the activation and mediation of the intrinsic blood-clotting cascade (Schachter, 1980).

Kallikreins are members of the serine-proteinase group of enzymes and all have a ‘trypsin-like’ primary specificity, with L-arginine peptide bonds being split preferentially to L-lysine peptide bonds (Fiedler & Leysath, 1979). Glandular kallikreins are particularly specific in their substrate requirements (Schachter, 1980), and in their action on high- and low-molecular-weight kininogens. It seems probable that additional or ‘secondary’ specificity arises from interactions of the substrate with the sequence of enzyme subsites adjacent to the primary specificity site (S_1) (Schechter & Berger, 1967). The peptide sequence of high-molecular-weight kininogen in the vicinity of the C-terminal sequence of the excised kallidin moiety is:

–Pro-Phe-Arg-Ser-Val-Gln–

Abbreviations used: Ac, *N*-acetyl; OMe, methyl ester; ·HCl, hydrochloride; Bz, *N*^α-benzoyl; OEt, ethyl ester

* To whom correspondence and reprint requests should be directed.

the Arg-Ser bond being susceptible to cleavage. The interactions of this hexapeptide with porcine pancreatic kallikrein have been shown to comprise the interactions essential for the kallikrein-catalysed release of the C-terminus of the kinin in kininogen (Fiedler & Leysath, 1979). The object of the present study was a kinetic investigation of the S_2 and S_3 subsites (Schechter & Berger, 1967), which are believed to interact with L-phenylalanine (P_2) and L-proline (P_3) residues of the substrate. In a previous study the interactions at S_2 , and to a lesser extent those at S_3 , were shown to affect enzymic activity (Fiedler & Leysath, 1979), but a detailed systematic investigation of these interactions was not made. The synthetic L-arginine methyl ester substrates Ac-Arg-OMe·HCl, Ac-Phe-Arg-OMe·HCl and Ac-Pro-Phe-Arg-OMe·HCl, have been used to investigate the overall interactions at S_2 and S_3 . The existence of any hydrogen-bonded interactions at the latter subsites has been investigated using the 'stripped' substrates Ac-Gly-Arg-OMe·HCl and Ac-Gly-Gly-Arg-OMe·HCl. The 'selectively stripped' substrates Ac-Gly-Phe-Arg-OMe·HCl and Ac-Pro-Gly-Arg-OMe·HCl have been used both to investigate specific interactions of L-phenylalanine with S_2 and L-proline with S_3 respectively and also to investigate the possibility of any subsite-subsite interactions occurring with the full substrate, Ac-Pro-Phe-Arg-OMe·HCl. The acetyl *N*-terminal blocking group has been used for reasons given in a previous study (Green & Tomalin, 1976).

The results for porcine pancreatic kallikrein have been compared with those obtained from a similar kinetic investigation using human plasma kallikrein I (Levison & Tomalin, 1982), in an attempt to clarify some of the differences between the two enzymes, which, although sharing the same name, exhibit markedly different substrate specificities (Schachter, 1980).

Materials and methods

Materials

Porcine pancreatic kallikrein [5.7 units/mg of protein (biuret)] (1 unit of activity hydrolysed 1 μ mol of Bz-L-Arg-OEt/min at pH 8.7 at 25°C) and 4-methylumbelliferone were from Sigma (London) Chemical Co., Poole, Dorset BN17 17NH, U.K. 4-Methylumbelliferyl *p*-guanidinobenzoate was synthesized as described by Hill (1979). Ac-Arg-OMe·HCl, Ac-Phe-Arg-OMe·HCl, Ac-Pro-Phe-Arg-OMe·HCl, Ac-Gly-Arg-OMe·HCl, Ac-Gly-Gly-Arg-OMe·HCl, Ac-Gly-Phe-Arg-OMe·HCl and Ac-Pro-Gly-Arg-OMe·HCl were synthesized from their constituent amino acids (Levison & Tomalin, 1982).

Methods

Active-site titration. The operational molarity of

the stock enzyme solutions was determined by spectrofluorimetric active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate, a Perkin-Elmer-Hitachi 204 spectrofluorimeter being used as described by Jameson *et al.* (1973).

Kinetic measurements. The progress of ester hydrolysis was monitored on a Radiometer pH-stat comprising a pH-meter (PHM 62), titration unit (TTT 60c), automatic burette (ABU 13), titration assembly (TTA 60) and recorder (REC 61/REA 160). The reaction vessel (enclosed in a water jacket) was maintained at $37 \pm 0.1^\circ\text{C}$ by circulation from a Grant LE8 thermostatically controlled water bath. All measurements were carried out under an atmosphere of CO_2 -free N_2 , saturated with water. The pH-electrode was calibrated against two standard buffers (Perrin & Dempsey, 1974). Alkali solutions were prepared by diluting a portion of carbonate-free 25 M-NaOH solution into previously boiled-out distilled water (Vogel, 1961). The absolute molarities of titrant solutions were determined by titration against solutions of potassium hydrogen phthalate (Vogel, 1961). Stock substrate solutions were prepared in 0.1 M-NaCl, and various concentrations were prepared by dilution with 0.1 M-NaCl to a final volume of 10 ml. Adjustment to pH 8.00 (37°C) was made with 0.15 M-NaOH. Porcine pancreatic kallikrein in 0.1 M-NaCl (1 mg/ml, 20 μ l) was added and the uptake of alkali at pH 8.00 recorded as a function of time. After correction for non-enzymic hydrolysis, initial velocities were computed by using a curve-fitting procedure involving orthogonal polynomials (Green, 1975). Values of k_{cat} and K_m were computed by using the method of Wilkinson (1961).

Product-partitioning studies were carried out by using Ac-Phe-Arg-OMe·HCl and Ac-Pro-Phe-Arg-OMe·HCl in the presence of 2 M-methanol (Seydoux & Yon, 1967, 1971) methods similar to those described above being used.

Results and discussion

Kinetics of porcine pancreatic kallikreins

The mechanism of action of serine proteinases is known to involve acylation and deacylation of an active-site serine residue by the acyl portion of the substrate according to the following scheme:

Here ES' is the acyl enzyme, in this case an *N*-aminoacyl-L-arginyl-kallikrein. In order to determine whether the rate-limiting step is acylation or deacylation, kinetic measurements were carried out in the presence and absence of a nucleophile that

competes with water in the deacylation process. If acylation is the rate-limiting step, it can be shown that the observed value of K_m remains constant, whereas the observed value of k_{cat}^{-1} is a linear function of nucleophile concentration. On the other hand, if deacylation is the rate-limiting step, the observed value of K_m is a linear function of nucleophile concentration, whereas the observed value of k_{cat} remains constant. If, however, there is no distinct rate-limiting step (i.e. $k_{+2} \approx k_{+3}$), then the observed values of both constants vary with nucleophile concentration (Seydoux & Yon, 1967). Table 1 gives the results of such a study with Ac-Phe-Arg-OMe·HCl and Ac-Pro-Phe-Arg-OMe·HCl in the presence and absence of 2M-methanol as nucleophile (Seydoux & Yon, 1971). Since the observed values of K_m and k_{cat}^{-1} increase in the presence of 2M-methanol, it can be concluded that there is no distinct rate-limiting step and that $k_{+2} \approx k_{+3}$. It seems reasonable to draw the same conclusions for the other substrates. It is therefore not possible to identify directly the observed values of k_{cat} with either the rate constant for acylation (k_{+2}) or deacylation (k_{+3}). The observed values of k_{cat} and K_m must be related to the rate constants in the above scheme by the following expressions:

$$k_{cat} = (k_{+2}k_{+3})/(k_{+2} + k_{+3}) \quad (1)$$

$$K_m = k_{+3}(k_{-1} + k_{+2})/k_{+1}(k_{+2} + k_{+3}) \quad (2)$$

Kinetic results for all seven substrates are given in Table 2. It can be seen that the k_{cat} and K_m values for the extended substrates differ markedly from those for Ac-Arg-OMe·HCl, indicating that the P_2 and P_3 residues are interacting with subsites in the enzyme. Taking the first three substrates in Table 2, it can be seen that significant increases occur in k_{cat} , from 21.5 to 282 s⁻¹ on extension of the *N*-amino-acyl chain from Ac-Arg-OMe·HCl to Ac-Gly-Gly-Arg-OMe·HCl. These changes must be the result of hydrogen-bonded subsite interactions in the S_2 – S_4 region of the enzyme. By far the largest increase occurs on extension of Ac-Gly-Arg-OMe·HCl to Ac-Gly-Gly-Arg-OMe·HCl, indicating that hydrogen-bonded interactions in the S_3 – S_4 region have an important effect on catalytic efficiency. Effects of this type have been reported for both bovine β -trypsin (EC 3.4.21.4) (Green & Tomalin, 1976) and bovine chymotrypsin A_a (EC 3.4.21.1) (Hill & Tomalin, 1981). The K_m values remain fairly constant for all three substrates in the range 0.2–0.3 mM.

Substitution of L-phenylalanine for glycine at P_2 in Ac-Gly-Arg-OMe·HCl, to give Ac-Phe-Arg-OMe·HCl, has the most marked influence on the catalytic constants, k_{cat} being raised 54-fold from 30.1 s⁻¹ to 1620 s⁻¹ and K_m being reduced from 0.22 mM to 0.04 mM. There is clearly a strong hydrophobic interaction at subsite S_2 . The same

Table 1. Kinetic constants for the hydrolysis of Ac-Phe-Arg-OMe·HCl and Ac-Pro-Phe-Arg-OMe·HCl in the presence and absence of methanol, catalysed by porcine pancreatic kallikrein at 37°C, pH 8.00, in 0.1 M-NaCl

Substrate	[Methanol]		k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)
	(M)	K_m (mM)		
Ac-Phe-Arg-OMe·HCl	0	0.040 ± 0.005	1620 ± 80	40500
	2	0.099 ± 0.009	1040 ± 50	10400
Ac-Pro-Phe-Arg-OMe·HCl	0	0.062 ± 0.009	1840 ± 110	29700
	2	0.141 ± 0.013	1180 ± 50	8300

Table 2. Kinetic constants for the hydrolysis of L-arginine methyl ester substrates catalysed by porcine pancreatic kallikrein and human plasma kallikrein I at 37°C, pH 8.00, in 0.1 M-NaCl

Substrate	Porcine pancreatic kallikrein*				Human plasma kallikrein I†		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	No. of runs	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)
Ac-Arg-OMe·HCl	0.343 ± 0.043	21.5 ± 1.6	63	16	1.22 ± 0.34	81 ± 18	66
Ac-Gly-Arg-OMe·HCl	0.220 ± 0.014	30.1 ± 1.0	137	20	0.560 ± 0.025	40.0 ± 1.2	72
Ac-Gly-Gly-Arg-OMe·HCl	0.261 ± 0.012	282 ± 7	1080	21	0.180 ± 0.007	40.0 ± 0.7	221
Ac-Phe-Arg-OMe·HCl	0.040 ± 0.005	1620 ± 80	40500	25	0.160 ± 0.014	33.9 ± 1.4	207
Ac-Gly-Phe-Arg-OMe·HCl	0.067 ± 0.006	2320 ± 100	34700	22	0.150 ± 0.006	34.1 ± 0.6	222
Ac-Pro-Gly-Arg-OMe·HCl	0.154 ± 0.011	88 ± 4	570	23	0.0270 ± 0.0011	39.4 ± 0.4	1460
Ac-Pro-Phe-Arg-OMe·HCl	0.062 ± 0.009	1840 ± 110	30000	22	0.0140 ± 0.0009	42.2 ± 0.7	2900

* The present work.

† Levison & Tomalin (1982).

effect is observed when a similar substitution is made in Ac-Gly-Gly-Arg-OMe·HCl, to give Ac-Gly-Phe-Arg-OMe·HCl, both of which have the additional hydrogen-bonded interaction to the Ac-Gly moiety at S_3 - S_4 . In this case $k_{cat.}$ is raised from 282 s^{-1} to 2320 s^{-1} and K_m is reduced from 0.261 mM to 0.067 mM .

On substitution of L-proline for glycine at P_3 in Ac-Gly-Gly-Arg-OMe·HCl, to give Ac-Pro-Gly-Arg-OMe·HCl, the opposite effect is observed for $k_{cat.}$, which is reduced 3-fold from 282 s^{-1} to 88 s^{-1} . The corresponding value of K_m is reduced from 0.261 mM to 0.154 mM . Substitution of L-proline for glycine in Ac-Gly-Phe-Arg-OMe·HCl, to give Ac-Pro-Phe-Arg-OMe·HCl, also reduces the observed value of $k_{cat.}$ in this case from 2320 s^{-1} to 1840 s^{-1} . In this case the corresponding values of K_m remain about the same. From values of $k_{cat.}/K_m$ it is concluded that interaction of an L-proline residue at the S_3 subsite has a marked overall negative effect on the catalytic efficiency of the enzyme.

Comparison of human plasma and porcine pancreatic kallikreins

It has been shown that, for porcine-pancreatic-kallikrein-catalysed ester hydrolysis, neither acylation nor deacylation of the enzyme is rate-limiting. This is in contrast with the results for a kinetic study of human plasma kallikrein I with similar substrates (Levison & Tomalin, 1982), where it has been shown that deacylation of the enzyme is rate-limiting.

A direct comparison of the kinetic constants obtained for the hydrolysis of the seven extended *N*-aminoacyl-L-arginine methyl ester substrates by the two enzymes is given in Table 2. A number of striking features are apparent. Firstly, the range of $k_{cat.}$ values for the pancreatic enzyme from 21.5 s^{-1} to 2320 s^{-1} is in complete contrast with the limited range of $k_{cat.}$ ($= k_{+3}$) values from 34.1 s^{-1} to 81 s^{-1} for the plasma enzyme. Also the substrate (Ac-Arg-OMe·HCl) which gives the lowest value of $k_{cat.}$ for the pancreatic enzyme gives the highest $k_{cat.}$ value for the plasma enzyme. The two enzymes also behave differently with substrates which have side chains at P_2 and P_3 . For the pancreatic enzyme the subsite interactions which promote the most efficient catalysis (i.e. largest values of $k_{cat.}/K_m$) are those with L-phenylalanine at P_2 , whereas this substitution has a relatively small effect with the plasma enzyme. On the other hand insertion of L-proline at P_3 reduces the catalytic efficiency of the pancreatic enzyme but gives the largest increase in catalytic efficiency for the plasma enzyme.

It is clear that kallikreins from these two different sources differ in their kinetic characteristics with specific extended substrates. Although the enzymes

share a common physiological substrate (high-molecular-weight kininogen), it is perhaps reasonable to suggest that, in view of their differing physiological roles and different kinetic characteristics, the enzymes be classified distinctly.

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