Peptide thioesters and 4-nitroanilides as substrates for porcine pancreatic kallikrein

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A series of 14 4-nitroanilide substrates and 17 thioester substrates have been used to measure kinetic constants with porcine pancreatic kallikrein. All of the substrates have a P₁ arginine residue. The 4-nitroanilide substrates consist of seven P₂-glycine and seven P₂-phenylalanine tripeptides. As expected from previous results, the phenylalanine series substrates were generally 100-fold 'better' than those in the glycine series. The S₃ subsite was found to 'prefer' lysine or phenylalanine, whereas glutamic acid in this position was distinctly unfavourable. The thioester substrates consisted of various thioester derivatives of arginine as well as 12 dipeptides. These substrates exhibited k_{cat}/K_m values generally 1000 times higher than the P₂-phenylalanine 4-nitroanilides. With the thioesters, a P₂ phenylalanine or tryptophan residue yielded the best substrates, but some of the simple derivatives of arginine were nearly as good. A comparison of the kinetic constants of the thioester substrates between the porcine enzyme and human plasma kallikrein provides further evidence that these enzymes have a similar preference for bulky P₂ residues, but otherwise are quite different enzymes. The thioester substrates are nearly as reactive as oxygen ester substrates such as acetylphenylalanylarginine methyl ester for the porcine enzyme [Levison & Tomalin (1982) *Biochem. J.* **203**, 299–302; Fiedler (1983) *Adv. Exp. Med. Biol.* **156A**, 263–274], and owing to the greater ease in assaying with the thioesters, they should find use in routine assays for the glandular kallikreins.

Porcine pancreatic kallikrein is a member of a group of glandular kallikreins (EC 3.4.21.35) that catalyse the release of vasodilator peptides or kinins from protein precursors known as kininogens (Han *et al.*, 1978; Fiedler *et al.*, 1981; Fiedler, 1983). Porcine glandular kallikreins are isolated from various sources such as the kidney, pancreas, salivary gland and submandibular gland. These enzymes are glycoproteins of relatively low M_r

(25000–40000) and are serine proteinases of trypsin-like specificity (Fiedler, 1979; Schachter, 1980). They are unusual, since, in spite of their specificity, they are known to cleave preferentially at a methionyl bond instead of at an adjacent lysyl bond during release of the decapeptide kallidin from kininogen (Fiedler, 1983). Trypsin cleaves the lysyl bond rather than the methionyl bond, thus releasing the nonapeptide bradykinin. Another unusual phenomenon observed with these enzymes is their low activity towards proteins such as casein. Under the same conditions, trypsin is approx. 200 times more active against casein as pancreas kallikrein. These extraordinary features have led to studies of the substrate specificity of porcine pancreatic kallikrein with a number of synthetic peptide substrates. The majority of the substrates tested have been ethyl and methyl esters of arginine or lysine and peptides containing these residues (Levison & Tomalin, 1982*b*). Chloromethyl ketone inhibitors designed as either natural or synthetic substrate analogues have been succes-

Abbreviations used: NMec, aminomethylcoumarin; Boc, *t*-butoxycarbonyl; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Nan, 4-nitroanilide; NPGb, 4-nitrophenyl-4'-guanidinobenzoate; -SBzl, benzyl thioester (-SCH₂C₆H₅); -S*Pe-i*, isopentyl thioester [-SCH₂CH₂CH(CH₃)₂]; -S*Pe-s*, *s*-pentyl thioester [-SCH₂CH(CH₃)CH₂CH₃]; -S*Bu-i*, isobutyl thioester [-SCH₂CH(CH₃)₂]; Z-, benzyloxycarbonyl. The nomenclature used for the individual amino acid residues of a substrate (P₁, P₂, etc.) and for the subsites of the enzyme (S₁, S₂, etc.) is that of Schechter & Berger (1967).

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fully investigated as well (Fiedler *et al.*, 1977; Kettner & Shaw, 1978).

The present study has sought to extend the previous studies by presenting the kinetic constants (k_{cat} and K_m) for the reaction of porcine pancreatic kallikrein with a large number of synthetic substrates. The substrates used were either thioesters (17 substrates) or 4-nitroanilides (14 substrates) of amino acids and peptides containing arginine residues. The results presented here are compared with the previously reported results with other substrates, as well as our results obtained with human plasma kallikrein and the thioester substrates (McRae *et al.*, 1981). Plasma kallikrein belongs to a group of blood-clotting enzymes and is thought to be a completely different enzyme (Fiedler *et al.*, 1981). The data is also viewed in terms of the recently determined X-ray structures of porcine pancreatic kallikrein (Bode *et al.*, 1983) and the complex of pancreatic kallikrein with bovine pancreatic trypsin inhibitor (Chen & Bode, 1983).

Experimental

Materials

The synthesis of the substrates has been previously reported by McRae *et al.* (1981) (thioesters) and Tanaka *et al.* (1983) (4-nitroanilides).

Porcine pancreatic kallikrein was prepared by the method of Kutzbach & Schmidt-Kastner (1972) and was kindly provided by Dr. Hans Fritz and Dr. Franz Fiedler of the University of Munich, Munich, Federal Republic of Germany. The enzyme was titrated with both benzyl 4-guanidinothiobenzoate (Cook & Powers, 1983) and NPGB. The thioester titrant gave a lower rate of titrant hydrolysis after the burst and was used to obtain the active-site concentration of the enzyme for the 4-nitroanilide kinetics. For the thioester kinetics, which were run earlier, NPGB was used as the titrant. Both titrants give comparable results for the enzyme concentration, although the burst size was more easily determined for the thioester titrant with this enzyme.

Methods

Nitroanilide kinetics. The rates of enzymic hydrolysis were monitored at 410 nm in a Beckman model 25 spectrophotometer. The assays were conducted by adding 50 μl of enzyme solution to a polystyrene cuvette containing 2.0 ml of buffer and substrate solution. All glassware was treated with trimethylsilyl chloride to prevent adherence of the enzyme on to the glass surfaces. Kinetic constants were determined from the initial rates of hydrolysis using Lineweaver-Burk plots. An ϵ_{410} value of $8800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used for 4-nitroaniline (Er-

lander *et al.*, 1961). Duplicate or triplicate determinations at each of four to six substrate concentrations were employed. In several cases, the substrate solubility was much smaller than K_m and only k_{cat}/K_m could be determined. Correlation coefficients were greater than 0.99 in all cases.

Thioester kinetics. The rates of hydrolysis of the thioesters were measured with 4,4'-dithiodipyridine (Castillo *et al.*, 1979). The following conditions were employed: 0.10 M-Hepes buffer, pH 7.5, 0.01 M- CaCl_2 , 9.8% (v/v) dimethyl sulphoxide and 30°C. To a cuvette containing 1.0 ml of substrate solution was added 150 μl of 2.0 mM-4,4'-dithiodipyridine in the same buffer with the same dimethyl sulphoxide concentration. After 'zeroing' the instrument, 50 μl of enzyme solution was added to the cuvette and the increase in A_{324} was monitored in a Beckman model 35 spectrophotometer. After obtaining initial rates, 20 μl of concentrated trypsin solution (about 80 μM stock solution in 1.0 M-HCl) was added to rapidly complete the hydrolysis, and the substrate concentration was calculated from the final absorbance reading. An ϵ_{324} value of $19800 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used (Grassetti & Murray, 1967). The kinetic constants were determined by the Lineweaver-Burk method and are based on duplicate or triplicate determinations at four to six separate substrate concentrations. Correlation coefficients were greater than 0.99 in all cases.

Results and discussion

The kinetic parameters for the reaction between porcine pancreatic kallikrein and the 4-nitroanilide and thioester substrates are presented in Tables 1 and 2 respectively. In order to facilitate comparison between porcine pancreatic kallikrein and human plasma kallikrein, the k_{cat}/K_m values for the human enzyme are presented as well as a ratio (porcine/human) of their k_{cat}/K_m values.

Nitroanilide substrates

The tripeptide 4-nitroanilide substrates clearly demonstrate the strong preference of the enzyme for a bulky P_2 side chain, in this case phenylalanine. The phenylalanine-series peptides were on average 100-fold better substrates than the glycine series. The difference is similar to that previously obtained with this enzyme and a series of esters of the general formula Ac-Xaa-Arg-OMe (Levison & Tomalin, 1982b; Fiedler, 1983). In this case, also, the P_2 -phenylalanine peptides had k_{cat}/K_m values of the order of 100 times greater than had those with a P_2 glycine residue. The explanation for this preference is understandable in terms of the X-ray structure of the enzyme active site. Trp-215 and Tyr-99 of kallikrein can interact with the aromatic ring of a substrate with a P_2

Table 1. *Kinetic constants for the hydrolysis of tripeptide nitroanilide substrates by porcine pancreatic kallikrein*
The reaction mixture contained 0.10M-Hepes buffer, pH 7.5, 10mM-CaCl₂, 10% (v/v) dimethyl sulphoxide and 10nM-porcine pancreatic kallikrein, and was incubated at 25°C.

Substrate	[Substrate] range (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Z-Ala-Gly-Arg-Nan	1.00–0.50			19
Z-Asn-Gly-Arg-Nan	1.06–0.27			13
Z-Glu-Gly-Arg-Nan	1.00–0.17			19
Z-Lys-Gly-Arg-Nan	1.00–0.25			21
Z-Phe-Gly-Arg-Nan	0.39–0.098			41
Z-Pro-Gly-Arg-Nan	0.99–0.25			24
Z-Ser-Gly-Arg-Nan	0.98–0.25			23
Z-Ala-Phe-Arg-Nan	0.098–0.016	1.6	0.61	2700
Z-Asn-Phe-Arg-Nan	0.11–0.018			1700
Z-Glu-Phe-Arg-Nan	0.10–0.025			360
Z-Lys-Phe-Arg-Nan	0.10–0.013	2.7	0.31	8700
Z-Phe-Phe-Arg-Nan	0.10–0.026	0.34	0.05	7000
Z-Pro-Phe-Arg-Nan	0.10–0.013			3800
Z-Ser-Phe-Arg-Nan	0.098–0.012	0.58	0.21	2800

Table 2. *Kinetic constants for the hydrolysis of synthetic thioester substrates by porcine pancreatic kallikrein and human plasma kallikrein (McRae et al., 1981)*

The reaction mixture contained 0.10M-Hepes buffer, pH 7.5, 0.01 M-CaCl₂, 9.8% (v/v) dimethyl sulphoxide and 3.4–4.8 nM-porcine pancreatic kallikrein (determined by titration with NPGb), and was incubated at 30°C.

Substrate	[Substrate] range (mM)	Porcine pancreatic kallikrein			Human plasma kallikrein	k_{cat}/K_m (porcine) k_{cat}/K_m (human)
		K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)	
Boc-Arg-SBu-i	0.13–0.67	0.16	41	250000	860000	0.29
oc-Arg-SPe-i	0.12–1.2	0.068	28	410000	270000	1.52
Boc-Arg-SPe-s	0.16–0.64	0.14	53	380000	210000	1.81
Boc-Arg-SBzl	0.27–0.54	0.043	220	5000000	410000	12.2
Z-Arg-SBu-i	0.23–0.46	0.043	200	4600000	690000	6.67
Z-Gly-Arg-SBu-i	0.15–0.74	0.20	28	140000	820000	0.17
Z-Ala-Arg-SBu-i	0.10–0.52	0.49	46	93000	390000	0.24
Z-Val-Arg-SBu-i	0.12–0.40	0.16	62	380000	360000	1.06
Boc-Met-Arg-SBu-i	0.17–0.84	0.045	13	300000	400000	0.75
Z-Phe-Arg-SBu-i	0.074–0.18	0.027	340	13000000	2200000	5.91
Z-Trp-Arg-SBzl	0.060–0.20	0.042	530	13000000	2200000	5.91
Z-Pro-Arg-SBu-i	0.13–0.63	0.34	70	200000	84000	2.38
Z-Ser-Arg-SBu-i	0.13–0.65	0.24	12	52000	370000	0.14
Z-Thr-Arg-SBu-i	0.099–0.25	1.4	71	51000	200000	0.26
Z-Asn-Arg-SBu-i	0.035–0.18	0.18	37	200000	59000	3.39
Z-Glu-Arg-SBu-i	0.12–0.48	1.4	24	17000	8200	0.12
Z-Lys-Arg-SBu-i	0.11–0.57	0.16	16	110000	810000	0.14

phenylalanine or tryptophan residue. The P₂ aromatic ring would insert into a wedge-shaped gap between the aromatic rings of Trp-215 and Tyr-99 and form an almost perfect 'sandwich' of aromatic rings (Chen & Bode, 1983).

The S₃ position was mapped with the 4-nitroanilide substrates by substituting a variety of residues at P₃. The enzyme showed a preference for a lysine or a phenylalanine residue in this position. It should be noted that the lysine may be favourable not for its positive charge but instead

may be fitting into the S₃ site in a folded fashion so that it resembles a phenylalanine residue. The P₃ residue can make contact with the peptide-bond backbone of kallikrein as well as Tyr-99 (Chen & Bode, 1983). A P₃ glutamic acid residue resulted in a distinctly unfavourable substrate.

The k_{cat}/K_m values for the methyl ester substrates were much larger than those of the 4-nitroanilide substrates reported here. However, a valid comparison is not possible, since the former were determined at 37°C and at a pH of 8.0, whereas the

latter were determined at 25°C and at a pH of 7.5 (Levison & Tomalin, 1982b; Fiedler, 1983). In addition, the rate-limiting step may be different for the two types of substrate, and, for methyl ester substrates, no single step appears to be rate-limiting (Levison & Tomalin, 1982b). Other 4-nitroanilide substrates that have been measured with this enzyme include those reported by Claeson *et al.*, (1978). In that report, relative k_{cat}/K_m values were generally given along with some K_m and V_{max} values; however, k_{cat} values were not reported, so comparison with our substrates is not possible on the basis of k_{cat}/K_m . Likewise, some fluorogenic substrates, such as Z-Phe-Arg-NMec, Pro-Phe-Arg-NMec and Z-Pro-Phe-Arg-NMec (Iwanaga *et al.*, 1979), were also reported, but without the k_{cat} values that would allow direct comparison with the 4-nitroanilide substrates.

Thioester substrates

Once again it was evident that the bulky side chains of phenylalanine and tryptophan were favourable at P₂. The equivalent k_{cat}/K_m values for the two substrates Z-Phe-Arg-SBu-*i* and Z-Trp-Arg-SBzl was somewhat surprising, since with the methyl ester substrates a tryptophan residue at P₂ was 2.6-fold 'worse' than a phenylalanine at this position (Fiedler, 1983). However, this is probably due to the different thiol leaving groups. The k_{cat} values for thioesters of Boc-Arg showed a 5-fold range (Table 2), which indicates the deacylation is not rate-limiting. Thus variation in the thiol leaving group would be expected to affect the catalytic constants and a large variation of k_{cat}/K_m values for the simple arginine thioesters was observed. A P₁' thiobenzyl ester was clearly preferred over the alkyl esters, although the blocking group is evidently involved as well (compare Boc-Arg-SBu-*i* and Z-Arg-SBu-*i*). The P₁' residue in the kallikrein-pancreatic-trypsin-inhibitor complex points toward a narrow channel that could presumably accommodate large P₁' groups. Significant P' effects have previously been noted with amide substrates and porcine pancreatic β -kallikrein (Fiedler, 1983).

With porcine pancreatic kallikrein the thioester substrates demonstrated the greatest degree of selectivity yet observed with these substrates with trypsin-like serine proteinases. The k_{cat}/K_m values varied 760-fold from the best substrates, Z-Phe-Arg-SBu-*i* and Z-Trp-Arg-SBu-*i*, to the worst substrates, Z-Glu-Arg-SBu-*i*. Apparently a negative charge is not favourable at S₂ (thioester results) or at S₃ (4-nitroanilide results). Another result of interest is that the methionine-containing peptide binds to the enzyme as well as the best substrates.

This is notable in the light of the cleavage of the methionyl bond by this enzyme in kininogen, a feature that has been puzzling, since an adjacent lysyl bond remains intact. The methionine-containing substrate, although binding quite well, has one of the lowest k_{cat} values measured with this series. However, only cleavage at the arginyl bond was assayed, so it is uncertain if the Met-Arg bond was cleaved.

A comparison of the k_{cat}/K_m values with those for human plasma kallikrein indicates some similarities between the two enzymes. The P₂-tryptophan and -phenylalanine peptides were the best with both enzymes, although their margin of superiority over P₂-glycine peptides was much lower for the human plasma enzyme than with the porcine enzyme. The maximum k_{cat}/K_m measured with the human enzyme was significantly lower than that measured with the porcine enzyme. These observations are in general agreement with those of Levison & Tomalin (1982a). As with the porcine enzyme, the P₂-glutamic acid peptide was the worst substrate of the series. Overall, the two enzymes were quite different, as is evident when looking at the ratio (porcine/human) of the k_{cat}/K_m values.

The thioester substrates are nearly as sensitive as the oxygen ester substrates when compared directly. The best ester substrate, Ac-Phe-Arg-OME, with a k_{cat}/K_m of 40 500 000 M⁻¹ s⁻¹ (Levison & Tomalin, 1982b) is three times better than the best thioester substrate. As with the 4-nitroanilide substrates, the comparison is not completely valid, owing to differences in temperature and pH between the two assays. If the thioester kinetics were measured at the higher pH and temperature, their sensitivity would probably equal that of the oxygen esters. Factors which may introduce errors in the oxygen-ester assay with a pH-stat, such as CO₂ dissolution in buffers and inhibitory heavy-metal contamination due in part to the calomel electrode (Fiedler *et al.*, 1981) are completely avoided with the spectrophotometric thioester assay. In addition, detection of thiol release can be observed with at least an order of magnitude greater sensitivity, owing to the high absorption coefficient of 4-thiopyridone. These features, in conjunction with the greater ease of using a spectrophotometer over a pH-stat, should render the thioester substrates generally preferable for assaying the glandular kallikreins.

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