Determinants of the unusual cleavage specificity of lysyl-bradykinin-releasing kallikreins

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Kinetic data for the hydrolysis by human tissue kallikrein of fluorogenic peptides with *o*-aminobenzoyl-Phe-Arg (Abz-FR) as the acyl group and different leaving groups demonstrate that interactions with the S'_1, S'_2 and S'_3 subsites are important for cleavage efficiency. In addition, studies on the hydrolysis of fluorogenic peptides with the human kininogen sequence spanning the scissile Met-Lys bond [Abz-M-I-S-L-M-K-R-P-*N*-(2,4-dinitrophenyl)ethylenediamine] and analogues with different residues at positions P'_1, P'_2 and P'_3 showed that (a) the presence of a proline residue at P'_3 and the interactions with the tissue kallikrein-binding sites S_2 to S'_2 are determinants of Met-Lys bond cleavage and (b) residues P_3, P_4 and/or P_5 are important for cleavage efficiency. The substitution of phenylalanine for methionine or arginine in substrates with scissile Met-Lys or Arg-Xaa bonds demonstrated that lysyl-bradykinin-releasing tissue kallikreins also have a primary specificity for phenylalanine. The replacement of arginine by phenylalanine in (D)P-F-R-*p*-nitroanilide (pNA) produced an efficient and specific chromogenic substrate, (D)P-F-F-pNA, for the lysyl-bradykinin-releasing tissue kallikreins as it is resistant to plasma kallikrein and other arginine hydrolases.

INTRODUCTION

Tissue (or glandular) kallikreins (EC 3.4.21.35) are serine proteinases involved in kininogen processing. They release lysyl-bradykinin (Pierce and Webster, 1961) by the highly specific cleavage of two dissimilar peptide bonds, Arg-Ser and Met-Lys, in bovine and human kininogens (Habermann, 1966; Han et al., 1976; Lottspeich et al., 1984) and Arg-Thr and Leu-Lys in horse kininogen (Sugo et al., 1981). The only exception known so far is the tissue kallikrein from rat which does not cleave the Met-Lys bond, but releases bradykinin by cleavage of the Lys-Arg and Arg-Arg bonds in bovine and rat kininogens respectively (Alhenc-Gelas et al., 1981; Kato et al., 1985).

Tissue kallikreins have been suggested to have an important role in the control of blood pressure and in the local regulation of blood flow [see review by Bhoola et al. (1992)]. These enzymes have also been associated with tissue inflammatory reactions such as those seen in asthma, rhinitis and arthritis [see review by Polosa (1993)]. On the other hand, there is evidence that these enzymes may process precursor molecules other than kininogens, including prolactin in the pituitary gland (Powers, 1993) and prorenin (Sealey et al., 1978; Derkx et al., 1979; Yokosawa et al., 1979). Apart from their possible roles in relevant physiological processes, understanding the mechanism of limited kininogen proteolysis by tissue kallikreins is of interest, because these enzymes are usually considered to be a prototype for several other kallikrein gene family members involved in the processing of prohormones (Taylor et al., 1970) and growth factor precursors (Thomas et al, 1981).

The development of specific inhibitors of tissue kallikreins and other kallikrein-like enzymes depends on detailed information about their substrate–subsite interactions. Some of these determinants were identified in previous studies on the hydrolysis by pig, horse and rat tissue kallikreins of the arginyl and lysyl bonds in synthetic substrates. The following observations were made: (a) arginine in position P_1 [nomenclature of Schechter and Berger (1967)] is preferred over lysine (Fiedler, 1987); (b) the phenylalanine side chain binds best to the S_2 subsite of the enzyme (Fiedler, 1987; Chen and Bode, 1983; Araujo-Viel et al., 1988); (c) D-amino acid residues at P_3 interact favourably with human and horse kallikreins (Kettner and Shaw, 1981; Oliveira et al., 1987; Araujo-Viel et al., 1988); (d) proline in P'_2 impairs the cleavage by horse and pig, but not by rat tissue kallikrein, and arginine in P'_1 or P'_2 increases the efficiency of cleavage by all three kallikreins (Prado et al., 1983, 1986; Chagas et al., 1991, 1992). Little is known about the determinants of the hydrolysis of the Met-Lys bond because of the resistance of the Met-Xaa linkage in short synthetic peptides to cleavage by tissue kallikreins (Araujo-Viel et al., 1981; Fiedler, 1983).

The hydrolytic activity of tissue kallikreins at the Met-Lys bond is very unusual as these serine proteases have been well characterized as arginine hydrolases. In order to gain more information on the determinants of the cleavage specificity of lysyl-bradykinin-releasing kallikreins, we have studied the effects of S'_1 to S'_3 interactions on the hydrolysis of Arg-Ser and Met-Lys bonds by human tissue kallikrein. For these studies, we have synthesized intramolecularly quenched fluorogenic peptides with the human kininogen sequences that flank the susceptible Met-Lys and Arg-Ser bonds. These model substrates, as well as peptides with analogous sequences, yielded important information on the determinants of this unusual cleavage specificity of tissue kallikreins, including data on the efficient hydrolysis of the Phe-Xaa bond in some of the synthetic peptide substrates.

MATERIALS AND METHODS

Enzymes

Homogeneous human urinary kallikrein and rat urinary kallikrein obtained as described by Shimamoto et al. (1980) and Chao

Abbreviations used: Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; pNA, *p*-nitroanilide; Tos, *p*-toluenesulphonyl. The peptides in this paper are identified by the letter of the P_1 residue in the cleavage site (R, M, F, A) and a number.

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and Margolius (1979) respectively were a generous gift from Professor J. Chao, Medical University of South Carolina, SC, U.S.A. Porcine pancreatic kallikrein (1956 kallikrein units/mg of protein), prepared by Bayer A. C., Wuppertal, Germany, was kindly supplied by Dr. F. Fiedler, University of Munich, Munich, Germany. Dr. C. A. M. Sampaio, of the Department of Biochemistry, Escola Paulista de Medicina, São Paulo, Brazil, kindly supplied the sample of human plasma kallikrein, which was isolated as described previously (Oliva et al., 1982). Horse tissue kallikrein was purified to homogeneity as described by Giusti et al. (1988). Molar concentrations of the enzyme solutions were determined by active-site titration with 4-nitrophenyl-4-guanidinobenzoate by the methods of Fiedler et al. (1972) and Sampaio et al. (1984).

Peptide substrates

Previously described procedures were used for the synthesis, purification and analysis of the intramolecularly quenched fluorogenic peptide substrates (Chagas et al., 1991) and of peptidyl-*p*-nitroanilide (pNA) (Juliano and Juliano, 1985). The peptides containing glutamine as C-terminal residue were synthesized by solid-phase peptide synthesis as follows: N^{α}-Fmoc-Glu(αCO)-*N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) was obtained and attached to benzhydrylamino resin through *p*-{(*R*,*S*)- α-[1-(9*H*-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzylphenoxyacetic acid as a linker and the peptides were synthesized by Fmoc methodology using the multiple automated peptide synthesizer PSSM-8 (Shimadzu Co.). Methionine-containing peptides, before purification by h.p.l.c., were submitted to a 48 h incubation at 37 °C with 0.1 M dithiothreitol to reduce the methionine sulphoxide introduced during synthesis. Peptides and fragments for h.p.l.c. identification of hydrolysis products were prepared with either methionine or oxidized methionine residues in order to allow the detection by h.p.l.c. of any oxidation product formed under the assay conditions. The stock solutions of the peptide substrates were prepared in 1 mM HCl containing no more than 10% dimethylformamide.

H.p.l.c. analysis of the synthetic fluorogenic substrates and their hydrolysis products

Peptide solutions (80–150 μM) in 20 mM Tris/HCl, pH 8.0 or 9.0, were incubated with the different proteinases at 30 °C. Samples (20 μl) of the substrate and incubation mixtures were periodically removed for h.p.l.c. analysis until 100% hydrolysis was reached. The hydrolysis products were separated by h.p.l.c. and submitted to amino acid analysis. The scissile bonds were deduced from the sequences of the substrate fragments. The h.p.l.c. conditions used for the analytical procedure were: (a) 0.1 M NaH_2PO_4 (pH 4.2) as solvent A and acetonitrile/water (9:1, v/v) as solvent B and (b) 0.1% trifluoroacetic acid in water (solvent A) and acetonitrile/water (9:1, v/v) containing 0.1% trifluoroacetic acid as solvent B. Separations were performed at a flow rate of 1 ml/min using a Novapack C18 column (3.9 mm × 150 mm). For peptide purification, an Ultraprep C18 column (150 mm × 21.2 mm; 10 μm particles) was used and gradient elution was performed with 0.1% trifluoroacetic acid (solvent A) and acetonitrile/water (9:1 v/v) (solvent B) at a flow rate of 3 ml/min. In all cases, elution was followed by u.v. absorption (214 nm) and by fluorescence ($\lambda_{emission}$ = 420 nm and $\lambda_{excitation}$ = 320 nm).

Enzyme assays

Hydrolysis of the peptidyl-pNA at 30 °C in 0.05 M Tris/HCl, pH 9.0, containing 1 mM EDTA was photometrically measured as previously described (Oliveira et al., 1987) by determination of the 4-nitroaniline released after the addition of enzyme to 2 ml of reaction buffer. Hydrolysis was carried out for 5–10 min and the initial hydrolytic rates were calculated from the slope of the time-dependent absorbance curves extrapolated to zero time.

Hydrolysis of the fluorogenic peptide substrates at 30 °C in 0.02 M Tris/HCl, pH 9.0, containing 1 mM EDTA was monitored by measuring the fluorescence at $\lambda_{emission}$ = 420 nm and $\lambda_{excitation}$ = 320 nm in an Hitachi F-2000 spectrofluorimeter, as previously described (Chagas et al., 1991).

The standard hydrolysis conditions were strictly maintained for different substrates. Low buffer concentrations were used because the activities of tissue kallikreins can be impaired by high concentrations of cations (Oliveira et al., 1987; Araujo-Viel et al., 1988). EDTA was added to avoid proteinase inhibition by heavy metals. The enzyme concentration varied from 0.016 nM, for the best substrates, to 4 nM for the less susceptible ones. In most of the cases, the concentration of the substrates ranged from 10 times less than the K_m to 10 times greater than the K_m. The kinetic parameters were calculated as described by Wilkinson (1961). For substrates with K_m lower than 0.2 μM, higher substrate concentrations were used and the $k_{cat.}/K_m$ values were confirmed from determinations under pseudo-first-order conditions.

Inhibition studies

Enzyme assays were conducted at 30 °C in 20 mM Tris/HCl containing 1 mM EDTA, at pH 9.0, using (D)P-F-R-pNA and aminobenzoyl (Abz)-F-R-S-S-R-EDDnp or Abz-F-R-S-R-EDDnp as substrates. Three inhibitor concentrations for each one of four substrate concentrations were used. The substrate concentration intervals used corresponded to 1–10 K_m and the limit for substrate hydrolysis was 10%. The K_i values were calculated from Dixon plots (Dixon, 1972) and/or from the $K_{i_{app.}}$ and the substrate K_m values (Salvesen and Nagase, 1989). The type of inhibition was determined from Lineweaver–Burk plots as described by Segel (1974).

RESULTS

Hydrolysis by human tissue kallikrein of Arg-Xaa bonds in fluorogenic peptides with Abz-F-R as the acyl group and different leaving groups

Tabies 1–3 present the kinetic data for hydrolysis by human tissue kallikrein of internally quenched fluorogenic peptides, which varied in their content of hydrophilic (serine), hydrophobic aliphatic (leucine), aromatic (phenylalanine) and basic (arginine) residues at positions P'_1, P'_2 and P'_3. H.p.l.c. analysis of the hydrolysates of all the substrates of the R series (Tables 1–3) demonstrated that Arg-Xaa was the only bond cleaved.

The model peptide for these fluorogenic substrates, Abz-F-R-S-S-R-EDDnp (peptide R1), has the sequence of human kininogen at the C-terminal side of bradykinin and was hydrolysed by human tissue kallikrein (K_m = 0.19 μM and $k_{cat.}$ = 1.35 s^{-1}). The glutamine residue in the analogue R2 (Table 1) does not correspond to the P'_4 residue in human kininogen but was introduced as a requirement for the solid-phase synthesis of the substrates with systematic variations at the P'_1, P'_2 and P'_3 positions. The addition of glutamine to the C-terminus of peptide R1 increased with K_m and did not change the $k_{cat.}$ values (peptide R2, Table 1).

Table 1 Kinetic parameters for the hydrolysis by human tissue kallikrein of fluorogenic peptides with different residues at P'_1 (X)

Hydrolysis conditions were 30 °C, 20 mM Tris/HCl/1 mM EDTA, pH 9.0. Values are means ± S.D.

Peptides	Substrates	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
	Abz-F-R-X-S-R-Q-EDDnp			
R2	Abz-F-R-S-S-R-Q-EDDnp	1.5 ± 0.4	1.6 ± 0.2	1067
R3	Abz-F-R-L-S-R-Q-EDDnp	0.6 ± 0.1	1.5 ± 0.1	2500
R4	Abz-F-R-R-S-R-Q-EDDnp	1.3 ± 0.3	7.1 ± 0.2	5462
R5	Abz-F-R-F-S-R-Q-EDDnp	0.4 ± 0.2	0.8 ± 0.1	2000
	Abz-F-R-X-P-EDDnp			
R6	Abz-F-R-R-P-EDDnp	0.13 ± 0.02	0.06 ± 0.01	462
R7	Abz-F-R-S-P-EDDnp	1.40 ± 0.20	0.22 ± 0.01	157
R8	Abz-F-R-A-P-EDDnp	1.20 ± 0.20	0.041 ± 0.004	34
	Abz-F-R-X-V-EDDnp			
R9	Abz-F-R-R-V-EDDnp	0.06 ± 0.01	0.45 ± 0.02	7500
R10	Abz-F-R-S-V-EDDnp	0.90 ± 0.10	0.88 ± 0.01	987
R11	Abz-F-R-L-V-EDDnp	0.32 ± 0.01	0.006 ± 0.001	19

Table 2 Kinetic parameters for the hydrolysis by human tissue kallikrein of fluorogenic peptides with different residues at P'_2 (X)

Hydrolysis conditions were 30 °C, 20 mM Tris/HCl/1 mM EDTA, pH 9.0. Values are means ± S.D.

Peptides	Substrates	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
	Abz-F-R-S-X-R-Q-EDDnp			
R2	Abz-F-R-S-S-R-Q-EDDnp	1.50 ± 0.40	1.6 ± 0.2	1067
R12	Abz-F-R-S-L-R-Q-EDDnp	0.18 ± 0.03	0.40 ± 0.02	2222
R13	Abz-F-R-S-R-R-Q-EDDnp	0.12 ± 0.02	1.18 ± 0.08	9833
R14	Abz-F-R-S-F-R-Q-EDDnp		Not hydrolysed	
	Abz-F-R-S-X-EDDnp			
R15	Abz-F-R-S-S-EDDnp	0.47 ± 0.06	0.53 ± 0.02	1128
R7	Abz-F-R-S-P-EDDnp	1.40 ± 0.20	0.22 ± 0.01	157
R10	Abz-F-R-S-V-EDDnp	0.90 ± 0.10	0.88 ± 0.01	978
R16	Abz-F-R-S-R-EDDnp	0.04 ± 0.01	1.01 ± 0.07	25250

Table 3 Kinetic parameters for the hydrolysis by human tissue kallikrein of fluorogenic peptides with different residues at position P'_3 (X)

Hydrolysis conditions were 30 °C, 20 mM Tris/HCl/1 mM EDTA, pH 9.0. Values are means ± S.D.

Peptides	Substrates	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
	Abz-F-R-S-S-X-Q-EDDnp			
R2	Abz-F-R-S-S-R-Q-EDDnp	1.5 ± 0.40	1.60 ± 0.20	1067
R17	Abz-F-R-S-S-L-Q-EDDnp	1.1 ± 0.20	0.61 ± 0.04	554
R18	Abz-F-R-S-S-F-Q-EDDnp	3.0 ± 0.20	0.69 ± 0.02	230
R19	Abz-F-R-S-S-S-Q-EDDnp	2.2 ± 0.92	1.50 ± 0.03	681

Substitutions at P'_1

(1) Abz-F-R-X-S-R-Q-EDDnp (X = S, L, R or F). The kinetic parameters for the hydrolysis by human tissue kallikrein of peptides R2 and R5 (Table 1) demonstrate a preference of the enzyme S'_1 subsite for arginine over serine, leucine and phenylalanine and was characterized by a higher $k_{cat.}/K_m$ value for the peptide with arginine in P'_1 (peptide R4).

(2) Abz-F-R-X-P-EDDnp and Abz-F-R-X-V-EDDnp (X = R, S, A or L). The preference for arginine in P'_1 was also demonstrated by the lower K_m values for the hydrolysis by human kallikrein 1 of the tetrapeptides R6 and R9 which have respectively Arg-Pro and Arg-Val as the leaving group.

Substitutions at P'_2

(1) Abz-F-R-S-X-R-Q-EDDnp (X = S, L, R or F). In the hydrolysis by human tissue kallikrein of these peptides with substitutions at the P'_2 position (R2, R12, R13 and R14 in Table

Table 4 Hydrolysis by human tissue kallikrein of fluorogenic peptides with the human kininogen sequence flanking the scissile Met-Lys bond (peptides M1 and M2) and analogues

Hydrolysis conditions were 30 °C, 20 mM Tris/HCl/1 mM EDTA, pH 9.0. Values are means ± S.D. L.R.C., low rate of cleavage; see the text for hydrolysis conditions. N.H., no hydrolysis observed with [E] = 7 nM and [S] = 4 μM. Arrows (⇓) indicate the cleavage sites.

Peptides	Substrates	P_2		P_1		P'_1		P'_2		P'_3		P'_4	K_m (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
M1	Abz-	L	–	M	⇓	K	–	R	–	P			1.1 ± 0.1	0.07 ± 0.01	64
M2	Abz-M-I-S-	L	–	M	⇓	K	–	R	–	P			0.7 + 0.1	0.33 ± 0.02	471
F1	Abz-	L	–	F	⇓	K	–	R	–	P			0.9 ± 0.1	2.0 ± 0.1	2222
F2	Abz-M-I-S-	L	–	F	⇓	K	–	R	–	P			0.8 ± 0.2	4.20 ± 0.04	5250
M3	Abz-M-I-S-	L	⇓	M(O)	⇓	K	–	R	–	P				L.R.C.	
A1	Abz-M-I-S-	L	–	A	⇓	K	⇓	R	–	P				L.R.C.	
M4	Abz-M-I-S-	L	–	M	⇓	R	⇓	R	–	P				L.R.C.	
M5	Abz-M-I-S-	L	–	M	⇓	A	–	R	–	P				L.R.C.	
M6	Abz-M-I-S-	L	–	M	⇓	K	–	R(Tos)	–	P				L.R.C.	
M7	Abz-M-I-S-	L	–	M	⇓	K	–	R	–	P	–	Q	2 ± 1	0.33 ± 0.01	150
M8	Abz-M-I-S-	L	–	M	⇓	K	–	K	–	P	–	Q	0.9 ± 0.1	1.42 ± 0.08	1578
M9	Abz-M-I-S-	L	–	M	⇓	K	–	S	–	P	–	Q	0.37 ± 0.04	0.04 ± 0.01	108
M10	Abz-M-I-S-	L	–	M	⇓	K	–	L	–	P	–	Q	9 ± 2	0.02 ± 0.01	2
M11	Abz-M-I-S-	L	–	M	–	K	–	F	–	P	–	Q		N.H.	
M12	Abz-M-I-S-	L	–	M	–	K	–	P	–	P	–	Q		N.H.	
M13	Abz-M-I-S-	L	–	M	⇓	K	⇓	R	–	S	–	S		L.R.C.	

2), the highest $k_{cat.}/K_m$ value was shown by peptide R13 (X = R), which had a 12-fold lower K_m value than peptide R2. The substrate R12 (X = L) was hydrolysed with a similarly low K_m value but its $k_{cat.}$ value was 4-fold lower than that of R2. Peptide R14 (X = F) was resistant to hydrolysis by kallikrein 1.

(2) Abz-F-R-S-X-EDDnp (X = S, P, V or R). The data in Table 2 show that arginine at P'_2 in peptide R16 led to a significant decrease in K_m and an increase in $k_{cat.}$ values which resulted in a 22-fold higher $k_{cat.}/K_m$ than for the peptide with serine at P'_2 (R15). The tetrapeptide R7 (X = P) showed the lowest susceptibility to human tissue kallikrein in this peptide series, and the $k_{cat.}/K_m$ value for substrate R10 (X = V) did not differ from that of peptide R15.

Substitutions at P'_3

(1) Abz-F-R-S-S-X-Q-EDDnp (X = R, L, F or S). The kinetic data in Table 3 demonstrate a lower susceptibility to human kallikrein 1 of the substrates with leucine, serine (peptides R17 and R19) and particularly phenylalanine (peptide R18) at position P'_3 than peptide R2 (X = R).

Hydrolysis by human tissue kallikrein of fluorogenic peptides with the human kininogen sequence flanking the scissile Met-Lys bond and their analogues

H.p.l.c. analysis of the hydrolysates of peptides Abz-L-M-K-R-P-EDDnp (M1) and of the longer homologue Abz-M-I-S-L-M-K-R-P-EDDnp demonstrated that the Met-Lys bond was the only one cleaved by human, horse and pig tissue kallikrein. As expected the bradykinin-releasing enzymes, rat tissue and plasma kallikreins, trypsin and plasmin, cleaved only the Lys-Arg bond. The kinetic data in Table 4 demonstrate that the increase in peptide size (M1 and M2) significantly increased the susceptibility of the Met-Lys bond to human tissue kallikrein.

The cleavage site(s) in this series of peptides with different P_1 P'_4 residues were determined by amino acid analysis of the hydrolysis products separated by h.p.l.c. and are indicated in Table 4.

Substitutions at P_1

(1) Abz-L-F-K-R-P-EDDnp (F1) and Abz-M-I-S-L-F-K-R-P-EDDnp (F2). The Phe-Lys bond in these peptides was the only bond susceptible to human, horse and pig tissue kallikreins. The $k_{cat.}$ values for the hydrolysis of these peptides by human kallikrein 1 were surprisingly higher than the values for the substrates with methionine at P_1 (Table 4).

(2) Abz-M-I-S-L-M(O)-K-R-P-EDDnp (M3). Incubation of a 60 μM peptide solution with 2.5×10^{-4} μM human tissue kallikrein at pH 9.0 for approx. 15 h led to a 33 % hydrolysis of the peptide from which the hydrolysis products were identified as being K-R-P-EDDnp (approx. 19 %) and M(O)-K-R-P-EDDnp (14 %). For the lower homologous peptide, Abz-L-M(O)-K-R-P-EDDnp, a 100-fold higher enzyme concentration was used and after a 15 h incubation 34 % of the substrate was hydrolysed via cleavage of the Met(O)-Lys and Leu-Met bonds. The higher K_i values for both peptides with an oxidized methionine residue (Table 5) indicate less efficient binding of this residue to the S_1 subsite of the tissue kallikrein.

(3) Abz-M-I-S-L-A-K-R-P-EDDnp (A1). This peptide was also very resistant to tissue kallikrein. At [S] = 30 μM and [E] = 1×10^{-2} μM, only 10 % hydrolysis was detected after a 5 h incubation. Similar amounts of Lys-Arg-Pro-EDDnp and Arg-Pro-EDDnp were found, indicating cleavage at both the Ala-Lys and Lys-Arg bonds. The K_i value for this peptide also indicates a less efficient binding to the enzyme (Table 5).

Substitutions at P'_1

(1) Abz-M-I-S-L-M-R-R-P-EDDnp (M4). Two bonds, Met-Arg and Arg-Arg, were cleaved in this peptide by the tissue kallikrein, but the rates of hydrolysis were low. At a substrate concentration of 60 μM, 30 % of the substrate was hydrolysed overnight, and the hydrolysis products, identified by h.p.l.c., corresponded to

Table 5 Inhibition of human tissue kallikrein by the fluorogenic peptide Abz-M-I-S-L-M-K-R-P-EDDnp (M2) and analogues with different residues at P_1 to P'_2

Assays conditions were 30 °C, 20 mM Tris/HCl/1 mM EDTA, pH 9.0. (D)P-F-R-pNa or Abz-F-R-S-S-R-EDDnp were used as substrates and [E] = 0.06 nM.

Peptides	P_2	P_1	P'_1	P'_2	P'_3	K_i (μM)
M2	– L	– M	– K	– R	– P	0.5
M3	– L	– M(O)	– K	– R	– P	3.5
A1	– L	– A	– K	– R	– P	2.2
M4	– L	– M	– R	– R	– P	0.5
M5	– L	– M	– A	– R	– P	8.5
M6	– L	– M	– K	– R(Tos)	– P	5.0

cleavage of 21 and 9% of the Met-Arg and Arg-Arg bonds respectively. The substitution of arginine for lysine at the P'_1 position resulted in a different cleavage pattern as well as lower susceptibility to human tissue kallikrein. However, no difference in the K_i value for peptides M2 and M4 (Table 5) was observed.

(2) Abz-M-I-S-L-M-A-R-P-EDDnp (M5). The Met-Ala bond in this peptide was the only bond cleaved by the kallikrein, but its susceptibility to the enzyme was very low. Using a substrate concentration of 7 μM, equivalent to 10 times the K_m value for peptide M2 (Table 4), the initial hydrolysis rate was 100-fold lower than the $k_{cat.}$ for the Met-Lys bond cleavage. The higher K_i value obtained with this peptide as an inhibitor of human tissue kallikrein (Table 5) indicates that replacement of lysine by alanine resulted in less efficient substrate–enzyme binding.

Substitutions at P'_2

More systematic variations were carried out at P'_2 (peptides M7 to M12) because the residue at this position was shown to be critical for the susceptibility to tissue kallikreins (Chagas et al., 1991, 1992). The presence of a glutamine residue in peptides M7 to M12 is a strategy that allows the solid-phase synthesis of any internally quenched peptide and results in a 3-fold increase in the K_m (compare M2 with M7).

(1) Abz-M-I-S-L-M-K-R(Tos)-P-EDDnp (M6). Blocking of the P'_2 arginine guanidine group by tosylation resulted in a lower rate of Met-Lys bond cleavage by kallikrein. At a substrate concentration of 10 μM, the hydrolytic rate was six times lower than the $k_{cat.}$ for the original peptide. The increase in K_i value that resulted from blocking of the P'_2 arginine in the peptide Abz-M-I-S-L-M-K-R-P-EDDnp (Table 5) indicates that the guanidine group in P'_2 arginine makes an important contribution to substrate–enzyme binding.

(2) Abz-M-I-S-L-M-K-X-P-Q-EDDnp (X = R, K, S, L, F or P). The presence of lysine at P'_2 (peptide M8) lowered the K_m and increased the $k_{cat.}$ values of its hydrolysis by human kallikrein 1 (Table 4) in comparison with the unsubstituted sequence (peptide M7). When a serine residue was inserted, a 10-fold decrease in K_m and a lower $k_{cat.}$ value were observed (compare peptides M7 and M9). Hydrophobic residues such as leucine, phenylalanine and proline (peptides M10, M11 and M12) at the P'_2 position were very unfavourable for Met-Lys bond hydrolysis by human tissue kallikrein.

Substitutions at P'_3

(1) Abz-M-I-S-L-M-K-R-S-S-EDDnp (M13). This peptide, without proline at P'_2, was cleaved at the Lys-Arg (40%) and Met-Lys (20%) bonds after incubation of a 30 μM solution with 65 nM human kallikrein 1 for 2 h. The resistance of the Arg-Ser bond was expected on the basis of the presence of the lysine residue at the P_2 position.

Table 6 Kinetic parameters for the hydrolysis by human tissue kallikrein of substrates with arginyl or phenylalanyl scissile bonds

Hydrolysis conditions were 30 °C, 20 mM Tris/HCl/1 mM EDTA, pH 9.0. Values are means ± S.D.

Peptides	Substrates	K_m (μM)	$k_{cat.}$ s^{-1}	$k_{cat.}/K_m$ ($s^{-1} \cdot mM^{-1}$)
Abz-F-X-S-R-EDDnp				
R16	X = Arg	0.04 ± 0.01	1.01 ± 0.07	25250
F3	X = Phe	0.45 ± 0.08	1.65 ± 0.02	3667
(D)P-F-X-pNa				
R20	X = Arg	11 ± 2	1.3 ± 0.3	118
F4	X = Phe	39 ± 4	18.0 ± 1.0	462

Substitution of phenylalanine for arginine in tissue kallikrein substrates

The unexpected susceptibility of the Phe-Lys bond in peptides F1 and F2 led us to study the substitution of phenylalanine for arginine in the chromogenic substrate (D)P-F-R-pNA (R20) and in Abz-F-R-S-R-EDDnp (peptide R16), the best substrate so far described for tissue kallikreins (Chagas et al., 1991). The kinetic parameters in Table 6, determined under the same conditions of pH and ionic strength, show that (D)P-F-F-pNA (peptide F4) is a better substrate than (D)P-F-R-pNA (peptide R20) for human tissue kallikrein. However, the substitution of phenylalanine for arginine in the peptide Abz-F-R-S-R-EDDnp (R16) led to a less efficient substrate, Abz-F-F-S-R-EDDnp (peptide F3), as a result of a 10-fold increase in the K_m value (Table 6). All these Phe-Xaa-containing peptides are also hydrolysed by horse and pig kallikrein 1 but are resistant to human plasma kallikrein, rat tissue kallikrein, rat tonin, T-kininogenase and trypsin when tested at a (D)P-F-F-pNA or Abz-F-F-S-R-EDDnp concentration of 150 μM and an enzyme concentration of up to 200 nM.

DISCUSSION

Cleavage of Arg-Xaa bonds

The kinetic data for the hydrolysis by human tissue kallikrein of the Arg-Xaa bond in the series of peptides in Tables 1 and 2 confirm the favourable interaction of arginine with the tissue

kallikrein subsites S'_1 and S'_2 as previously described for the horse and pig enzymes (Araujo-Viel et al., 1988; Chagas et al., 1991). A similar preference of the S'_3 subsite of human tissue kallikrein for arginine was also demonstrated in the present work by the high $k_{cat.}/K_m$ value for peptide R2, which has arginine at P'_3 (Table 3). The low $k_{cat.}/K_m$ values for peptides R6–R8 in Table 1 confirm the low susceptibility of arginine bonds with proline at P'_2 to the kallidin-liberating tissue kallikreins (Prado et al., 1986; Araujo-Viel et al., 1988) and is also in accordance with the resistance of the Lys-Arg bonds in kininogens to these enzymes. On the other hand, peptides with proline in the P'_2 position were previously described as substrates for human plasma kallikrein (Chagas et al., 1991) and for rat tissue kallikrein (Chagas et al., 1992).

Cleavage of the Met-Lys bonds

The low $k_{cat.}/K_m$ values for the hydrolysis of peptide M1 by human tissue kallikrein (Table 4) is in agreement with the previously reported resistance of G-L-M-K-bradykinin to horse tissue kallikrein (Araujo-Viel et al., 1981) and of S-L-M-K-bradykinin to pig tissue kallikrein (Fiedler, 1983). The higher $k_{cat.}/K_m$ value for the hydrolysis of peptide M2 by human tissue kallikrein (Table 4) shows the influence of further interactions with residues in positions P_3 to P_5 on the efficiency of the Met-Lys bond cleavage. These interactions apparently do not play a role in Arg-Ser bond cleavage as no differences in the kinetic parameters for the hydrolysis of P-F-R-S-V-Q and bradykinin-S-V-Q were found by Fiedler (1987).

The resistance of the Lys-Arg bond, and consequently the cleavage by tissue kallikreins of the Met-Lys bond in kininogen, were suggested to be due to a poor S'_2–Pro interaction based on X-ray crystallography data (Chen and Bode, 1983) and on kinetic data for the hydrolysis of synthetic peptides with valine or proline in the P'_2 position (Prado et al., 1983, 1986). Our present data on the non-specific cleavage of Lys-Arg and Met-Lys bonds in the peptide Abz-M-I-S-L-M-K-R-S-S-EDDnp (M13) and the resistance of Abz-M-I-S-L-M-K-P-P-Q-EDDnp (M12) to hydrolysis by human tissue kallikrein confirm the importance of proline in orientating the cleavage of the kininogen Met-Lys bond. In addition, as observed for Arg-Xaa bond cleavage, the substitution of arginine at P'_2 for hydrophobic or aromatic residues such as leucine or phenylalanine (peptides M10 or M11 respectively) impairs the Met-Lys bond cleavage (Table 4).

The guanidine group in the P'_2 arginine makes an important contribution to the binding of the substrate to the tissue kallikreins as demonstrated by the low affinity of peptide M6, which has a tosylated guanidine group, for human tissue kallikrein (Table 5). This finding is in agreement with the very low K_m values for the hydrolysis of Abz-F-R-S-R-EDDnp by human (see above) and other tissue kallikreins (Chagas et al., 1991, 1992).

Cleavage of the Phe-Lys bond

The replacement of Met^5 in Abz-M-I-S-L-M-K-R-P-EDDnp (peptide M2) and Met^2 in the shorter homologue (peptide M1) by phenylalanine resulted in interesting novel substrates with Phe-Lys as the only bond susceptible to the tissue kallikreins. The $k_{cat.}/K_m$ values for the hydrolysis of these substrates are significantly higher than those for peptides M1 and M2 with Met-Lys as the susceptible bond (Table 4).

The replacement of arginine by phenylalanine in peptide R16 resulted in lower $k_{cat.}/K_m$ values from the hydrolysis of Abz-F-F-S-R-EDDnp (peptide F3) by human tissue kallikrein (Table 6). On the other hand, the replacement of arginine by phenylalanine in (D)P-F-R-pNA (peptide R20, Table 6) resulted in a substrate [(D)P-F-F-pNA, peptide F4, Table 6] with a $k_{cat.}$ value one order of magnitude higher. Similar data were obtained with horse and pig tissue kallikreins (results not shown). In addition, an interesting and useful observation was the resistance of the Phe-Xaa bonds in these substrates to other arginine hydrolases, such as human plasma and rat tissue kallikreins, tonin, trypsin and T-kininogenase.

All the substrates developed in the present study for Phe-Xaa bond cleavage by tissue kallikreins have either phenylalanine or leucine at the P_2 position. We have, on the other hand, verified that horse and pig tissue kallikreins hydrolyse the Phe-pNA bond of angiotensin-II-pNA which has proline at P_2, although the efficiency is very low (results not shown). These results suggest that Phe-Xaa bond cleavage by tissue kallikreins has the same requirement for bulky and hydrophobic residues at P_2 as that demonstrated for Arg-Ser bond cleavage.

These results indicate that the benzene ring of phenylalanine can fit very adequately into the S_1 subsite of tissue kallikreins. This conclusion agrees with the observations reported elsewhere in which (a) the X-ray data for porcine pancreatic kallikrein complexed with pancreatic trypsin inhibitor show the sandwiching of the hydrophobic lysine side chain methylene groups by the main-chain segments 215–216 and 191–192 at the entrance of the enzyme's S_1 primary pocket (Chen and Bode, 1983) and (b) the same sandwiching for the benzamidine phenyl ring was observed in the three-dimensional structure of benzamidine-porcine pancreatic kallikrein crystals (Bode et al., 1983).

The surprising efficiency of tissue kallikreins in hydrolysing (D)P-F-F-pNA (peptide F4), the Phe-Lys bond in peptides F1 and F2 and the Phe-Ser bond in F3 led us to speculate on the existence of natural substrates, other than kininogens, the specificity of which could be determined by a pair of bulky hydrophobic residues. This possibility is well supported by the known structure of the P_3–P'_1 reactive centre (Lys-Phe-Phe-Ser) of kallistatin, where the Phe-Ser bond is hydrolysed (Chai et al., 1993). Tissue kallikreins therefore deserve more detailed investigation, particularly with regard to the subsite determinants when phenylalanine, methionine or other hydrophobic amino acid residues occur together at the P_1 and P_2 positions of the substrates.

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