Evaluation of the extent of the binding site in human tissue kallikrein by synthetic substrates with sequences of human kininogen fragments

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We have synthesized internally quenched peptides spanning the Met³⁷⁹-Lys³⁸⁰ or Arg³⁸⁹-Ser³⁹⁰ bonds of human kininogen (hkng) that flank lysyl-bradykinin and have studied the kinetics of their hydrolysis by human tissue kallikrein. The kinetic data for the hydrolysis of the Met-Lys bond in substrates with an N-terminal extension showed that interactions up to position residue P₁₀ contribute to the efficiency of cleavage. In contrast, there were no significant variations in the kinetic data for the hydrolysis of substrates with C-terminal extensions at sites P'₄ to P'₁₁. A similar pattern was observed for the cleavage of substrates containing an Arg-Ser bond because substrates extended up to residue P₆ were hydrolysed with the highest k_{cat}/K_m values in the series, whereas those extended to P'₁₁ on the C-terminal side had a lower susceptibility to hydrolysis. Time-course studies of hydrolysis by human and porcine tissue kallikreins of a Leu³⁷³ to Ile³⁹³ human kininogen fragment containing *o*-aminobenzoic

acid (Abz) at the N-terminus and an amidated C-terminal carboxyl group Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Ser-Arg-Ile-NH₂ (Abz-[Leu³⁷³-Ile³⁹³]-hkng-NH₂) indicated that the cleavage of Met-Lys and Arg-Ser bonds in the same molecule occurs via the formation of independent enzyme-substrate complexes. The hydrolysis of Abz-F-R-S-S-R-Q-EDDnp [where EDDnp is *N*-(2,4-dinitrophenyl)ethylenediamine] and Abz-M-I-S-L-M-K-R-P-Q-EDDnp by human tissue kallikrein had maximal k_{cat}/K_m values at pH 9–9.5 for both substrates. The pH-dependent variations in this kinetic parameter were almost exclusively due to variations in k_{cat} . A significant decrease in k_{cat}/K_m values was observed for the hydrolysis of Arg-Ser and Met-Lys bonds in the presence of 0.1 M NaCl. Because this effect was closely related to an increase in K_m , it is likely that sodium competes with the positive charges of the substrate side chains for the same enzyme subsites.

INTRODUCTION

Tissue kallikreins (EC 3.4.21.35) comprise a subfamily of trypsin-like serine proteinases, widely distributed in animal tissues, with closely related structures and sharing common biological features, including a highly specific kininogenase activity. In limited proteolysis of kininogens of high and low molecular mass by tissue kallikreins, the vasoactive decapeptide Lys-bradykinin (Lys-Bk) is released by cleavage at the Met³⁷⁹-Lys³⁸⁰ and Arg³⁸⁹-Ser³⁹⁰ bonds [1]. The human kininogen sequence from residue 370 to 399, using the human prekininogen numbering [2], is -Cys³⁷⁰-Gln-Pro-Leu-Gly-Met-Ile-Ser-Leu-Met³⁷⁹-Lys³⁸⁰-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg³⁸⁹-Ser³⁹⁰-Ser-Arg-Ile-Gly-Glu-Ile-Lys -Glu-Glu³⁹⁹.

Rat tissue kallikrein [3] is the only exception in this class of proteolytic enzymes because it cleaves the Lys³⁸⁰-Arg³⁸¹ bond in kininogen, thus releasing bradykinin [4]. The resistance of this bond to hydrolysis by the other tissue kallikreins is essentially related to the presence of Pro³⁸² because substrates containing this amino acid at P'₂ (Schechter and Berger nomenclature [5]) are hardly hydrolysed by tissue kallikreins [6,7]. The preference for hydrolysing the Met³⁷⁹-Lys³⁸⁰ bond by tissue kallikreins has been attributed to the favourable secondary interactions of Leu at position P₂ as well as of Lys and Arg at P'₁ and P'₂ respectively [8]. However, the peptides (Gly,Ser)-Leu-Met-Lys-Bk were shown to be hydrolysed by tissue kallikreins with low efficiency [9,10], and the fluorogenic peptide Abz-MISLMKRP-EDDnp, although still a poor substrate for human-tissue kallikrein, was hydrolysed with a higher k_{cat} than the lower homologue Abz-LMKRP-EDDnp [8]. We have also observed that human tissue

kallikrein more efficiently hydrolyses short substrates containing arginine or phenylalanine at P₁ compared with those with methionine at the same position [8]. These results suggest that the hydrolysis of the Met³⁷⁹-Lys³⁸⁰ bond of kininogen requires greater interaction of the substrate with the extended kallikrein binding site than does the hydrolysis of the Arg³⁸⁹-Ser³⁹⁰ bond.

In spite of the two distinct cleavages necessary to release Lys-Bk, tissue kallikrein does not have two different active sites [11]. Evidence from reaction time-course and competition experiments indicate that both hydrolyses occur in the same enzyme-substrate complex [12,13]. Nevertheless the real mechanism of this unusual double cleavage remains unknown. Information on this reaction is of interest because tissue kallikrein is usually considered as a prototype for several other kallikrein gene family members involved in the processing of prohormones [14] and growth factor precursors [15].

To determine the extent of the human tissue kallikrein binding site required for efficient hydrolysis of Met³⁷⁹-Lys³⁸⁰ and Arg³⁸⁹-Ser³⁹⁰ bonds we have synthesized internally quenched fluorescent peptides of different sizes that span these bonds in human kininogen and have studied the kinetics of their hydrolysis by human tissue kallikrein. As previously reported, *o*-aminobenzoic acid (Abz) and *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) were employed as donor-acceptor pair at the N- and C-terminus of the peptides, respectively [7,8]. We have also synthesized a Leu³⁷³ to Ile³⁹³ fragment of human kininogen containing Abz at the N-terminus and an amidated C-terminal carboxyl group (Abz-[Leu³⁷³-Ile³⁹³]-hkng-NH₂). This fragment was used to study the time course of the cleavage of Met-Lys and Arg-Ser bonds in the same molecule by human and porcine tissue kallikreins. The

Abbreviations used: Abz, *o*-aminobenzoic acid; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; hkng, human kininogen; Bk, bradykinin; Abz-[Leu³⁷³-Ile³⁹³]-hkng-NH₂, Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Ser-Arg-Ile-NH₂.

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effects of salt concentration on the pH-specificity-constant (k_{cat}/K_m) curves for the hydrolysis of these bonds by human tissue kallikrein were also determined.

MATERIALS AND METHODS

Enzymes

Homogeneous preparations of human tissue kallikrein obtained by the method of Shimamoto et al. [16] were kindly provided by Dr. J. Chao of the Medical University of South Carolina, Charleston, SC, U.S.A. Porcine pancreatic kallikrein (form B) prepared by Bayer A. C., Wuppertal, Germany, was further purified [17] and was generously supplied by Dr. F. Fiedler, University of Munich, Germany. The molar concentrations of enzyme solutions were determined by active-site titration with 4-nitrophenyl-4-guanidinobenzoate [18].

Synthetic substrates

The intramolecularly quenched fluorogenic peptides have EDDnp attached to glutamine, a necessary result of the solid-phase peptide synthesis strategy employed, the details of which are provided elsewhere [19]. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the fluoren- α -ylmethoxycarbonyl procedure. The peptides Abz-Leu-Met-Lys-Arg-Pro-EDDnp and Abz-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-EDDnp were synthesized by a solution method as previously described [8]. Amino acid analyses were carried out in a Beckman 6300 amino acid analyser after hydrolysis in 6 M HCl with 5% (v/v) phenol at 110 °C for 48 h. The final deprotected peptides were purified by semipreparative HPLC on an Econosil C-18 column (10 μ m, 22.5 mm $\times$ 250 mm) and with two solvent systems: (A) trifluoroacetic acid (TFA)/H₂O (1:1000, v/v) and (B) TFA/acetonitrile (ACN)/H₂O (1:900:100 by vol.). The column was eluted at a flow rate of 5 ml/min with a 10% (or 30%) to 50% (or 60%) gradient of system B over 30 or 45 min. Analytical HPLC was performed with a C-R7A Shimadzu UV-visible-light detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μ m, 4.6 mm $\times$ 150 mm), which was eluted with solvent systems A and B above at a flow rate of 1.7 ml/min and a 10–80% gradient of B over 15 min. The HPLC column eluates were monitored by their absorbance at 220 nm and by fluorescence emission at 420 nm after excitation at 320 nm.

Fluorometric enzyme assay

Hydrolysis of the fluorogenic peptide substrates at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA was followed by measuring the fluorescence at $\lambda_{\text{em}} = 420$ nm and $\lambda_{\text{ex}} = 320$ nm in a Hitachi F-2000 spectrofluorimeter. The 1 cm path-length cuvette containing 2 ml of the substrate solution was placed in the thermostated cell compartment for 5 min before the enzyme solution was added, and the increase in fluorescence with time was continuously recorded for 10 min. The slope was converted into mol of substrate hydrolysed per min based on the fluorescence curves for standard peptide solutions before and after total enzymic hydrolysis. Provided that HPLC analysis of the different fluorogenic substrates did not show any significant contamination, their concentration in solution could be determined from the fluorescence obtained after total tryptic hydrolysis. The concentration of a standard solution of Abz-Phe-Arg-OH, used for calibration of the fluorimeter measurements, was calculated on the basis of the amount of *p*-nitroaniline released from Abz-Phe-Arg-pNA by trypsin as

measured by the absorbance at 405 nm. The enzyme concentrations for initial rate determinations were chosen so as to hydrolyse less than 5% of the substrate present. The kinetic parameters were calculated according to Wilkinson [20]. The effect of pH on the hydrolysis of the fluorogenic peptide substrates was determined at 37 °C with either 20 mM sodium phosphate buffer (for pH 6–8) or 20 mM Tris/HCl buffer (for pH ~ 8); both buffers contained 1 mM EDTA. In experiments investigating the influence of salt on the reaction, 0.1 M NaCl was added to the assay mixture.

Kininogenase assay

The products arising from the hydrolysis of Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Ser-Arg-Ile-NH₂ (Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂) by human and porcine tissue kallikreins were identified and quantified by HPLC on a Novapack C-18 column (3.2 mm $\times$ 150 mm) equilibrated with 0.1% H₃PO₄ (solvent A). The column was eluted at a flow rate of 1.7 ml/min with a 0–10% gradient of solvent B (90% ACN, 0.1% H₃PO₄, v/v) over 5 min followed by a 10–80% gradient of the same solvent over 15 min. The elution profile was determined at 220 nm and by fluorescence at 420 nm and after excitation at 320 nm. Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂ (10 μ M) was incubated with either human or porcine tissue kallikrein (final concn. 8 nM) in buffers containing 20 mM Na₂HPO₄ and 1 mM EDTA for the pH range 6.5–7.0, or 20 mM Tris/HCl and 1 mM EDTA for pH 9.0 at 30 °C. Aliquots (40 μ l) were removed from these assay mixtures at 20 min intervals after the addition of enzyme and mixed with 60 μ l of 0.1% (v/v) H₃PO₄ to stop the reaction. Standard solutions of the substrate and of authentic synthetic peptides corresponding to the products of substrate hydrolysis were chromatographed under the same conditions. The fluorescent fragments produced by hydrolysis were quantified by determining the area of the corresponding peak in the elution profile.

RESULTS

Hydrolysis of fluorogenic peptides containing Met-Lys or Arg-Ser bonds

Internally quenched fluorescent peptides with an increasing number of residues on the amino (Table 1) and carboxyl (Table 2) sides of the Met³⁷⁹-Lys³⁸⁰ bond of human kininogen were synthesized. Met instead of Cys was introduced in peptide V (Table 1) to avoid complications with peptide polymerization arising from oxidation of the cysteine-SH group. In peptide VII (Table 2), Gly was substituted for Phe to avoid cleavage of the Arg-Gln bond by tissue kallikrein.

The kinetic data for the hydrolysis by human tissue kallikrein of N- and C-terminal extended Met-Lys bond-containing peptides of human kininogen are shown in Tables 1 and 2, respectively. The substrates containing the Arg³⁸⁹-Ser³⁹⁰ bond of human kininogen are shown in Table 3. In view of the favourable effects of Arg at positions P₁ to P₃ for the hydrolysis of Abz-FRSSRQ-EDDnp by human tissue kallikrein [8], we synthesized analogues of this peptide containing Asp or Glu at each of the amino acid positions from P₁ to P₄ to determine the effect of a negatively charged substrate side chain on subsequent cleavage. The kinetic data for the hydrolysis of these substrates by human-tissue kallikrein are shown in Table 4.

Hydrolysis of Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂

The time-course of hydrolysis by human tissue kallikrein of peptide Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂, which contains both Met-

Table 1 Kinetic data for the hydrolysis by human tissue kallikrein of N-terminal extended fluorogenic peptides (Abz-peptidyl-EDDnp) spanning the Met³⁷⁹-Lys³⁸⁰ bond of human kininogen (Indicated by arrow)Hydrolysis was carried out for 10 min at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA. Values are means $\pm$ S.D.

No.	Substrates														K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
	P ₁₀	P ₉	P ₈	P ₇	P ₆	P ₅	P ₄	P ₃	P ₂	P ₁ ↓	P' ₁	P' ₂	P' ₃	P' ₄			
I									L	M	K	R	P	Q	2.3 $\pm$ 1.0	0.04 $\pm$ 0.01	17
II						M	I	S	L	M	K	R	P	Q	2.2 $\pm$ 0.9	0.3 $\pm$ 0.07	136
III				L	G	M	I	S	L	M	K	R	P	Q	2.2 $\pm$ 0.3	3.9 $\pm$ 0.2	1772
IV		Q	P	L	G	M	I	S	L	M	K	R	P	Q	4.2 $\pm$ 0.5	7.1 $\pm$ 0.6	1690
V	M	Q	P	L	G	M	I	S	L	M	K	R	P	Q	1.1 $\pm$ 0.1	5.4 $\pm$ 0.4	4909

Table 2 Kinetic data for the hydrolysis by human tissue kallikrein of C-terminal extended fluorogenic peptides (Abz-peptidyl-EDDnp) spanning the Met³⁷⁹-Lys³⁸⁰ bond of human kininogen (Indicated by arrow)Hydrolysis was carried out for 10 min at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA. Values are means $\pm$ S.D.

No.	Substrates																K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
	P ₅	P ₄	P ₃	P ₂	P ₁ ↓	P' ₁	P' ₂	P' ₃	P' ₄	P' ₅	P' ₆	P' ₇	P' ₈	P' ₉	P' ₁₀	P' ₁₁			
II	M	I	S	L	M	K	R	P	Q								2.2 $\pm$ 0.4	0.3 $\pm$ 0.07	136
VI	M	I	S	L	M	K	R	P	P	G	F	S	Q				2.2 $\pm$ 0.6	0.6 $\pm$ 0.05	273
VII	M	I	S	L	M	K	R	P	P	G	F	S	P	G	R	Q	2.9 $\pm$ 0.6	0.9 $\pm$ 0.1	310

Table 3 Kinetic data for the hydrolysis by human tissue kallikrein of fluorogenic peptides (Abz-peptidyl-EDDnp) spanning the Arg³⁸⁹-Ser³⁹⁰ bond of human kininogen (Indicated by arrow)Hydrolysis was carried out for 10 min at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA. Values are means $\pm$ S.D.

No.	Substrates																				K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM \cdot s^{-1}$)	
	P ₉	P ₈	P ₇	P ₆	P ₅	P ₄	P ₃	P ₂	P ₁ ↓	P' ₁	P' ₂	P' ₃	P' ₄	P' ₅	P' ₆	P' ₇	P' ₈	P' ₉	P' ₁₀	P' ₁₁				
VIII				G	F	S	P	F	R	S	S	R	Q									0.4 ± 0.04	7.5 ± 0.3	18750
IX	R	P	P	G	F	S	P	F	R	S	S	R	Q									0.7 ± 0.07	5.3 ± 0.1	7571
X				G	F	S	P	F	R	S	S	R	I	G	E	I	K	E	E	Q		6.7 ± 1.3	3.0 ± 0.3	448

Table 4 Kinetic data for the hydrolysis by human tissue kallikrein of fluorogenic analogues of Abz-FRSSRQ-EDDnp (Indicated by arrow)Hydrolysis was carried out for 10 min at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA. Values are means $\pm$ S.D.

No.	Substrates						K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
	P ₂	P ₁ ↓	P' ₁	P' ₂	P' ₃	P' ₄			
XI*	F	R	S	S	R	Q	1.5 $\pm$ 0.4	1.6 $\pm$ 0.2	1067
XII	F	R	D	S	R	Q	16.4 $\pm$ 2	0.2 $\pm$ 0.02	12
XIII	F	R	S	D	R	Q	5.7 $\pm$ 0.6	0.4 $\pm$ 0.05	70
XIV	F	R	S	S	D	Q	8.3 $\pm$ 0.9	1.1 $\pm$ 0.6	133
XV	F	R	S	S	R	E	1.2 $\pm$ 0.3	0.18 $\pm$ 0.01	150

* Data from reference [8].

Lys and Arg-Ser bonds in the same molecule, was performed at three different pH values (6.5, 7.0 and 9.0). The products of hydrolysis were separated by HPLC and the elution profiles were determined by UV absorbance and by fluorimetry as described in

Materials and Methods. Figure 1a shows a typical HPLC profile for the digestion of Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂ by human tissue kallikrein in which the fragments (peptides 1, 2, 4 and 5), and the intact substrate (peptide 3) were detected. The fragment

Figure 1 Products of the hydrolysis of Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂ by human (a) and porcine (b) tissue kallikreins

After a 10, 30 or 60 min incubation of enzyme (8 nM) and substrate (10 μ M) at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA, aliquots of the reaction mixture were applied to a Novapak C-18 column (3.2 mm $\times$ 150 mm) previously equilibrated with solvent A [$\text{H}_3\text{PO}_4/\text{H}_2\text{O}$ (1/100 v/v)]. The reaction products were eluted from the column with a 0–10% (v/v) gradient of solvent B [H_3PO_4 /acetonitrile/ H_2O (1/900/100 v/v)] over 5 min and 10–80% over 15 min at a flow rate of 1.7 ml/min. The elution profiles (i)–(iii) correspond to reaction times for both enzymes of 10, 30 and 60 min respectively.

[Lys(Bk)-Ser-Ser-Arg-Ile-NH₂] that corresponds to the single hydrolysis of the Met-Lys bond was not detected, possibly because the Arg-Ser bond was preferentially cleaved. The time-course reactions of the hydrolysis by human tissue kallikrein of Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂ at pH 6.5 and 7.0 (results not shown) were slower but with a similar pattern to that at pH 9.0,

Figure 2 pH dependence of the hydrolysis of Abz-FRSSRQ-EDDnp (a) and Abz-MISLMKRPQ-EDDnp (b) by human tissue kallikrein at 37 °C in the absence (■) and presence (□) of 0.1 M NaCl

and the accumulation of the fragment Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys(Bk) was also demonstrated. Porcine pancreatic kallikrein hydrolysed Abz-[Leu³⁷³-Ile³⁸³]-hkng-NH₂ at the Arg-Ser bond only, even at pH 9.0 (Figure 1b). Peptides I, II, IV and VII were tested as substrates for this enzyme to verify the

Table 5 Kinetic data for the hydrolysis by porcine tissue kallikrein of fluorogenic peptides (Abz-peptidyl-EDDnp) spanning the Met³⁷³-Lys³⁸⁰ bond of human kininogen (indicated by arrow)

Hydrolysis was carried out for 10 min at 30 °C in 20 mM Tris/HCl, pH 9.0, containing 1 mM EDTA. Values are means $\pm$ S.D. Peptides XVII and XVIII were synthesized by solution methods as previously described [8].

No.	Substrates																			K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (mM $\cdot$ s) ⁻¹
	P ₉	P ₈	P ₇	P ₆	P ₅	P ₄	P ₃	P ₂	P ₁ ↓	P' ₁	P' ₂	P' ₃	P' ₄	P' ₅	P' ₆	P' ₇	P' ₈	P' ₉	P' ₁₀	P' ₁₁		
XVII								L	M	K	R	P								4.7 $\pm$ 0.1	0.08 $\pm$ 0.004	17
XVIII					M	I	S	L	M	K	R	P								5.9 $\pm$ 1.2	0.2 $\pm$ 0.02	34
VII	M				I	S	L	M	K	R	P	P	G	F	S	P	G	R	Q	0.5 $\pm$ 0.1	0.07 $\pm$ 0.01	140
IV	Q	P	L	G	M	I	S	L	M	K	R	P	Q							3.9 $\pm$ 0.6	0.4 $\pm$ 0.04	103

resistance of the Met-Lys bond to cleavage. The kinetic data for these peptides are shown in Table 5.

Effect of pH on Arg-Ser and Met-Lys bond hydrolysis by human-tissue kallikrein

The hydrolysis of Abz-F-R-S-S-R-Q-EDDnp and Abz-M-I-S-L-M-K-R-P-Q-EDDnp by human-tissue kallikrein was tested over a pH range of 5 to 10. Maximal k_{cat}/K_m values were obtained at pH 9–9.5 for both substrates (Figures 2a and 2b). The variations in this kinetic parameter with pH are almost exclusively due to k_{cat} , because the K_m values did not change significantly in the pH range studied (results not shown). Similar results were obtained with larger substrates such as peptides V and X (results not shown). Figure 2 also shows the effect of NaCl on the k_{cat}/K_m –pH curves.

DISCUSSION

The kinetic data for the hydrolysis by human tissue kallikrein of substrates that span the Met³⁷⁹-Lys³⁸⁰ bond of human kininogen and have an extended N-terminus (Table 1) show that interactions up to position P₁₀ contribute to the efficiency of cleavage, as demonstrated by the higher k_{cat} values for the hydrolysis of the larger substrates (compare peptides I to V). In contrast, in comparison with peptide II, which contains the minimal C-terminal extension for the correct and detectable hydrolysis by human tissue kallikrein [8], no significant variations in the kinetic data were observed for the hydrolysis of substrates whose structures include the amino acids of Bk at positions P'₅ to P'₁₀ (Table 2).

The substrates spanning the Arg³⁸⁹-Ser³⁹⁰ bond of human kininogen (Table 3), and which contain the bradykinin sequence at positions P₁ to P₉ (peptides VIII and IX) were hydrolysed by human tissue kallikrein with k_{cat}/K_m values higher than that of peptide X, whose sequence corresponds to that of peptide VIII but with a C-terminal extension up to P'₁₁. The lower susceptibility of peptide X to hydrolysis by human tissue kallikrein resulted from a significant increase in the K_m value, indicating that the presence of the sequence -G-E-I-K-E-E- impairs adequate binding of this peptide to the enzyme's active site.

Peptides Abz-FRDSRQ-EDDnp, Abz-FRSDRQ-EDDnp and Abz-FRSSDQ-EDDnp (peptides XII to XIV) were poorly hydrolysed by tissue kallikrein and had lower k_{cat} and higher K_m values than peptide XI. These results contrast with the high susceptibility to human tissue kallikrein hydrolysis of the substrates Abz-FRRSRQ-EDDnp, Abz-FRSRRQ-EDDnp and Abz-FRSSRQ-EDDnp [8], in which Arg was replaced by Asp at positions P'₁ to P'₃. A significant decrease in the k_{cat} value was also observed by the substitution of Glu for Gln (peptide XV, Table 4). Therefore the enzyme subsites S'₁ to S'₄ possibly contain anionic moieties that accept positively charged side chains while repelling the negative groups.

In contrast to previous observations suggesting that tissue kallikrein releases Lys-Bk from kininogen through a single enzyme-substrate complex [12,13], the accumulation of the fragment Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-(Bk), resulting from the preferential hydrolysis of the Arg-Ser bond in Abz-[Leu³⁷³-Ile³⁹³]-hkng-NH₂, indicates that cleavages of Arg-Ser and Met-Lys bonds occur through the formation of two independent enzyme-substrate complexes. The fragment Abz-Leu-Gly-Met-Ile-Ser-Leu-Met-Lys-(Bk) was undetectable only in experiments in which a 10-fold higher enzyme concentration was used and in which the high velocity of Arg-Ser and Met-Lys bond cleavage prevented the isolation of any intermediate fragment. Thus if the

release of Lys-Bk by human tissue kallikrein occurs through a single enzyme-substrate complex, it most probably depends on interactions with parts of the kininogen molecule other than the Leu³⁷³-Ile³⁹³ segment.

Porcine pancreatic kallikrein hydrolysed Abz-[Leu³⁷³-Ile³⁹³]-hkng-NH₂ only at the Arg-Ser bond (Figure 1b). The resistance of the Met-Lys bond to cleavage by porcine pancreatic kallikrein was also observed with the longer internally quenched fluorescent peptides (IV and VII) whose k_{cat} values for hydrolysis by the porcine enzyme (Table 5) were significantly lower than those obtained with human-tissue kallikrein. These results indicate that the specificities for the cleavage of Arg-Ser and Met-Lys bonds in human kininogen by human tissue kallikrein are determined by long enzyme-substrate interactions, involving mainly the enzyme S₁-S₁₀ subsites and the P₁-P₁₀ residues of the substrates. These observations are in accordance with those recently reported for contribution of an extended interaction site in rat tissue kallikrein peptidase activity [21]. In addition, for Arg-Ser bond cleavage the Bk sequence extends from positions P₁ to P₉ in kininogen and because the structure of bradykinin is the same in all mammalian species so far studied, the specificity for the recognition of kininogens by tissue kallikrein is determined by 8 to 10 amino acids located on the N-terminal side of the Met-Lys bond.

The pH profiles for the hydrolysis of Abz-F-R-S-S-R-Q-EDDnp and Abz-M-I-S-L-M-K-R-P-Q-EDDnp by human tissue kallikrein over the pH range 5 to 10 were similar, indicating that the different protonation states of the enzyme did not discriminate between the basic side chain of Arg and the neutral side chain of Met at the P₁ position of the substrates. The pH range of maximal k_{cat}/K_m values obtained for hydrolysis of these substrates are comparable with the maximal pH range of kinin release from human high- and low-molecular-mass kininogens by human urinary kallikrein [22].

A significant decrease in the k_{cat}/K_m values was observed after the addition of 0.1 M NaCl, particularly for the hydrolysis of the Arg-Ser bond in the peptide Abz-F-R-S-S-R-Q-EDDnp. This decrease was due mainly to an increase in K_m . These observations agree with previously reported data for the hydrolysis of short chromogenic peptide substrates by horse and porcine tissue kallikreins [23]. Thus sodium possibly competes with the positively charged guanidine and amino side chains of Arg and Lys in these substrates for the above-mentioned anionic moiety at the enzyme subsites S₁ to S'₄.

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