Characterization of the Thermolysin-like Cleavage of Biologically Active Peptides by *Xenopus laevis* Peptide Hormone Inactivating Enzyme†

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ABSTRACT: Peptide hormone inactivating endopeptidase (PHIE) is a metalloendopeptidase which was isolated from the skin granular gland secretions of *Xenopus laevis* [Carvalho, K. M., Joudiou, C., Boussetta, H., Leseney, A. M., & Cohen, P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 84–88]. This peptidase exhibits a thermolysin-like character and hydrolyzes bonds on the amino terminus of hydrophobic amino acids, performing cleavage of Xaa–Phe, Xaa–Leu, Xaa–Ile, Xaa–Tyr, and Xaa–Trp doublets. When the enzyme recognized a doublet of hydrophobic amino acids such as Phe^6–Phe^7 of somatostatin-14, Phe^7–Phe^8 of substance P, Phe^4–Leu^5 of [Leu^5,Arg^6]enkephalin, and Tyr^4–Ile^5 of angiotensin II, cleavage occurred preferentially between these residues. The use of selectively modified carboxy-terminal octapeptide fragments of atrial natriuretic factor (ANF) indicated that the enzyme tolerates as substrates only peptides bearing a P′1 bulky hydrophobic amino acid residue. Although a P′1 hydrophobic residue was a necessary condition, it was found in a number of peptides that all potential cleavage sites were not recognized by the enzyme. These data suggested that this metalloendoprotease requires for its thermolysin-like activity a preferred conformation of the peptide chain. Kinetic results obtained using a series of related substrates derived from biologically active peptides of the atrial natriuretic factor, tachykinin, and enkephalin families indicated the presence of an extended binding site accommodating at least six amino acid residues, in contrast to thermolysin (EC 3.4.24.4) and neutral endopeptidase (NEP; EC 3.4.24.11), which hydrolyze shorter homologous peptides. Since PHIE hydrolyzed the Lys^{12}-Ile^{13}bond in PGLa, a major *X. laevis* skin secretion antimicrobial component (K_m = 28 μM), it is inferred that this novel enzyme, which is distinct from angiotensin-converting enzyme (ACE; EC 3.4.15.1), from meprin (EC 3.4.24.18), and from other, presently known, thermolysin-like metalloendoproteases, may play a role in the *in vivo* inactivation of biologically active peptides.

Enzymes that inactivate biologically active peptides are of great interest since they may regulate the physiological action of such messengers [reviewed in Erdös and Skidgel (1989)]. There is evidence, indeed, for their role in controlling the degradation of neuropeptides *in vivo*. For example, whereas angiotensin-converting enzyme (ACE; EC 3.4.15.1) is implicated in the angiotensin–renin system (Wywrat & Patchett, 1985), neutral endopeptidase (NEP; enkephalinase; EC 3.4.24.11) was proposed to be involved in the inactivation of both enkephalins by a Gly^3–Phe^4 cleavage (Malfroy et al., 1978; Hersh, 1982) and of the atrial natriuretic factor (Stephenson & Kenny, 1987; Sonnenberg et al., 1988; Olins et al., 1989; Bralet et al., 1990; Schwartz et al., 1990; Lecomte et al., 1990; Gros et al., 1990) by a Cys^7–Phe^8 hydrolysis (Kenny & Stephenson, 1988) although a minor cleavage was observed at the Ser^{25}–Phe^{26} bond (Olins et al., 1989). Other possible substrates for this enzyme were also tentatively proposed (Matsas et al., 1983; Hersh, 1984; Vijayaraghavan et al., 1990b). Selective cleavage of atrial natriuretic factor (ANF) at Ser^{25}–Phe^{26} abolishes the bioactivity of this diuretic–natriuretic and hypotensive peptide [Thibault et al., 1984; reviewed in Schwartz et al. (1990)]. Moreover, in vascular smooth muscle cells, removal of the C-terminal tripeptide of ^{125}I-labeled ANF is not blocked by specific enkephalinase inhibitor, suggesting that a peptidase different from NEP is responsible for the turnover of endogeneous ANF (Johnson et al., 1989).

We have previously reported the purification, from *Xenopus laevis* skin secretion, of a peptide hormone inactivating endopeptidase (PHIE), a novel metalloendopeptidase with a thermolysin-like specificity (Carvalho et al., 1992). This enzyme cleaves specific peptide bonds in several regulatory peptides such as ANF, substance P, angiotensin II, neuromedin B, somatostatin-14, litorin, and bradykinin. Therefore, PHIE was considered as a serious candidate for controlling activity of these peptides, and it was proposed to play a role in the balance of hormonal systems, since the K_m values measured *in vitro* for a series of peptide substrates were found within the physiological range (Carvalho et al., 1992).

In order to elucidate the regulation mechanisms in which metalloendopeptidases are implicated, a detailed knowledge of their specificity and of the mode of action is necessary (Devault et al., 1988; Vijayaraghavan et al., 1990a; Wolz et al., 1991). In this report, we describe an improved procedure for the purification of PHIE and a study of its substrate specificity toward three different peptide families (ANF, tachykinins, and enkephalins). The data demonstrate that

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PHIE is a novel metalloendopeptidase distinguishable from bacterial thermolysin (Matthews et al., 1972) and from all presently described matrix-degrading metalloproteinases (Matrisian, 1990) and that it possesses properties different from those of both cell-surface, zinc-dependent hydrolases like ACE (Skidgel et al., 1987; Soubrier et al., 1988) and NEP (Kerr & Kenny, 1974a,b; Almenoff & Orlowski, 1984; Turner, 1987; Devault et al., 1987; Malfroy et al., 1987, 1988; Letartre et al., 1988) and from those of tetrameric meprin (Beynon et al., 1981; Kenny & Ingram, 1987; Kounnas et al., 1991; Dummermuth et al., 1991; Johnson & Hersh, 1992). Moreover, the observations made with related peptides of various lengths and homologous sequences indicate that the active site of this metallopeptidase requires an extended hexapeptide motif in a particular conformation.

MATERIALS AND METHODS

Materials

(19–28)ANF and related peptides, [Leu^5]enkephalin-related peptides, and [D-Arg^8]kermit (Kuks et al., 1989) were synthesized according to the solid-phase method (Merrifield, 1963), using an NPS 4000 semiautomated multisynthesizer (Neosystem, Strasbourg, France). Peptides were purified and analyzed by procedures including amino acid composition (Chang et al., 1983) using a Pico Tag station (Waters) and fast atomic bombardment mass spectrometry as previously described (Plevrakis et al., 1989; Nicolas et al., 1986).

α-ANF(5–28) from rat, PGLa, [Arg^0,Leu^5]enkephalin, and [Ala^{26}](21–28)ANF were purchased from Neosystem (Strasbourg, France). Substance P and its fragments, [Leu^5]-enkephalin, [Met^5]enkephalin, were from Sigma (St Louis, MO). Neurokinin A, RP 67580, CP-96345 (racemic product), β-(1–40) amyloid, and related peptides were obtained from Rhône-Poulenc Rorer Recherche-Développement (Vitry/Seine, France). Thermolysin from *Bacillus thermoproteolyticus* was from Serva (Le Perray-en-Yvelines, France). Neutral endopeptidase was kindly donated by Dr. G. Boileau (Montréal, Canada) and the tissue inhibitor of metalloproteinase (TIMP; Faucher et al., 1989) by Dr. A. Crespo (Rhône-Poulenc Rorer, Vitry/Seine). All other chemicals were commercially available reagent grade.

Enzyme Preparation

X. laevis peptide hormone inactivating endopeptidase (PHIE) was purified using an improved procedure based on a previously described method (Carvalho et al., 1992). Exudates were obtained from 40 *X. laevis*, diluted with 50 mM Tris-HCl buffer, pH 7.5, to a total volume approximating 250 mL, and dialyzed exhaustively against the same buffer. The dialyzed material was subjected to ion-exchange chromatography (DEAE Trisacryl M, IBF, Villeneuve-La-Garenne, France) and eluted with 50 mM Tris-HCl, pH 7.5. The enzyme emerged in the void volume. The active fractions were pooled, concentrated using Centriprep-30 concentrators (Amicon, Epernon, France), dialyzed against 10 mM potassium phosphate, pH 7.5, and applied on an hydroxyapatite Ultrogel column (IBF, Villeneuve-La-Garenne, France) equilibrated in 10 mM potassium phosphate, pH 7.5. The column was eluted with a 10–500 mM potassium phosphate, pH 7.5, gradient. Enzymatically active fractions were pooled, concentrated, desalted, and then, after addition of 1.5 M sodium chloride and 0.05% Nonidet P-40 (final concentrations), further fractionated by hydrophobic absorption chromatography on ω-aminoethylagarose (Sigma, St Louis, MO). Elution was carried out with a linear gradient ranging from 1.5 to 0 M sodium chloride in 0.05% Nonidet P-40 and 10 mM potassium phosphate, pH 7.5. Fractions containing metalloendopeptidase activity were eluted with 200 mM sodium chloride in 10 mM potassium phosphate, pH 7.5, and then collected, desalted, and concentrated. All columns were run at 4 °C. The purified enzyme was stored at 4 °C in 0.05% Nonidet P-40 and 10 mM potassium phosphate, pH 7.5. Under such conditions, the PHIE activity was stable for over 3 months. The enzyme preparation was found electrophoretically homogeneous under both nondenaturing and denaturing conditions using the Phast System (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) as described previously (Carvalho et al., 1992).

PHIE Assays

Hydrolysis of Peptides. All peptides were checked as possible substrates or inhibitors of PHIE. The routine assay mixture (final volume, 20 μL) contained 50 mM Tris-HCl, pH 7.5, 2–15 nmol of test peptide, and a standard aliquot of the pure enzyme known to hydrolyze 1 pmol of [Leu^5,Arg^6]-enkephalin per minute. The reaction at 37 °C was initiated by the addition of the enzyme. Samples were removed at successive times, and the reaction was stopped by heating 10 min at 100 °C. The entire sample was then fractionated at room temperature by reverse-phase HPLC (Milton Roy-Thermo Instruments, Orsay, France) on a Nucleosil 5-μm C18 column, 146 × 4.5 mm (SFCC-Shandon, Eragny, France), with the following linear gradients of acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 1 mL/min: 2–30% (over 15 min), 2–15% (over 15 min), or 10–20% (over 15 min) for ANF-related peptides, 2–40% (over 20 min) for [Leu^5]-enkephalin-related peptides and for [Met^5]enkephalin, 2–45% (over 20 min) for substance P-related peptides including neurokinin A and for β-(25–35)amyloid related peptides, 2–17% (over 26 min) for β-(1–40)amyloid protein, and finally 2–45% for PGLa (over 30 min). Isocratic elution (11% acetonitrile) was used for (24–28)ANF and (25–28)ANF. The absorbance of both substrates and products was monitored at 214 nm. Emerging peaks were collected, and the amino acid composition of the corresponding peptide fragments was determined to identify the cleavage site. [D-Arg^8]Kermit was used as a diaminobenzylthiocyanate (DABITC) derivative form as described (Kuks et al., 1989). All peptides were water soluble at 1 mg/mL except some amyloid and substance P fragments. β-(1–40)Amyloid was dissolved in 1 mM sodium hydroxyde, β-(31–35)amyloid and the β-(31–35)amyloid amide in 3 mM HCl, (5–11)substance P, (6–11)substance P, and (8–11)substance P in 25 mM acetic acid, and (7–11)substance P in 50 mM acetic acid. In these cases, controls were run in order to evaluate the possible effect of the particular solvents used on substance P cleavage by PHIE. No inhibition of the kinetic of cleavage was observed under the experimental conditions of the assay.

Kinetic Determinations. K_m and V_{max} values were determined under the following conditions: peptide substrates were incubated at final concentrations ranging from 8 to 60 μM ([Arg^0,Leu^5,Arg^6]enkephalin), 50 to 125 μM [β-(1–40)amyloid peptide], 50 to 300 μM ([Leu^5,Arg^6]enkephalin, [Leu^5,Lys^6]enkephalin, [Leu^5,Ile^6]enkephalin, [Arg^0,Leu^5]-enkephalin, PGLa), 100 to 400 μM (substance P, [Trp^{26}](21–28)ANF), 240 to 740 μM [(23–28)ANF, (22–28)ANF, (21–28)ANF, (20–28)ANF, [Leu^{26}](21–28)ANF, [Tyr^{26}](21–28)ANF, [Ile^{26}](21–28)ANF, (6–11)substance P], 40 to 100 μM [(19–28)ANF], 40 to 400 μM [(5–28)ANF, (5–11)sub-

stance P], 200 to 600 μM (neurokinin A), or 40 to 120 μM ([D-Arg^8]kermit) with the same final enzyme concentration (as described above) in 50 mM Tris-HCl, pH 7.5, at 37 °C. Incubations were stopped when, at times ranging from 30 min to 2 h, less than 10% of the substrates was digested. Where no significant degradation was observed after 2 h, longer incubation times (8–15 h) were used. The substrates and products were separated by HPLC. K_m and V_{max} were determined from initial velocity measurements plotted vs various substrate concentrations using the Lineweaver–Burk representation. In experiments involving substrates with oxidizable residues like Cys or Met, highly purified peptides were used and rapidly tested after the purification step and the shortest possible incubation times were used. Since no k_{cat} could be measured, relative efficacies were expressed in arbitrary units. They allow a comparison of the various peptide substrates within a set of experiments run with aliquots of the same enzyme preparation.

For inhibitors, the K_i values were measured by the following procedure: substance P, the reference, was incubated, at 37 °C, at different concentrations (ranging from 100 to 400 μM) with PHIE, both in the absence and in the presence of a peptide or nonpeptide inhibitor (40–500 μM) as described above. Initial velocities of the Gly^9–Leu^{10} bond hydrolysis were determined from the rate of formation of the amino-terminal substance P (1–9) fragment for both the control assay and that run in the presence of inhibitor. Inhibition constants were determined from the apparent K_m according to the Michaelis–Menten equation.

For the experiments with metal ions, PHIE activity was tested by the routine enzyme assay using [D-Arg^8]kermit (1 nmol) as substrate in the presence of a standard aliquot of PHIE (see above). The amount of cleavage (68%) obtained after 1 h of incubation was taken as 100% basis. For inhibition experiments, the incubation was preceded by preincubation of the enzyme with 375 μM *o*-phenanthroline for 30 min, and then the substrate was added. Under these conditions, 90% inhibition of the reference activity was attained. For reactivation experiments, the same standard assay was run, but, after preincubation, removal of the chelator (*o*-phenanthroline) was performed by 50-fold dilution of the sample followed by filtration and concentration through a Centriprep-30 ultrafiltration cartridge. Then, either Zn^{2+} (10 or 50 μM $ZnSO_4$), Ca^{2+} (10 or 50 μM $CaCl_2$), or Mn^{2+} (10 or 50 μM $MnCl_2$) was added to the assay, and the extent of cleavage was evaluated as usual after 1 h of incubation.

Tissue Inhibitor Metalloproteinase (TIMP) Assay

TIMP was purified from cells of calf aorta medial tissue maintained in culture (Faucher et al., 1989). The inhibitor was tested on the collagenolytic activity from a cell culture medium of rat alveolar macrophages activated by phagocytosis using labeled collagen. To evaluate TIMP activity on PHIE, the inhibitor (in a final concentration range from 50 μg·L^{-1} to 50 mg·L^{-1}) was added in the routine endopeptidase assay using substance P as substrate (see above).

Thermolysin and Neutral Endopeptidase Assays

Either thermolysin (160 μDMC-U) or NEP (30 ng) were incubated at 37 °C in Tris-HCl, pH 7.5, 50 mM final concentration, with either (24–28)ANF (14 nmol), (7–11)substance P (16 nmol), or [Leu^5]enkephalin (18 nmol) at 37 °C in a total volume of 16 μL. The reaction was stopped by heating at 100 °C at different incubation times. After centrifugation, the entire mixture was then analyzed by HPLC to assess product formation.

RESULTS

PHIE has previously been shown to possess a thermolysin-like character and to produce peptide bond cleavage on the amino terminus of hydrophobic residues of various biologically active peptides (Carvalho et al., 1992). In order to characterize this activity and to determine the kinetic parameters, we have used a pure preparation of PHIE to study its interactions with a series of peptides related to (5–28)ANF, substance P, and [Leu^5]enkephalin.

Hydrophobic Character of P'1 Amino Acid in PHIE Substrates. The importance of the amino acid residue situated on the carboxyl terminus of the scissile peptide bond, i.e., residue P'1, was analyzed using peptides of the ANF family. The carboxy-terminal octapeptide of (5–28)ANF, which includes the Ser^{25}–Phe^{26} doublet, was first shown to be a substrate for the enzyme (see below; Table II). A series of (21–28)ANF derivatives were synthesized in which the carboxyl amino acid of the scissile peptide bond was substituted. When P'1 was either a tyrosine, tryptophan, phenylalanine, isoleucine, or leucine residue, cleavage occurred with comparable K_m values in the range 143–600 μM (Table I). Relative efficacies were also found in the range (3–25) × 10^{-9} L·min^{-1}. In contrast, when Phe^{26} was replaced by an hydrophobic amino acid with a shorter side chain, like valine or alanine, the resulting octapeptides, i.e., [Val^{26}](21–28)ANF and [Ala^{26}](21–28)ANF, were no substrates but behaved as weak inhibitors ($K_i \geq 200$ μM) (Table I). Experiments with nonpeptide antagonists of substance P (NK1) receptor, i.e., RP 67580 (Garret et al., 1991) and CP-96345 (Snider et al., 1991), were also conducted as inhibitors of the Gly^9–Leu^{10} cleavage of substance P by PHIE. These synthetic plurifunctional nonpeptide molecules bearing aromatic hydrophobic side chains were found to compete with comparable efficiencies (K_i values of 190 and 820 μM were, respectively, measured) (Table IVb).

Interestingly, when two hydrophobic residues were adjacent in a peptide, the enzyme was found to cleave predominantly between the amino acid doublet, and minor (or no) cleavage was observed on the NH_2 terminus of the first residue. This was noticeable in the case of the Phe^7–Phe^8 cleavage of substance P and Phe^6–Phe^7 hydrolysis in somatostatin-14 [results shown in Carvalho et al. (1992)]. Similar observations were also made in the case of Phe^4–Leu^5 cleavage in dynorphin A(1–6), [Leu^5,Lys^6]enkephalin, and [Leu^5,Ile^6]enkephalin as well as the Phe^4–Leu^5 cleavage in [Arg^0,Leu^5,Arg^6]enkephalin (Table III) and Phe^{19}–Phe^{20} cleavage of β-(1–40)amyloid peptide (Glenner & Wong, 1984) (Table IVb). In contrast, cleavage occurred on the NH_2 terminus of the hydrophobic amino acid doublet only when these moieties occupied the carboxy terminus of the peptide as in [Arg^0,Leu^5]enkephalin (Gly^4–Phe^5 cleavage; Table III) or in [D-Arg^8]kermit (Ser^{12}–Phe^{13} cleavage; Table V). Similarly, cleavage at the Gly^{20}–Leu^{21} site of (19–28)ANF and the lack of cleavage of the same motif in (20–28)ANF showed that amino-terminal position of potential scissile doublet was not favorable to hydrolysis. This indicated that this endopeptidase did not exhibit any carboxy- or amino-peptidase activity.

PHIE Requires a Minimal Peptide Length for Substrate. In order to obtain preliminary information on the enzyme active site, the minimal peptide chain length required for substrate degradation was determined. The kinetic parameters obtained for the enzyme reaction with a series of ANF

Table I: Characterization of the P'1 Amino Acid in PHIE Substrates. Kinetic Parameters of the Cleavage by *X. laevis* PHIE of (21–28)ANF and (21–28)ANF Derivatives Substituted in Position 26[a]

peptide		K_m (μM)	K_i (μM)	V_{max} (pmol·min^{-1})	V_{max}/K_m (×10^{-9} L·min^{-1})
(21–28)ANF	L G C N S ↓ F R Y	571		2	4
[Leu^{26}](21–28)ANF	L G C N S ↓ L R Y	408		6	15
[Tyr^{26}](21–28)ANF	L G C N S ↓ Y R Y	600		2	3
[Trp^{26}](21–28)ANF	L G C N S ↓ W R Y	200		5	25
[Ile^{26}](21–28)ANF	L G C N S ↓ I R Y	143		2	14
[Val^{26}](21–28)ANF	L G C N S V R Y		200		
[Ala^{26}](21–28)ANF	L G C N S A R Y		ND		

[a] The arrow indicates the point of cleavage as determined by amino acid composition of the two generated fragments, separated by HPLC. When no arrow is shown, this indicated that the substrate was recovered intact at the end of the incubation period (see Materials and Methods for details). K_m and V_{max} were measured as described under Materials and Methods. Peptide substrates were incubated at final concentrations ranging from 240 to 740 μM [(21–28)ANF, [Leu^{26}](21–28)ANF, [Tyr^{26}](21–28)ANF, [Ile^{26}](21–28)ANF] and 100 to 400 μM [[Trp^{26}](21–28)ANF] with PHIE in 50 mM Tris-HCl, pH 7.5, at 37 °C. Since no k_{cat} could be measured, the efficacy values displayed in this table are expressed in arbitrary units and, as such, are purely comparative. When the peptide was not substrate, its capacity to inhibit the cleavage of substance P (as reference substrate) by PHIE was measured as the K_i value. [Val^{26}](21–28)ANF was tested at 500 μM. ND, not determined. K_i for [Ala^{26}](21–28)ANF was not determined because of its weak inhibitory capacity (K_i > 1 mM).

Table II: Kinetic Parameters of (5–28)ANF and ANF-Related Peptide Cleavage by *X. laevis* PHIE[a]

peptide		K_m (μM)	K_i (μM)	V_{max} (pmol·min^{-1})	V_{max}/K_m (×10^{-9} L·min^{-1})
(5–28)ANF	SSCFGGRIDRIGAQSG ↓ L G C N S ↓ F R Y	50		5	100
(19–28)ANF	SG ↓ L G C N S ↓ F R Y	91		2	22
(20–28)ANF	G ↓ L G C N S ↓ F R Y	513		4	8
(21–28)ANF	L G C N S ↓ F R Y	571		2	4
(22–28)ANF	G C N S ↓ F R Y	208		12	58
(23–28)ANF	C N S ↓ F R Y	370		1	3
(24–28)ANF	N S F R Y		410		
(25–28)ANF	S F R Y		420		
[Cys^{29}](24–29)ANF	N S ↓ F R Y C	ND		ND	ND
[des-Tyr^{28}](22–27)ANF	G C N S ↓ F R	ND		ND	ND
[Pro^{23}](23–28)ANF	P N S ↓ F R Y	ND		ND	ND

[a] K_m and V_{max} were measured as described under Materials and Methods. Peptide substrates were incubated at final concentrations ranging from 240 to 740 μM [(23–28)ANF, (22–28)ANF, (21–28)ANF, (20–28)ANF], 40 to 100 μM [(19–28)ANF], and 40 to 400 μM [(5–28)ANF] with PHIE in 50 mM Tris-HCl, pH 7.5, at 37 °C. (24–28)ANF and (25–28)ANF were tested at 300 μM. The large arrows indicate the major point of cleavage. The smaller arrow represents a minor cleavage point. ND, not determined.

Table III: Kinetic Parameters of [Leu^5]Enkephalin and [Leu^5]Enkephalin-Related Peptide Cleavage by *X. laevis* PHIE[a]

peptide		K_m (μM)	K_i (μM)	V_{max} (pmol·min^{-1})	V_{max}/K_m (×10^{-9} L·min^{-1})
[Leu^5]enkephalin	Y G G F L		250		
[Leu^5,Arg^6]enkephalin	Y G G F ↓ L R	79		10	126
[Arg^0,Leu^5]enkephalin	R Y G G ↓ F L	133		7	53
[Arg^0,Leu^5,Arg^6]enkephalin	R Y G G F ↓ L R	11		16	1454
[Leu^5,Lys^6]enkephalin	Y G G F ↓ L K	200		13	65
[Leu^5,Ile^6]enkephalin	Y G G F ↓ L I	167		10	60

[a] K_m and V_{max} were measured as described under Materials and Methods. Peptide substrates were incubated at final concentrations ranging from 8 to 60 μM ([Arg^0,Leu^5,Arg^6]enkephalin) and 50 to 300 μM ([Leu^5,Arg^6]enkephalin, [Leu^5,Lys^6]enkephalin, [Leu^5,Ile^6]enkephalin, [Arg^0,Leu^5]enkephalin) with PHIE in 50 mM Tris-HCl, pH 7.5, at 37 °C. [Leu^5]Enkephalin was tested at 300 μM. The arrow indicates the point of cleavage as in Table I.

fragments, ranging from 4 to 24 amino acids, are summarized in Table II. The data clearly indicated that the minimal size of the peptide substrates was six amino acids. No cleavage was observed with smaller peptides: both (24–28)ANF and (25–28)ANF were not substrates but inhibited the cleavage of substance P at the Gly^9–Leu^{10} and Phe^7–Phe^8 doublets. The same conclusion was drawn from studies conducted with [Leu^5]enkephalin (Table III) and substance P-related peptides (Table IVa). All the hexapeptides of the enkephalin family ([Leu^5,Arg^6]enkephalin, [Arg^0,Leu^5]enkephalin, [Leu^5,Lys^6]-enkephalin and [Leu^5,Ile^6]enkephalin) and [Arg^0,Leu^5,Arg^6]-enkaphalin were hydrolyzed by PHIE, whereas the pentapeptide [Leu^5]enkephalin was an inhibitor (Table III). The cleavage of [Met^5,Arg^6]enkephalin and the lack of cleavage of [Met^5]enkephalin by PHIE (results not shown) were also consistent with these findings. Cleavage of peptides in the

Table IV: Kinetic Parameters of *X. laevis* PHIE Reaction with Substance P, Substance P Fragments, and Neurokinin A (a) and with β-(1–40)Amyloid, β-(1–40)Amyloid Derivatives, RP 67580, and CP 96345 (b)[a]

substrate or inhibitor		K_m (μM)	K_i (μM)	V_{max} (pmol·min^{-1})	V_{max}/K_m (×10^{-9} L·min^{-1})
(a)					
substance P	R P K P Q Q F↓F G ↓L M (NH_2)	20		3	150
(5–11)substance P	Q Q F↓F G ↓L M (NH_2)	124		5	40
(6–11)substance P	Q F↓F G ↓L M (NH_2)	143		0.3	2
(7–11)substance P	F F G L M (NH_2)		31		
(8–11)substance P	F G L M (NH_2)		280		
neurokinin A	H K T D S F V G ↓L M (NH_2)	420		1	2
(b)					
β-(1–40)amyloid	DAEFRHDSGYEVHHQKLVF F–	125		19	152
	AEDVG S N K G A I I G↓L MVGGVV				
β-(25–35)amyloid	G S N K G A I I G L M		41		
β-(31–35)amyloid	I I G L M		47		
β-(31-35)amyloid(NH_2)	I I G L M (NH_2)		47		
RP 67580			190		
CP 96345			820		

[a] K_m and V_{max} were measured as described under Materials and Methods. Peptide substrates were incubated at final concentrations ranging from 100 to 400 μM [substance P, (6–11)substance P], 40 to 400 μM [(5–11)substance P], 200 to 600 μM (neurokinin A), and 50 to 125 μM [β-(1–40)amyloid peptide] with PHIE in 50 mM Tris-HCl, pH 7.5, at 37 °C. (7–11)Substance P and (8–11)substance P were tested at 250 μM, RP 67580 and CP 96345 at 200 μM, and β-(31–35)amyloid peptide, β-(31–35)amyloid (NH_2) peptide and β-(25–35)amyloid peptide at 40 μM. The arrows indicate the point of cleavage as in Table II.

enkephalin family was observed when the amino acid extension was on either the amino or carboxy terminus (i.e., [Arg^0,Leu^5]-enkephalin and [Leu^5,Arg^6]enkephalin) and was independent of the nature of the additional residues. Either a basic (Arg^6,Lys^6) or a hydrophobic, isosteric, Ile^6 residue could be added in position 6 (P′2) (Table III). These conclusions were strengthened by comparable results obtained with the ANF family. Data in Table II showed that the addition of a single residue to the amino or carboxy terminus of the pentapeptide, i.e., (24–28)ANF derivative, as in the case of [Cys^{29}](24–29)ANF and of (23–28)ANF, transformed the inhibitor into a substrate for the enzyme. Cleavage was also observed when proline was positioned at the amino terminus ([Pro^{23}](23–28)ANF; Table II) indicating that no free amino group was necessary for substrate recognition. Similarly, cleavage occurred at the Ser^{25}–Phe^{26} bond when Tyr^{28} was deleted from the carboxy terminus, i.e., in the case of [des-Tyr^{28}](22–27)ANF.

Similar results were obtained in the series of tachykinins since both the tetrapeptide and pentapeptide substance P derivatives, i.e., the (8–11) and (7–11) fragments, were not cleaved but behaved as inhibitors of PHIE. Cleavage was observed at the Gly^9–Leu^{10} bond in either the (6–11) and (5–11) fragments of substance P (Table IVa).

This feature was illustrated by another example in the β-amyloid peptide (Glenner & Wong, 1984) family. The (31–35) pentapeptide fragment of β-amyloid peptide which contains the Gly^{33}–Leu^{34} potential cleavage site for PHIE was not recognized as substrate but behaved as an inhibitor (K_i = 47 μM; Table IVb). Interestingly, the (31–35) amidated fragment of this peptide which is homologous to (7–11) substance P (Yankner et al., 1990) was found to compete efficiently with substance P for its Gly^9–Leu^{10} cleavage. K_i for both peptides were 47 and 31 μM, respectively.

Together these results suggested that peptide substrates for PHIE have a minimal length of six residues. Moreover, they showed that if this peptide backbone size requirement was fulfilled, except in the case where the potential scissile doublet occupied either the carboxy- or amino-terminal position, cleavage occurred independently of its position within the peptide chain.

In general, it was observed that the larger the peptide substrate used, the lower the K_m value attained. Indeed, K_m values decreased from 370 to 91 μM when in the ANF series the cleavage motif was elongated on the NH_2 terminus from six [(23–28)ANF] to ten amino acid residues [(19–28)ANF] (Table II). The lowest value (50 μM) was attained with the full-length (5–28)ANF substrate (Table II). In line with this observation was the fact that K_i values also decreased when comparing the (8–11) and (7–11) fragments of substance P (K_i, respectively, of 280 and 31 μM). Moreover, the best apparent affinity was attained with the largest enkephalin derivative, i.e., the heptapeptide [Arg^0,Leu^5,Arg^6]enkephalin (K_m = 11 μM; Table III).

These data demonstrated that PHIE requires for its catalytic activity an hexapeptide extended substrate, a property comparable to that of some metallopeptidases like meprin (EC 3.4.24.18), which tolerates only extended peptides (Butler et al., 1987).

However, in some peptides containing more than six residues, potential cleavable bonds are resistant to PHIE hydrolysis. For example, truncation of amino- and carboxy-terminal segments of β-(1–40)amyloid, resulting in the (25–35) fragment, prevented hydrolysis of this peptide at the Gly^{33}–Leu^{34} bond (Table IVb). In the tachykinin family, only Gly^8–Leu^9, and not Ser^5–Phe^6, cleavage was observed in neurokinin A (Table IVa). Similarly, the Ala^{17}–Leu^{18} bond of PGLa (Andreu et al., 1985) remained intact while the Lys^{12}–Ile^{13} doublet was cleaved (Table V).

The results suggested that, in addition to the minimal hexapeptide length and to an adequate P′1 amino acid, cleavage by PHIE may require a favorable peptide chain conformation.

PHIE, a Novel Metalloendopeptidase. In this section we will discuss the data which led to the conclusion that PHIE possesses particular properties which distinguish this novel enzyme from other known, and presently characterized, metalloendopeptidases which cleave peptide bonds at hydrophobic residues.

Table V: Kinetic Parameters of PGLa and D-[Arg^8]Kermit Cleavage by *X. laevis* PHIE[a]

peptide		K_m (μM)	V_{max} (pmol·min^{-1})	V_{max}/K_m ($\times 10^{-9}$ L·min^{-1})
PGLa	GMASKAGAIAGK↓IAKVALKAL (NH_2)	28	24	857
[D-Arg^8]kermit	D V D E R D V R G F A S ↓ F L (NH_2)	120	1	8

[a] K_m and V_{max} were measured as described under Materials and Methods. Peptide substrates were incubated at final concentrations ranging from 40 to 120 μM ([D-Arg^8]kermit) and 50 to 300 μM (PGLa) with PHIE in 50 mM Tris-HCl, pH 7.5, at 37 °C. The arrow indicates the point of cleavage as in Table I.

Table VI: Comparison of Thermolysin, Neutral Endopeptidase, and PHIE Reactivity toward Three Different Families of Pentapeptides: (7–11)Substance P, [Leu^5]Enkephalin, and (24–28)ANF[a]

	hydrolysis by		
peptide substrates	PHIE	neutral endopeptidase	thermolysin
(7–11)substance P	–	+	+
[Leu^5]enkephalin	–	+	+
(24–28)ANF	–	–	+

[a] The reactivity of three substrates, i.e., the (7–11) fragment of substance P, [Leu^5]enkephalin, and the (24–28) carboxy terminal segment of ANF toward three metallopeptidases, thermolysin, neutral endopeptidase, and *X. laevis* PHIE, was tested. The assay was conducted as described under Materials and Methods. When cleavage occurred, this was observed in (7–11)substance P at the Gly^9–Leu^{10} bond, in [Leu^5]enkephalin at the Gly^3–Phe^4 bond, and in (24–28)ANF at the Ser^{25}–Phe^{26} bond as determined by HPLC separation followed by identification of the corresponding generated fragments. The sign minus indicates that, under these experimental conditions, the peptide was recovered intact at the end of the incubation period.

The metallopeptidase character of the enzyme activity was previously shown by its marked sensitivity to EDTA, EGTA, and mainly to *o*-phenanthroline (Carvalho et al., 1992). This conclusion was reinforced by reactivation experiments of the enzyme by divalent cations, after inactivation (90% inhibition) by 375 μM *o*-phenanthroline. After removal of the chelator by 50-fold dilution of the sample followed by ultrafiltration, various divalent cations were tested for reactivation. It was found that either 10 or 50 μM Zn^{2+}, Ca^{2+}, or Mn^{2+} fully restored PHIE activity, confirming the essential role of this type of cation in the enzyme reaction.

Whereas a series of pentapeptide fragments [(24–28)ANF, [Leu^5]enkephalin, and (7–11)substance P] were not cleaved by PHIE but behaved as inhibitors of this enzyme, bacterial thermolysin (EC 3.4.24.4) produced cleavage at the level of the usual Ser^{25}–Phe^{26}, Gly^3–Phe^4, and Gly^9–Leu^{10} bonds, respectively (Table VI). NEP produced cleavage of the Gly^3–Phe^4 and Gly^9–Leu^{10} bonds in [Leu^5]enkephalin and (7–11)substance P but not of Ser^{25}–Phe^{26} in the pentapeptide derivative of ANF. Also, when compared with matrix metalloproteinases which are sensitive to tissue inhibitors like TIMPs (Matrisian, 1990), PHIE cleavage of substance P was insensitive to the action of this competitor (in a concentration range from 50 μg·L^{-1} to 50 mg·L^{-1}) whereas the tissue inhibitor was fully active on collagenase activity in control experiments. Finally, it is worth comparing PHIE with tetrameric meprin, another metalloendopeptidase with similar properties. PHIE exhibited distinct catalytic properties since it recognized hexapeptide substrates whereas meprin did not tolerate as substrates peptides shorter than octapeptides (Butler et al., 1987). Therefore, PHIE can be conclusively classified as a novel metalloendopeptidase with thermolysin-like activity and properties distinct from those of thermolysin, NEP (EC 3.4.24.11), meprin (EC 3.4.24.18), matrix metalloendoproteinases, and also from ACE (EC 3.4.15.1) (Carvalho et al., 1992).

PGLa, an antibacterial peptide present in skin secretion of *X. laevis* (Andreu et al., 1985) was cleaved by PHIE, with a low K_m (28 μM), at the Lys^{12}–Ile^{13} bond (Table V). This, together with the observation that in all peptide families presently described PHIE had great affinity for natural substrates [substance P, (5–28)ANF, and dynorphin A(1–6), 20, 50, and 79 μM, respectively], constitutes a good argument for its role *in vivo*.

DISCUSSION

The present data conclusively indicate that PHIE is a thermolysin-like endopeptidase possessing an hydrophobic S'1 subsite. They suggest the importance of a bulky hydrophobic side chain in the enzyme reaction. The inhibition of substance P cleavage by RP 67580 and CP-96345 reinforces the conclusion of a hydrophobic character of the enzyme S'1 subsite. Moreover, they show that although the enzyme could cleave between an Xaa–Xaa hydrophobic motif, as defined above; doublets of hydrophobic residues are preferentially hydrolyzed.

PHIE and several metalloendopeptidases have been proposed to be involved in the selective inactivation of peptide messengers. Some, like NEP, produce cleavage of peptide bonds on the amino side of hydrophobic amino acid residues and thus were classified under the generic term of thermolysin-like. Beside NEP, meprin, ACE, insulin-degrading enzyme, IDE (Müller et al., 1992), and matrix metalloproteinases seem to belong to related families although they exhibit quite distinct structural and enzymatical properties.

NEP (Turner, 1987), also called brain "enkephalinase" (Malfroy et al., 1978) and CALLA (common acute lymphocytic leukemia antigen), has generated considerable interest since opioid peptides, as well as other peptide messengers and small synthetic peptide substrates, were found to be hydrolyzed by this endopeptidase by a thermolysin-like cleavage (Almenoff et al., 1981; Matsas et al., 1983; Pozsgay et al., 1985). Clearly, the present data indicate that PHIE has properties that are similar to those of NEP and thermolysin but differs noticeably from these enzymes by a number of mechanistic features.

PHIE can be classified as distinct from NEP by the fact that it does not cleave small peptides, like the pentapeptide enkephalins, and that the canonical Xaa–Xaa hydrophobic or Xaa hydrophobic–Xaa hydrophobic sites can be cleaved only when present in a peptide backbone constitued of six, or more, amino acids. Moreover, it is clear that the action of both enzymes on the atrial natriuretic factor, i.e., (5–28)ANF, is different. Whereas NEP preferentially cleaves the Cys^7–Phe^8 bond situated in the cyclic portion of the peptide chain (Kenny & Stephenson, 1988), PHIE produces only a single cleavage at the level of Ser^{25}–Phe^{26} motif. Interestingly, some authors also reported a minor NEP cleavage of this bond (Olins et al., 1989) which in our hands was not observed with pure recombinant NEP when (24–28)ANF was used as substrate. Moreover, whereas both NEP and PHIE produced a major cleavage of substance P at the Gly^9–Leu^{10} bond (Matsas et

al., 1983), PHIE was unable, unlike NEP, to hydrolyze this peptide bond in fragments shorter than six amino acid residues. Since this difference was also observed with bacterial thermolysin, it is reasonable to conclude that PHIE possesses a thermolysin-like character with strict selectivity for hydrolyzing peptide bonds on the amino side of hydrophobic bulky side chain amino acid residues in an extended peptide substrate.

Finally, it should be recalled that the classical NEP inhibitors like phosphoramidon, thiorphan, retrothiorphan, kelatorphan, and acetorphan inhibited PHIE activity at concentrations 100–1000-fold higher than those required for their action on NEP (Carvalho et al., 1992). However, it is clear that since PHIE was inhibited by the classical NEP pentapeptide substrates of the enkephalin family, their respective active sites as well their structure may share some similarity. Elucidation of PHIE sequence will assess the extent of this homology. Noticeably, the fact that PHIE could be reactivated after inhibition by divalent cation chelators reinforces this assumption, although the exact nature of the actual cation present in the native form of the enzyme could not be established unequivocally.

To some extent, the endoproteolytic properties of PHIE could be compared with those of the matrix metalloproteinases (MMPs) and with those of meprin (EC 3.4.24.18). However, they display a number of striking differences which demonstrate that they belong to functionally and mechanistically distinct peptidase families. The matrix metalloproteinases, like collagenases, stromelysins, and punctuated metalloproteinase, act on the extracellular matrix and protein components of basal membrane. Since we tested in this study only peptide, and not protein, substrates, substrate specificity cannot be directly compared. But PHIE is clearly distinguishable from the MMPs by its resistance to the tissue inhibitor of MMPs (TIMP).

Whereas meprin (EC 3.4.24.18) has a strong preference for peptide bonds flanked by an hydrophobic residue but tolerates a negatively charged side chain in position P′1 (Butler et al., 1987), PHIE has a strict requirement for the hydrophobic character of P′1 residue. Moreover, dynorphin A(1–6), one of the best substrates for PHIE (K_m = 79 μM), is not hydrolyzed by meprin (Kenny & Ingram, 1987), which requires an extended octapeptide chain for substrates (Butler et al., 1987). In addition, although NEP may tolerate Ala (Pozsgay et al., 1986) or Val (Kerr & Kenny, 1974a) in P′1, it is noteworthy that PHIE could not cleave an ANF-related motif in which the Phe^{26} was replaced by Ala or Val but recognized these octapeptides as weak inhibitors. These features could well reflect some discrete differences in the nature and the size of the S′1 enzyme hydrophobic pocket.

Because of the biological importance of its identified substrates, ACE (EC 3.4.15.1) was intensively analyzed by various authors. This metallopeptidase is inhibited by low concentrations of captopril, a compound which has no effect on PHIE up to millimolar concentrations. Indeed, ACE was found to cleave both Phe^7–Phe^8 and Phe^8–Gly^9 bonds in substance P (Yokosawa et al., 1983) whereas PHIE cleaved the former but not the latter, the major cleavage being performed at the Gly^9–Leu^{10} level. Together, these data clearly established this enzyme as a novel metalloendopeptidase with distinct properties.

It is generally accepted that most endopeptidases exhibit a selectivity that is primarily governed by the nature of the amino acids linked by the hydrolyzed peptide bond and by the kind of interactions that they establish with the enzyme subsites. However, there is growing evidence that a higher order of peptide backbone organization may contribute in some fashion to protease selectivity. Indeed, β-turns (Brakch et al., 1989) and α-helices (Resnick et al., 1991), as examples, were shown to participate in substrate recognition by processing enzymes, and it can be anticipated that these secondary structure features are only part of the overall complexity of the catalytic process. The observations here reported unambigously indicated that the presence of the proteolytically cleavable canonical doublet, although a prerequisite, is not sufficient *per se*. Physicochemical approaches on the actual secondary and tertiary organization of these peptide substrates are needed to settle the basis for an understanding of these mechanisms and to evaluate the relative importance of the nature of the amino acid side chains and of the three-dimensional substrate conformations. However, it can be hypothesized that although it probably shares common features, the active site of PHIE may exhibit a different level of complexity than those for NEP and for ACE.

X. laevis skin secretions constitute an extraordinary "endocrine" and "neuroendocrine" model which molecular complexity has not been entirely elucidated [reviewed in Bevins and Zasloff (1990) and Nicolas et al. (1992)]. Its enzyme potential has only recently started to be deciphered (Mizuno et al., 1986; Mollay et al., 1986a,b; Kuks et al., 1989; Darby et al., 1991; Resnick et al., 1991). PHIE constitutes a new enzyme for which the actual physiological substrate(s) have not been unequivocally indentified. The question can be raised of the significance of PHIE in the batracian skin secretions. It is tempting to speculate that its presence in such a biological secretion, particularly rich in peptides, must correspond to a determined function. Indeed, the fact that PGLa, a well-established antimicrobial peptide marker of these secretions, was a good substrate for PHIE suggests a possible role of that enzyme in its inactivation. This must await the identification of the expected PGLa products, which in the case of magaininase (Resnick et al., 1991) were characterized (Gibson et al., 1986; Giovannini et al., 1987) in parallel with the *in vitro* properties of the enzyme. But, it can be tentatively proposed that PHIE might, because of a broader range of peptide selectivity, participate in the inactivation of a great number of peptide messengers, some not as yet identified. For now, it can be envisioned that its affinity and specificity for peptide substrates of higher organisms may indicate that there are closely related endopeptidases present in mammals. Preliminary observations on various human tumor cell lines suggesting the presence of enzymes of similar specificity (Delporte et al., 1992; Carvalho et al., 1993) may further support the idea that, together with PHIE, they may represent specimen of a novel endopeptidase family related to NEP and thermolysin.

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