An internally quenched fluorogenic substrate of prohormone convertase 1 and furin leads to a potent prohormone convertase inhibitor

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Based upon the observed cleavage of various peptidyl substrates by the recombinant prohormone convertases PC1 and furin, an intramolecularly quenched fluorogenic peptidyl substrate, (*o*-aminobenzoyl)-Lys-Glu-Arg-Ser-Lys-Arg-Ser-Ala-Leu-Arg-Asp-(3-nitro)Tyr-Ala, was synthesized. In spite of the distance (approx. 33 Å) separating the fluorescent donor/acceptor pair, the highly fluorescent *o*-aminobenzoyl group is efficiently quenched by long-range resonance energy transfer to the (3-nitro)Tyr moiety. Both recombinant human PC1 and human furin recognize and cleave specifically this substrate at the expected Arg-Ser site in a sensitive manner. The K_m values for

human PC1 and human furin were 17 μ M and 30 μ M respectively, with V_{max} values of 6.4 μ M/h and 18 μ M/h. These values differ significantly from those obtained when using a 7-amino-4-methylcoumarin-containing pentapeptidyl substrate where, for similar K_m values, the V_{max} values were much lower. The peptide sequence was used to synthesize another peptide incorporating a ketomethylene arginyl pseudopeptide bond. This compound proved to be a potent competitive inhibitor of both human PC1 and human furin, displaying K_i values of 7.2 μ M and 2.4 μ M respectively.

INTRODUCTION

Nowadays, it is customary to study the endoproteolytic activity and specificity of endoproteases by measuring the level of products resulting from the enzymic digestion of a suitable peptidyl and/or protein substrate. A number of technical approaches have been proposed and these can be classified into four major categories. The first includes the use of various chromogenic or fluorogenic substrates, such as peptidyl 7-amino-4-methylcoumarin (AMC) [1]. The second group relies on the release into solution of a radiolabelled reporter group from an immobilized substrate, as we [2–4] and others [5–7] have done in the past. The third group is based upon reverse-phase (RP)-HPLC in order to both separate the released peptides from the original substrate and quantify the amount released [8–10]. Finally, the last group involves the synthesis and use of intramolecular quenched fluorogenic substrates [11,12]. These fluorogenic substrates, allowing for the inclusion of amino acids on either side of the enzymically cleaved bond, are characterized by the presence of a fluorescent group at one end of the substrate and an acceptor group placed near the other end. According to the concept developed by Yaron et al. [11], the substrate fluorescence is initially quenched by intramolecular energy transfer between the donor and acceptor but is recovered following enzymic cleavage of any peptide bond between the two mutually interacting groups. The extent of fluorescence quenching and

hence the magnitude of the increase following cleavage is dependent upon the distance between the two groups and their spectral overlap. In recent years, numerous donor/acceptor pairs have been proposed; these include *o*-aminobenzoyl (Abz)/3-nitrotyrosine [12], 5-(2-aminoethyl)aminonaphthalene-1-sulphonyl (Edans)/4-(4-dimethylaminophenylazo)benzoyl (Dabcyl) [13], (7-methoxycoumarin-4-yl)acetyl (Mca)/2,4-dinitrophenyl (Dnp) [14], Abz/ethylenediamine dinitrophenyl (EDDnp) [15], Trp/Dansyl [16] and Trp/Dnp [17]. Amongst these, the Abz/3-nitrotyrosine pair appears to represent a good compromise in terms of ease of synthesis (by solid-phase peptide synthesis) and efficient quenching.

So far, members of the newly discovered prohormone convertase family (reviewed in [18–20]), to which belong human prohormone convertase 1 (hPC1) and human furin, have been studied in terms of proteolytic activity and specificity either using methods belonging to the first group described above or following analysis of cellular co-expression of enzymes together with various prohormone substrates. Whereas the use of small fluorogenic substrates is convenient, rapid and sensitive, since the released aromatic amine has a relatively high fluorescence quantum yield, it does not allow determination of the role played by amino acids located C-terminally to the scissile bond. Similarly, the use of cellular co-expression of enzyme and substrate renders difficult the determination of kinetic constants even though this technique has been used extensively to demonstrate

Abbreviations used: Abz, *o*-aminobenzoyl or anthranoyl; AMC, 7-amino-4-methylcoumarin; AOGO, 4-oxo-8-tosylguanidino-octanoic acid; Boc, *t*-butyloxycarbonyl; Bzl, benzyl; Dabcyl, 4-(4-dimethylaminophenylazo)benzoyl; Dnp, 2,4-dinitrophenyl; EDDnp, ethylenediamine dinitrophenyl; Edans, 5-(2-aminoethyl)aminonaphthalene-1-sulphonyl; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GEMSA, guanidinoethylmercaptosuccinic acid; HMP resin, 4-hydroxymethylphenoxymethyl resin; Mca, (7-methoxycoumarin-4-yl)acetyl; MCA, 4-methylcoumarin-1-yl-1-amine; m.u., mass unit; Nsu, *N*-succinimidyl; PAM resin, phenylacetamidomethyl resin; PC1, prohormone convertase 1 (the prefix h or m indicates human or murine PC1 respectively); hPTH, human parathyroid hormone; RP-HPLC, reverse phase HPLC; QFS, quenched fluorescent substrate; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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the cleavage specificity of both PC1 and furin [18]. Finally, *in vitro* studies using small peptidyl substrates [7,21], small peptidyl substrates containing a P1' substitution [22] and larger protein substrates such as human proinsulin [23], anthrax-protective antigen [24] and HIV envelope glycoprotein precursor (gp160) [25], all indicated that amino acids located C-terminally to the scissile bond, in addition to the presence of the basic amino acids and other factors, could play an important role during the cleavage process.

In this paper, we use a model peptide [hProPC1-(77-90)], corresponding to the junction between the pro and mature human PC1 enzyme in the zymogen molecule, in order to assess the usefulness of both an internally quenched fluorescent peptidyl substrate and an analogous peptide in which a ketomethylene pseudopeptide bond is introduced at the Arg-Ser cleavage site.

EXPERIMENTAL

Materials

For fluorometric assays, the pentapeptide pGlu-Arg-Thr-Lys-Arg-4-methylcoumaryl-1-amide (MCA) was purchased from Enzyme System Products, Livermore, CA, U.S.A. The synthetic peptides insulin-like growth factor-II (fragment 33-40), rabbit protein kinase inhibitor, fibroblast growth factor (fragment 106-120), protein kinase C substrate, des-His¹[Glu⁹]glucagon amide and myelin basic protein (fragment 68-82) were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The 7B2 peptide (fragment 156-171) was synthesized in this laboratory, as were all of the ProPC1 peptides according to previously described procedures [21,22]. The synthetic tridecapeptide [hProPTH-(-6-+7); hPTH = human parathyroid hormone] was kindly synthesized and purified by Dr. S. Sakakibara and Dr. T. Kimura (Peptide Institute, Osaka, Japan) and was a gift from Dr. G. N. Hendy (McGill University). The 9-fluorenylmethoxycarbonyl-*O*-*N*-succinimidyl (Fmoc-*O*-Nsu) and the preloaded Fmoc-Ala-4-hydroxymethylphenoxy (HMP) resin were purchased from Novabiochem, La Jolla, CA, U.S.A., while the *t*-butyloxycarbonyl (Boc)-anthranilic acid and the (3-nitro)Tyr were obtained from Bachem California, Torrance, CA, U.S.A. and Aldrich Chemical Co., Milwaukee, WI, U.S.A., respectively.

Peptide synthesis was carried out on an automated peptide synthesizer (Model 431A; Applied Biosystems, Mississauga, Ont., Canada) using procedures already described [21,22].

The two human endoproteases, hPC1 and human furin, were obtained from the medium of somatomammotroph GH₄C₁ cells following infection with the respective recombinant Vaccinia virus as described [21]. The two proteinases were partially purified through anion-exchange chromatography prior to use and their enzymic activities were determined using a fluorometric assay as reported [21].

Chemical synthesis of the internally quenched fluorogenic substrate (QFS-1)

The Fmoc-(3-nitro)Tyr was prepared by condensation between Fmoc-*O*-Nsu dissolved in acetonitrile and a solution of (3-nitro)Tyr in buffered triethylamine, pH 8.5, at room temperature; the reaction was followed by TLC using silica gel precoated aluminium sheet with a chloroform/methanol (4:1, v/v) elution system and continued until disappearance, at approx. 1 h, of the (3-nitro)Tyr. The final product (R_f 0.44) was purified and recovered using the protocol previously described [26] in an overall 95 % yield. The fast-atom bombardment (FAB)-MS [m/z 471 ($M^+ + Na$)] and the ¹H-NMR analyses confirmed its identity.

Following coupling of Fmoc-(3-nitro)Tyr to the Ala-HMP resin, elongation of the peptide chain was accomplished using Fmoc solid phase peptide chemistry until the last step, whereupon Boc-anthranilic acid was added. The peptide derivative was cleaved from the resin and deprotected by treating the resin for 3 h with reagent K [27], followed by lyophilization and repeated washings with ether. The crude material was purified by RP-HPLC, using first a semipreparative CSC-Exsil C₁₈ column (25 cm × 0.94 cm; Chromatography Sciences Co., St. Laurent, Québec, Canada) and then an identical analytical column (25 cm × 0.46 cm). The buffer system consisted of an aqueous 0.1 % (v/v) trifluoroacetic acid (TFA) solution and an organic phase consisting of acetonitrile also containing 0.1 % TFA (v/v). The elution was carried out with a linear gradient from 5 to 60 % organic phase in 60 min following a 5 min isocratic step at 5 % organic phase; the flow rate was either 2.0 ml/min (semipreparative) or 1.0 ml/min (analytical). The elution was monitored on-line by UV-absorbency at 225 nm, by measuring the fluorescence emitted at 420 nm in an automatically collected 1 ml fraction by excitation at 320 nm or with an on-line Fluorichrom (Varian Ass.) fluorescence detector. In the latter case, a combination of band filters (300-380 nm) was used for excitation of the fluorophor, whereas a sharp cut-off filter (> 400 nm) was used in the emission window. The purified peptide (R_t 28.5 min) was characterized by amino acid analysis yielding the following composition: Asx_{1.0} (1), Ser_{1.7} (2), Glx_{1.0} (1), Ala_{1.8} (2), Leu_{1.0} (1), Lys_{1.8} (2), Arg_{3.4} (3) and (3-nitro)Tyr_{1.0} (1), and by FAB-MS analysis yielding a molecular ion of m/z 1743.4 ($M^+ + H$) together with numerous identifiable fragmentation peaks.

As a side product of the synthesis of the internally quenched substrate, a peptide, named FS-1, containing the anthranilic acid but devoid of the (3-nitro)Tyr, was obtained and, upon an overnight incubation in the presence of hPC1, did not yield any further increase in fluorescence. Its structure was fully characterized by FAB-MS [m/z 1536 ($M^+ + H$)] and amino acid composition: Asx_{1.1} (1), Ser_{2.0} (2), Glx_{1.2} (1), Ala_{2.1} (2), Leu_{1.0} (1), Lys_{2.0} (2) and Arg_{3.3} (3).

Chemical synthesis of the isostere-containing inhibitor

The inhibitor was synthesized using the automated standard protocol optimized for Boc chemistry on a preloaded Asp-phenylacetamidomethyl (PAM) resin (Applied Biosystems, Mississauga, Ont., Canada). The α -protected ketomethylene amino acid, Boc-4-oxo-8-tosylguanidino-octanoic acid (AOGO), prepared as previously described [28], was incorporated into the polymer-bound peptide by coupling mediated by dicyclohexylcarbodi-imide/*N*-hydroxybenzotriazole. The cleaved and deprotected peptide was recovered following HF treatment. The crude material was purified to homogeneity using RP-HPLC as described above. The two major products were characterized by amino acid analysis and FAB-MS.

Digestion of synthetic peptidyl substrates

Incubations with the various peptidyl substrates were conducted overnight using a fixed amount of partially purified hPC1 and/or human furin at 25 °C in a total volume of 350 μ l; the final incubation mixtures contained 50 mM sodium acetate, 5.0 mM CaCl₂, pH 5.5, for hPC1; and 50 mM sodium acetate, 2.0 mM CaCl₂, pH 7.0, for furin. Following incubation, aliquots were acidified using 50 μ l of glacial acetic acid and analysed by RP-HPLC as previously described [21]. Identification and quantification of the released fragments were accomplished by performing amino acid analysis on each isolated fraction.

Determination of kinetic constants

Initially, the FS-1 peptide was used in order to derive a fluorescence/amount calibration curve. Later on, complete digestion of the substrate, QFS-1, was carried out by overnight incubation, and subsequent calculations were based upon the integrated peak area of the N-terminal fluorescent product after RP-HPLC separations. Incubations for kinetic constant determinations were typically carried out in the presence of various concentrations (based on weight and uncorrected for peptide content) of the substrate at 25 °C for 3.5 h before stopping the reaction by addition of 10 mM EDTA. In each case, the amount of enzymic activity used was similar: 0.90 μ M of AMC released/h with hPC1 and 1.01 μ M of AMC released/h with human furin, as determined using 50 μ M pGlu-Arg-Thr-Lys-Arg-MCA in 50 mM sodium acetate, pH 6.0 or 7.0, containing 5 or 2 mM CaCl₂. The values of K_m and V_{max} were determined using either the Enzfitter software (Elsevier Science Publishers, Amsterdam, Holland) or the hyperbole curve-fitting algorithm of the SigmaPlot for Windows V1.02 (Jandel Scientific, San Rafael, CA, U.S.A.).

The synthetic inhibitor at various concentrations (determined by amino acid analysis) was preincubated with hPC1 (2.5 μ M of AMC released/h using 66 μ M fluorogenic substrate) or furin (3.4 μ M of AMC released/h using 66 μ M fluorogenic substrate) for 30 min at 25 °C in 50 mM sodium acetate, pH 6.0 or 7.0, containing 5 or 2 mM CaCl₂ respectively. At the end of the preincubation period, the reaction was initiated by rendering the solution 66 and 133 μ M in pGlu-Arg-Thr-Lys-Arg-MCA. The amount of AMC released relative to the control (done in parallel but without inhibitor) was measured after a 2 h (hPC1) or 4 h (furin) period at 25 °C. All fluorescence measurements were made with a Perkin-Elmer MPF-3L spectrofluorimeter using an excitation wavelength set at 370 nm and an emission wavelength set at 460 nm. The K_i values were determined graphically as described [29].

RESULTS

Digestion of synthetic peptidyl substrates by hPC-1

In order to further assess the validity of using the ProPC1 model peptide, as done previously [21], we compared its cleavage efficiency with a variety of other synthetic peptides incorporating different putative cleavage sites. As can be seen in Table 1, partially purified recombinant hPC1 is able to cleave Lys-Arg-, Arg-Lys- and Arg-Arg-containing sites to various degrees. These results further confirm the data derived from *in vitro* studies of digestion of small fluorogenic substrates by recombinant PC1, and also indicate that, in addition to the pair of basic residues, the enzyme is able to discriminate between different sequences. As previously reported [22], it is worth noting that substitution of the P1' position using unnatural amino acids leads not only to reduced cleavage but also to competitive inhibition when enzymic activity is assayed using the fluorogenic substrate. In addition to the major cleavage reactions observed and listed in Table 1, further cleavages were sometimes observed and shown to be due to a carboxypeptidase activity able to remove C-terminal basic residues which can be inhibited by addition of guanidinoethylmercaptosuccinic acid (GEMSA) (results not shown). Based on these results, it appeared that both the tridecapeptide hProPTH-(−6+7) and the decapeptide hProPC1(80–88), also well recognized and cleaved by human furin (results not shown) represent good substrates for the further study of the enzymic activity of hPC1 and human furin.

Table 1 Cleavage of synthetic peptidyl substrates by recombinant hPC1

An identical amount of each peptidyl substrate, solely on a weight basis and not corrected for the peptide content, was incubated overnight at pH 5.5 as described in the Experimental section. The extent of cleavage was determined following RP-HPLC separation of the digest and quantification by amino acid analysis of the peptide content of each released fragment. The various residues (Xaa) contained in compound **IX** are described in ref. [22]. rProPC4A, rat prohormone convertase 4A; IGF-II, insulin-like growth factor-II; FGF, fibroblast growth factor.

Compound	Total number of residues	Putative cleavage site (−6 to +5)	Extent of cleavage (%)
I hProPTH-(−6+7)	13	KSVKKR↓SVSEI	100
II [Tyr ¹]mProPC1-(80–88)	10	YRSKR↓SVQKD	100
III [Tyr ¹ , Leu ⁸]mProPC1-(80–88)	10	YRSKR↓LVQKD	100
IV [D-Tyr ¹]rProPC4A-(76–91)	17	RRRVKR↓SLVVP	86
V IGF-II-(33–40)	8	SRVSRR↓SR	76
VI Protein kinase inhibitor	20	GRTGRR↓NAIHD	28
VII FGF-(106–120)	15	YRSRK↓YSSWY	23
VIII Protein kinase C substrate	9	VRKR↓TLRRL	21
IX [Tyr ¹ , Xaa ⁸]mProPC1-(80–88)	10	YRSKR↓XVQKD	5–15
X des-His ¹ [Glu ⁹]Glucagon-NH ₂	28	YLDSTR↓AQDFV	0
XI Myelin basic protein-(68–92)	15	PQKSQR↓SQDEN	0
XII m7B2 protein-(156–171)	16	YLQGKR↓LDNVV	0
XIII hPC1-(706–726)	21	KHRDDR↓LLQAL	0

Preparation and application of the internally quenched fluorescent substrate

Given that, at least for furin, the minimal recognition sequence is four residues including an Arg at both the P4 and P1 positions, we decided to synthesize a substrate in which the acceptor/donor pair were as far as possible from each other and yet still able to interact. Such compounds, in which the acceptor/donor pair are separated by more than 18 Å (approx. six amino acids), have been prepared and used successfully [12]. An identical approach was used herein to prepare a peptide incorporating both a fluorescent donor group, the anthranilic acid moiety, and an acceptor group, the (3-nitro)Tyr group, separated by 11 residues and encompassed within the peptidyl sequence corresponding to the native hProPC1 sequence (residues 77–90). This substrate, as shown in Figures 1(a) and 1(b), contains one substitution, namely Ser⁸⁹ to Tyr, when compared with the native sequence.

Upon incubation with partially purified recombinant hPC1 for 18 h, and as shown by separation of the products by RP-HPLC, the substrate is completely digested and its fluorescence spectrum indicates a 3.85-fold increase in fluorescence over that obtained using an identical amount of undigested substrate. This is shown in Figure 2, which illustrates the fluorescence of both the starting product and the final product resulting from complete digestion. As shown in Figure 3, this substrate is cleaved at the expected site, with production of the fluorescent N-terminal fragment Abz-Lys-Glu-Arg-Ser-Lys-Arg (peaks labelled c and c'); it is worth noting that a small amount of the C-terminally truncated Abz-Lys-Glu-Arg-Ser-Lys can sometimes be detected, since no GEMSA was added (results not shown). In addition, production of the non-fluorescent but UV-absorbing C-terminal fragment, Ser-Ala-Leu-Arg-Asp-(3-nitro)Tyr-Ala (peak labelled b) can be seen, as well as the fluorescent starting product (peaks labelled a and a').

As previously reported [12], the fluorescence of the undigested substrate (peak a') was expected, since the donor/acceptor pair are located at a significant distance from each other, thus lessening the quenching effect. Consequently the significant background fluorescence of the starting product (as seen in

Figure 1 Structural representations of the intramolecularly quenched fluorogenic substrate QFS-1 and its related isostere-containing inhibitor

(a) Amino acid sequence corresponding to the junction between the pro and mature human PC1 enzyme in the zymogen molecule (positions 77-90). (b) Amino acid sequence of the QFS-1 peptidyl substrate incorporating the donor/acceptor pair at positions 77 and 89. (c) Isostere-containing inhibitor with an Argψ(COCH₂)-Gly moiety replacing the Arg-Ser dipeptide and corresponding to positions 76-88 of the proPC1 zymogen molecule.

Figure 2) and the calibration curve based upon the fluorescence of the isolated side product (FS-1), Abz-Lys-Glu-Arg-Ser-Lys-Arg-Ser-Ala-Leu-Arg-Asp-Ala, a much longer peptide than the actual product, lead to imprecision in the determination of kinetic constants. Both problems can be circumvented by measuring initial velocities or performing stopped-time assays coupled with RP-HPLC and by the use of the proper peptide, Abz-Lys-Glu-Arg-Ser-Lys-Arg, for calibration purposes. Using the first method and deducing the background from the observed fluorescence, hPC1 exhibits an estimated K_m and V_{max} of $16 \pm 5 \mu\text{M}$ and $2.8 \pm 0.3 \mu\text{M/h}$ respectively. Using the second procedure we obtained more precise results (Figure 4a), namely a K_m and V_{max} of $17 \pm 2 \mu\text{M}$ and $6.4 \pm 0.3 \mu\text{M/h}$ (yielding a $V_{\text{max.}}/K_m$ ratio of 0.38) respectively for hPC1, compared with $30 \pm 7 \mu\text{M}$ and $18 \pm 2 \mu\text{M/h}$ ($V_{\text{max.}}/K_m$ of 0.60) for human furin. Interestingly, as shown in Figure 4(b) and indicated in Table 2, the pGlu-Arg-Thr-Lys-Arg-MCA substrate displayed similar K_m values for both furin ($5.9 \pm 0.7 \mu\text{M}$) and hPC1 ($23 \pm 3 \mu\text{M}$) but between 3- and 6-fold lower values of $V_{\text{max.}}/K_m$, with $V_{\text{max.}}$ values of $1.23 \pm 0.03 \mu\text{M/h}$ and $1.32 \pm 0.05 \mu\text{M/h}$ respectively. Similar results have also been obtained with furin and hPC1 digestion of the synthetic tridecapeptide hProPTH-(6-7), where com-

Figure 2 Fluorescence emission spectra of $35 \mu\text{M}$ undigested QFS-1 substrate and $35 \mu\text{M}$ N-terminal cleavage product

The fluorescence emission spectrum of undigested QFS-1 was obtained in assay buffer (solid line) and shows an emission maximum at 420 nm with a relative fluorescence intensity of 1.13. Following an overnight incubation of QFS-1 with hPC1, resulting in complete hydrolysis of the substrate as shown by RP-HPLC analysis (see Figure 3), the emission spectrum of the fluorescent N-terminal moiety was recorded (broken line) showing a relative fluorescence intensity of 4.34 at 420 nm, thus yielding a quenching factor of 3.84.

Figure 3 Representative RP-HPLC chromatograms showing the separation of the products of digestion of the internally quenched fluorogenic substrate QFS-1 by hPC1

The elution profile was obtained by monitoring UV absorbance at 225 nm and fluorescence emission with a Fluorichrom on-line detector. Identification of the peaks was accomplished following amino acid analysis of the recovered fraction and elution was carried out as described in the Experimental section. Thus peaks a and a' correspond to undigested QFS-1, peak b corresponds to the non-fluorescent C-terminal moiety and peaks c and c' correspond to the fluorescent N-terminal fragment.

parable low micromolar K_m values were determined together with V_{max} values even higher than those obtained here with the QFS-1 substrate (G. N. Hendy, H. P. J. Bennett, B. F. Gibbs, C. Lazure, R. Day and N. G. Seidah, unpublished work).

Figure 4 Determination of kinetic constants exhibited by hPC1 for QFS-1 following RP-HPLC and for the pentapeptidyl fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA

(a) Fluorescence data associated solely with the fluorescent N-terminal fragment were obtained following RP-HPLC separation and converted to $\mu\text{M}/\text{h}$ values by using the fluorescence value obtained following 100% cleavage by hPC1. (b) Determination of kinetic constants for the pentapeptidyl fluorogenic substrate was accomplished by measuring directly the fluorescence emitted at 460 nm by the released AMC group. In each case, the inset figure shows the linear release of either fluorescent product released from 35 μM QFS-1 (a) and from 35 μM pGlu-Arg-Thr-Lys-Arg-MCA (b) over a 5 h time period using the conditions described in the Experimental section.

Preparation and use of the isostere-containing inhibitor

We have shown recently that substitution of the P1' position by unnatural amino acids leads to peptides displaying inhibition at micromolar concentrations when assayed with human furin and/or hPC1 [22]. However, these peptides are also substrates, as they are slowly hydrolysed by both enzymes [22]. This study suggested that a scissile bond rendered resistant to proteolysis,

Figure 5 Representative RP-HPLC chromatogram showing the purification of the isostere-containing peptidyl inhibitor

The elution was carried out as described in the Experimental section and was monitored by measuring the UV absorbance at 225 nm. Each fraction was collected manually and identification of the products was accomplished by amino acid analysis and by FAB-MS. The proposed structures of the ketopiperazine-containing derivative present in the peak labelled A and of the isostere-containing inhibitor present in the peak labelled B are shown.

either by using other P1' substituents as we have recently done (A. Basak, C. Schmidt, A. A. Ismail, N. G. Seidah, M. Chrétien, and C. Lazure, unpublished work) or by modifying the P1-P1' peptide bond itself, may eliminate this problem and lead to a better inhibitor. Furthermore, based on the studies using the tridecapeptide hProPTH-(−6+7) and the QFS-1 substrate, it

Table 2 Kinetic constants of QFS-1 and pGlu-Arg-Thr-Lys-Arg-MCA determined on incubation with hPC1 and human furin

	QFS-1			pERTKR-MCA		
	K_m (μM)	V_{max} ($\mu\text{M}/\text{h}$)	V_{max}/K_m (h^{-1})	K_m (μM)	V_{max} ($\mu\text{M}/\text{h}$)	V_{max}/K_m (h^{-1})
hPC1	17 ± 2	6.4 ± 0.3	0.38	23 ± 3	1.32 ± 0.05	0.06
hfurin	30 ± 7	18 ± 2	0.60	5.9 ± 0.7	1.23 ± 0.03	0.21

Figure 6 Graphical determination of inhibitor constant (Dixon's plot)

The enzymic activity of human furin (●) and hPC1 (▲) towards *p*Glu-Arg-Thr-Lys-Arg-MCA was measured in the presence of [(D)-Tyr¹⁶, Arg¹⁷/(COCH₂)⁸³, Gly⁸⁴]hProPC1-(76–88) using 66 μM (curve S1) and 133 μM (curve S2) fluorogenic substrate concentrations.

appeared advantageous to use a reasonably long peptide sequence. Therefore we synthesized a peptide, the structure of which is shown in Figure 1(c), incorporating a ketomethylene pseudopeptide bond replacing the Arg-Ser bond using the recently proposed Boc-AOGO derivative [28]. As shown in Figure 5, two main products were obtained and further characterized by both amino acid analysis and FAB-MS. The early eluting one, labelled A, actually contained two components; one has an *m/z* of 516 mass units (m.u.) and corresponds to the truncated and capped peptide acetyl-Ala-Leu-Arg-Asp, whereas the other has an *m/z* of 795 m.u. and probably corresponds to a peptide containing a ketopiperazine structure formed upon cyclization of the Lys α-NH₂ with the ketone function of the ketomethylene moiety, as proposed earlier [30]. This cyclization is facilitated by the substitution of the carbonyl amide by the more electrophilic carbonyl ketone, which can further react with the free NH₂ of the next amino acid. The formation of such a cyclic piperazine derivative, whose structure supported by FAB-MS is shown in Figure 5, prevents any further elongation of the peptide chain. This product, when assayed with either hPC1 or human furin, did not display any inhibitory properties. The second peak, labelled B, exhibits the expected *m/z* of 1607 m.u. and has the correct amino acid composition. Its ability to act as an inhibitor for human furin and hPC1 was evaluated by monitoring the cleavage of the pentapeptidyl-MCA fluorogenic substrate *p*Glu-Arg-Thr-Lys-Arg-MCA. Thus, whereas it failed to discriminate against either enzyme, it proved nevertheless to be equally potent against each one, as can be seen from the Dixon's representations shown in Figures 6(a) and 6(b). Indeed, the

inhibitor constants are in the very low micromolar range, being 2.4 μM with furin and 7.2 μM with hPC1, and it proved entirely resistant to proteolysis (results not shown).

DISCUSSION

Based upon our earlier results with substrates and inhibitors encompassing the peptide sequence surrounding the activation site of the human proconvertase PC1, we have synthesized both a novel peptidyl substrate and an isostere-containing inhibitor for both hPC1 and human furin.

Whereas the rate of hydrolysis of a given substrate is often determined by the nature of the amino acids immediately adjacent to the scissile bond, there exist many instances where residues remote from the bond in either N-terminal or C-terminal orientation can greatly influence the efficiency of cleavage. As shown here (Table 1) and often observed in co-expression studies, prohormone convertases appear to exhibit such a discriminating power. Furthermore, whereas the short peptidyl-MCA substrates are well recognized (*K_m*s in the micromolar range), they appear not to be well cleaved as the overall catalytic efficiency (as assessed by the *V_{max}*/*K_m* ratio) is generally low. This aspect is further supported by the observations that (i) substitution of the P1' position by a bulky group can lead to a competitive inhibitor [22], (ii) the efficiency of cleavage is greater when using natural substrates such as proinsulin [23] or anthrax-protective antigen [24] and (iii) some synthetic peptides, such as the previously mentioned hProPTH-(−6+7) and hProPC1 peptide, exhibit relatively high turnover rates. In the present study, these aspects were confirmed by using a fluorescent internally quenched substrate which exhibits both good recognition and good cleavage characteristics. However, it must be said at this stage that the observed improvement in the catalytic efficiency over that observed with fluorogenic peptidyl-MCA substrates could be due to (i) the bulkiness of the MCA moiety, rendering it a poor leaving group, (ii) the stabilization of the substrate by elongation in the C-terminal direction, and/or (iii) the presence of as yet undefined specificity-determining residues in the C-terminal region of the substrate. Nevertheless, in the case of hPC1 and human furin, QFS-1 represents the best synthetic substrate so far reported, and thus could be a very efficient tool for further defining the importance of the residues occupying the P and P' positions. However, because of its relatively low quenching efficiency, as indicated in Figure 2, and possibly also because of quenching by internal absorption at high substrate concentrations as previously noted and reported for these kinds of compounds [12,31], in order to obtain precise values of *V_{max}*, it was found to be necessary to separate the digestion products by RP-HPLC. Nevertheless, because of its ease of synthesis, this substrate might represent the prototype of a series of analogues which, together with a portion-mixing library of internally quenched substrates [32] or a substrate phage library [33], could lead to a better understanding of the intricate substrate specificities of members of the convertase family.

Concomitantly, we also prepared an inhibitor containing a proteolysis-resistant bond and examined its inhibitory characteristics. During preparation of this compound, a significant amount of the end products corresponded to a cyclic piperazine derivative. As proposed [30], the addition of the Lys residue preceding the ketomethylene Arg pseudopeptide bond as the dipeptide Boc-Ser(Bzl)-Lys(Z)-OH would eliminate the tendency of the α-NH₂ group of the Lys to undergo intramolecular cyclization with the ketone moiety following acid removal of the Boc protecting group. Whereas the expected compound is equally

potent towards both human furin and hPC1, with inhibition constants (K_i) in the low micromolar range, it was not as strongly inhibitory as originally anticipated. In contrast, a similar inhibitor, the ketomethylene-containing hirudin [28,30], was active against thrombin in the nanomolar range, hinting that some structural features of the recognition peptidyl sequence are still eluding us. It is also worth noting that shorter peptides incorporating a pseudo-peptide bond, and based upon the sequence surrounding the cleavage site recognized by human furin in the gp160 viral coat protein, exhibited inhibition constants in the low micromolar to high nanomolar range [25]. It is therefore clear that successful development of peptidyl convertase inhibitors will rely on improvements in the chosen peptide sequence, and thus could benefit greatly from results based on analogues of the above-described substrate. The main advantage of this inhibitor remains that it could be used, as presently, for developing an affinity purification procedure that would allow gentle elution conditions.

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