Synthetic peptides derived from the prosegments of proprotein convertase 1/3 and furin are potent inhibitors of both enzymes

Ajoy BASAK*¹ and Claude LAZURE†

*Laboratory of Regional Protein Chemistry Center, Diseases of Ageing, Ottawa Health Research Institute (affiliated to the University of Ottawa), Loeb Building, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4E9, and †Neuropeptides Structure and Metabolism Laboratory, Institut de Recherches Cliniques de Montréal (affiliated to the University of Montréal), 110 Pine Avenue West, Montréal, Québec, Canada H2W 1R7

Proprotein convertases (PCs) are Ca^{2+} -dependent serine proteases of the subtilisin/kexin family which are known specifically to cleave propeptide and proprotein substrates at the C-terminal of R-X-(K/R)-R↓ to generate the relevant biologically active peptides. PCs are initially synthesized as enzymically inactive proenzyme forms where the prosegments play an important inhibitory role to the respective enzymes. Here we investigated whether synthetic peptides derived from the pro-region could also represent specific and potent inhibitors. Based upon sequence alignment, secondary structure analysis and hydrophilicity plot, a number of peptides ranging from 8 to 33 residues were selected. These included segments encompassing residues 55–62, 50–62, 39–62, 50–83, 55–83, 64–83 and 74–83 in the pro-mouse PC1/3 sequence and residues 54–62, 48–62 and 39–62 of the pro-human furin sequence. All peptides were prepared by solid-phase FastMoc chemistry, purified by reversed-phase HPLC and characterized by MS and amino acid analysis. These peptides

were tested *in vitro* for inhibitory activity towards recombinant mouse PC1/3 and human furin. Progress-curve and end-time kinetic analysis demonstrated that a number of these peptides, particularly those containing both the primary and the secondary processing sites, displayed strong inhibition of both enzymes with inhibition constants (K_i) in the high nanomolar range. Unlike the whole propeptide, these small synthetic peptide inhibitors exhibited either true competitive or mixed competitive inhibition, depending on the sequence. Our data revealed further the critical role of the last two basic amino acid residues (e.g. Lys⁸²-Arg⁸³ for the mouse PC1/3 sequence) of the prodomain in imparting a strong anti-convertase activity. The study also establishes the inhibitory potential of certain regions contained within the prosegment of the two convertases.

Key words: enzyme inhibitor, prodomain peptide, subtilase.

INTRODUCTION

A large variety of proteins are synthesized as precursors in the form of pre-proproteins. Whereas the presequences usually act as signal peptides for transport within the endoplasmic reticulum, thereby directing these proteins into the secretory pathway, the prosequences may serve different functions. For numerous proteolytic enzymes, it has been found that they act as intramolecular chaperone by catalysing the protein-folding process of, for example, cysteine and serine proteases [1,2]. In addition, prosequences may also serve in many cases to regulate the activity of their associated enzymes, as their removal through proteolytic cleavages is a prerequisite to excise the fully enzymically active enzymes. This was described initially in the middle of the 19th century for the aspartyl protease pepsin and later on for a variety of other enzymes belonging to various classes. In fact the prosegment has been shown to be a potent and specific inhibitor of its associated protease. This has been demonstrated with cathepsins B and L [3,4], papain [5], subtilisin [6,7], pepsin [8] and stromelysin [9]. This unique property of the prosegment can thus be seen as an additional strategy to regulate enzymic activity in delaying its onset until the moment when the enzyme needs to be fully functional.

All the seven known members of the family of kexin/subtilisin-like endopeptidases, namely proprotein convertase (PC) 1/3,

PC2, PC4, PC5/6, PC7/8/LPC, furin/PACE (paired-basic-amino-acid-converting enzyme) and PACE4 [10,11], are synthesized as enzymically inactive zymogen forms that are also known as proenzymes. It has been demonstrated that the propeptide of subtilisin BPN', a bacterial model for the eukaryotic convertases, is involved and necessary for proper folding of subtilisin [12], in addition to being a potent slow tight-binding inhibitor of the active enzyme [13]. Moreover, it has been shown that propeptide removal of kexin [14], furin [15] and PC2 [16] is a prerequisite for enzyme activity and/or transport out of ER [14]. Elegant studies based on chimaeric molecules have shown that, in certain cases, the propeptide can be interchanged, such as with PC1/3 and furin [17], leading to the correct expression of the recombinant enzymes. In other cases, such as proPC1/3 and proPC2, it is clearly not [15]. Studies with furin [18], Kex-2 [19,20], PC1/3 [21] and PC7 [22] have provided strong evidence for the inhibitory role of the pro-region, although another study [23] has indicated that C-terminal region of PCs can also be inhibitory to the cognate enzyme. However, besides one study by NMR [24], much of the mechanisms of action and the structural determinants of the pro-region, and also the sites of binding involved in the observed potent inhibition remain to be established. Similarly, since cells are capable of expressing simultaneously a number of convertases, the question of specificity of each pro-region for its cognate enzyme remains unclear. In fact, studies indicated that prodomains

Abbreviations used: PC, proprotein convertase; mPC, mouse PC1/3; Fmoc, fluorenyl-methoxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; pGlu, pyroglutamic acid; MCA, 4-methylcoumaryl-7-amide; RP-HPLC, reversed-phase HPLC; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; hFurin, human furin; pro-PTH, parathyroid hormone; QFS-8, (Abz-Gly-Ser-Arg-Lys-Lys-Arg↓Ser-Val-Ser-Glu-3-nitrotyrosine-Ser, where Abz is 2-aminobenzoic acid).

¹ To whom correspondence should be addressed (e-mail abasak@ohri.ca).

of PCs do not display any high degree of inhibitory specificity towards the cognate enzymes [20–22,25–28].

The present study attempts to address some of these issues by defining important residues within the prosegment sequence, which are critical for enzyme inhibition. This is accomplished by studying the interaction of various synthetic peptides derived from the pro-regions of PC1/3 and furin with enzymically active PC1/3 and furin. The present paper describes in detail the design, synthesis, molecular characterization and comparative inhibitory properties towards PC1/3 and furin of a number of such synthetic peptides and some specific mutants. A preliminary account of a part of this study was reported earlier [26].

MATERIALS AND METHODS

All fluoren-9-ylmethoxycarbonyl (Fmoc)-protected amino acid derivatives (L-form), coupling reagents and solvents for peptide synthesis were purchased from Applied Biosystems (Mississauga, Ont., Canada), Calbiochem. (San Diego, CA, U.S.A.), Armand Frappier Institute (Laval, Québec, Canada) and Richelieu Biotechnologies (Montréal, Québec, Canada). The fluorogenic substrate pyroglutamic acid (pGlu)-Arg-Thr-Lys-Arg-4-methylcoumaryl-7-amide (MCA) was purchased from Peptides International (Louisville, KY, U.S.A.).

Peptide synthesis

Synthesis of all peptides were performed on an automated Applied Biosystems model 431A peptide synthesizer using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate ('HBTU')/*N*-hydroxybenzotriazole ('HOBT')-mediated FastMoc chemistry. In the case of sequence-related peptides, these were prepared by a single run of synthesis, removing part of the resin [*p*-hydroxymethylphenoxymethyl polystyrene ('HMP') resin] at the appropriate step and continuing the synthesis until the next desired length. The following side-chain-protecting groups were used: *t*-butyloxycarbonyl ('Boc') for Lys; 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg; *t*-butyl for Ser, Thr, Asp and Tyr; and trityl for His, Asn and Gln. The synthesis was terminated before the removal of the *N*-terminal Fmoc-protecting group, which was finally removed following treatment with 20% piperidine in *N*-methylpyrrolidone. The bound peptides were then cleaved from the resin and fully deprotected by treatment for 3–4 h at room temperature with reagent K as described in [29]. In certain cases, due to the high content of protecting groups, particularly the Pmc of Arg residues, it was necessary to repeat the deprotection procedure for a second time.

Peptide purification and chemical characterization

The crude peptides were purified by reversed-phase HPLC (RP-HPLC) using a semi-preparative CSC Exsil C₁₈ (25 cm × 1.0 cm; Chromatography Speciality Co., St-Laurent, Québec, Canada) [29]. The buffer system consisted of an aqueous 0.1% (v/v) trifluoroacetic acid solution and an organic phase of acetonitrile also containing 0.1% (v/v) trifluoroacetic acid. The elution was carried out by using 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 adjusted to 2.0 ml/min. The separation was monitored by UV absorbance at 225 nm, whereas the inhibitory properties of selected chromatogram peaks were assayed by incubating an aliquot for 150 min and overnight with each enzyme in the presence of fluorogenic substrate. In

all cases, the identified peptides were purified further under the same conditions using a CSC Exsil C₁₈ analytical column (25 cm × 0.46 cm) and a flow rate of 1.0 ml/min. The peptide purity and concentration were determined by quantitative amino acid analysis following 18–24 h hydrolysis in the presence of 5.7 M HCl *in vacuo* at 110 °C on a Beckman autoanalyser model 6300 with post-column ninhydrin detection system loaned to us generously by the Canadian National Research Council and coupled to a Varian DS604 integrator/plotter. The identity of each purified peptide was confirmed by mass-spectral analysis using either an MS-50 HMTCTA instrument operating in fast-atom bombardment mode (University of Montréal, Québec, Canada) or a matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) Tofspec MicroMass instrument using 4-hydroxycinnamic acid as matrix (University of Waterloo, Ontario, Canada).

Peptide I, mouse PC1/3 (mPC1/3) (55–62): 8-mer, Ser-Ala-Leu-His-Ile-Thr-Lys-Arg; RP-HPLC, *R*_t = 28.5 min (29% acetonitrile); MS, *m/z* 925 (M + H)⁺. Peptide IA, mPC1/3 (55–62A)⁵⁸: 8-mer, Ser-Ala-Leu-Ala⁵⁸-Ile-Thr-Lys-Arg; RP-HPLC, *R*_t = 22.9 min (23% acetonitrile); MS, *m/z* 860 (M + H)⁺. Peptide II, mPC1/3 (50–62): 13-mer, Arg-Arg-Ser-Arg-Arg-Ser-Ala-Leu-His-Ile-Thr-Lys-Arg; RP-HPLC, *R*_t = 23.9 min (24% acetonitrile); MS, *m/z* 1635 (M + H)⁺. Peptide III, mPC1/3 (39–62): 24-mer, Asn-His-Tyr-Leu-Phe-Lys-His-Lys-Ser-His-Pro-Arg-Arg-Ser-Arg-Arg-Ser-Ala-Leu-His-Ile-Thr-Lys-Arg; RP-HPLC, *R*_t = 21.1 min (21% acetonitrile); MS, *m/z* 3025 (M + H)⁺. Peptide IIIA, mPC1/3 (39–62A)^{40,45,48,58}: 24-mer, Asn-Ala⁴⁰-Tyr-Leu-Phe-Lys-Ala⁴⁵-Lys-Ser-Ala⁴⁸-Pro-Arg-Arg-Ser-Arg-Arg-Ser-Ala-Leu-Ala⁵⁸-Ile-Thr-Lys-Arg; RP-HPLC, *R*_t = 30.2 min (30% acetonitrile); MS, *m/z* 2812 (M + H)⁺. Peptide IV, mPC1/3 (50–83): 34-mer, Arg-Arg-Ser-Arg-Arg-Ser-Ala-Leu-His-Ile-Thr-Lys-Arg-Leu-Ser-Asp-Asp-Arg-Val-Thr-Trp-Ala-Gln-Gln-Tyr-Glu-Lys-Glu-Arg-Ser-Lys-Arg; RP-HPLC, *R*_t = 33.9 min (34% acetonitrile); MS, *m/z* 4258 (M + H)⁺, 4240 (M-H₂O), 4100 (M-Arg)⁺. Peptide V, mPC1/3 (55–83): 29-mer, Ser-Ala-Leu-His-Ile-Thr-Lys-Arg-Leu-Ser-Asp-Asp-Arg-Val-Thr-Trp-Ala-Gln-Gln-Tyr-Glu-Lys-Glu-Arg-Ser-Lys-Arg; RP-HPLC, *R*_t = 33.7 min (34% acetonitrile); MS, *m/z* 3546 (M + H)⁺, 3528 (M-H₂O)⁺. Peptide VI, mPC1/3 (64–83): 20-mer, Ser-Asp-Asp-Asp-Arg-Val-Thr-Trp-Ala-Gln-Gln-Tyr-Glu-Lys-Glu-Arg-Ser-Lys-Arg; RP-HPLC, *R*_t = 29.0 min (29% acetonitrile); MS, *m/z* 2526 (M + H)⁺. Peptide VII, mPC1/3 (74–83): 10-mer, Gln-Gln-Tyr-Glu-Lys-Glu-Arg-Ser-Lys-Arg; RP-HPLC, *R*_t = 19.0 min (19% acetonitrile); MS, *m/z* 1351 (M + H)⁺, 1334 (M-H₂O). Peptide VIII, human furin (hfurin) (54–62): 9-mer, Ser-Pro-His-Arg-Pro-Arg-His-Ser-Arg; RP-HPLC, *R*_t = 14.6 min (15% acetonitrile); MS, *m/z* 1128 (M + H)⁺. Peptide IX, hfurin (48–62): 15-mer, Val-Thr-Lys-Arg-Ser-Leu-Ser-Pro-His-Arg-Pro-Arg-His-Ser-Arg; RP-HPLC, *R*_t = 20.4 min (20% acetonitrile); MS, *m/z* 1814 (M + H)⁺. Peptide X, hfurin (39–62): 24-mer, Asp-Tyr-Tyr-His-Phe-Trp-His-Arg-Gly-Val-Thr-Lys-Arg-Ser-Leu-Ser-Pro-His-Arg-Pro-Arg-His-Ser-Arg; RP-HPLC, *R*_t = 38.4 min (38% acetonitrile); MS, *m/z* 3075 (M + H)⁺.

Treatment of peptide IV [mPC1/3 (50–83)] with carboxypeptidase E

Peptide IV (20 μg) was dissolved in 50 mM sodium acetate buffer, pH 6.0 (95 μl), and then treated with carboxypeptidase E [30] (5 μl; estimated concentration ≈ 1 μg/ml; a generous gift from Dr Lloyd Fricker, Albert Einstein College, Bronx, New York, U.S.A.) at room temperature for 4 h and then overnight at 4 °C. The material was then purified by RP-HPLC using a C₁₈ analytical column (Phenomenex) under the conditions described

above. The collected peak ($R_t = 34.5$ min) was lyophilized and characterized by MS and amino acid composition as IV_{ctdd} (where ctdd is C-terminal double deletion) [mPC1/3 (50–81)] 32-mer, Arg-Arg-Ser-Arg-Arg-Ser-Ala-Leu-His-Ile-Thr-Lys-Arg-Leu-Ser-Asp-Asp-Asp-Arg-Val-Thr-Trp-Ala-Glu-Gln-Gln-Tyr-Glu-Lys-Glu-Arg-Ser; MS, m/z 3975 ($M + H$)⁺ and amino acid analysis Arg_{6.64} {7}, Ser_{3.80} {4}, Ala_{1.58} {2}, Leu_{2.0} {2}, His_{1.1} {1}, Ile_{1.13} {1}, Thr_{1.76} {2}, Lys_{1.88} {2}, Asp_{3.29} {3}, Val_{1.0} {1}, Trp_{0.59} {1}, Glu/Gln_{4.72} {5} and Tyr_{1.20} {1}.

Recombinant enzymes

Initially the recombinant endoproteases human PC1/3 and hfurin were obtained from the secreted culture media of somatomammotroph GH₄C₁ cells following infection with respective cDNAs encoding vaccinia viruses. The collected media were concentrated, dialysed and partially purified through anion-exchange chromatography prior to use as described in [31]. In a later part of the study, the purified 71 kDa mPC1/3 form, obtained from medium following 36 h infection of *Spodoptera frugiperda* cells with *Autographa californica* nuclear polyhedrosis virus-infected cDNA of mPC1/3, was used [32].

Enzymic assays and kinetic analysis

All *in vitro* enzymic assays were performed using either initial rate assays and/or end-time assays at room temperature in a final volume of 100 μ l in 96-well flat-bottom white plates (Micro-fluor; Dynatec). For hfurin, the final assay conditions were 50 mM sodium acetate buffer, pH 7.0, in the presence of 1 mM CaCl₂, whereas for mPC1/3 the conditions were 50 mM sodium acetate, pH 6.0, in the presence of 5 mM CaCl₂. Unless stated otherwise, all assays were performed using a final concentration of 100 μ M pGlu-Arg-Thr-Lys-Arg-MCA substrate. The release of the fluorescent 7-amino 4-methyl coumarin ('AMC') was quantified on line with a PerkinElmer model LS50B spectrofluorimeter using excitation and emission wavelengths of 370 ± 5 and 460 ± 3 nm respectively. For measurement of both K_i (inhibition constant) and IC₅₀ (the concentration necessary to achieve 50 % inhibition of enzymic activity) values, the inhibitor concentrations were varied over a range wide enough to yield residual activities of 25–75 % of the control value. The various kinetic parameters were evaluated using Enzfitter software (Elsevier-Biosoft, Cambridge, U.K.). In the case of K_i being derived from the IC₅₀ value, the following equation, applicable for reversible and competitive inhibitors, was used: $K_i = IC_{50} / (1 + [S]/K_m)$, where [S] represents the substrate concentration and K_m is the Michaelis–Menten constant [32]. The K_m values used were 20 μ M for human PC1 [31], 8.0 μ M for mPC1 [21,32] and 5.9 μ M for hfurin [33] respectively.

Determination of the K_i using the internally quenched fluorogenic substrate QFS-8 (Abz-Gly-Ser-Arg-Lys-Lys-Arg↓Ser-Val-Ser-Glu-3-nitrotyrosine-Ser, where Abz is 2-aminobenzoic acid) [34], derived from the sequence of the processing site of proparathyroid hormone (pro-PTH), was done essentially as described previously [34].

Synthetic peptides as substrates of mPC1/3 and hfurin

Each of the peptides IV–VII (20 μ g) was incubated separately at room temperature with enzymically active hfurin or mPC1/3 (5 μ l) in 50 mM sodium acetate buffer (100 μ l) containing 2 mM

CaCl₂ at pH 7.0 for furin and 5 mM CaCl₂ at pH 6.0 for mPC1. Aliquots were withdrawn at intervals of 1 h until 4 h and analysed on RP-HPLC using the conditions as described above, in order to examine any possible proteolytic cleavage of the peptides.

RESULTS

Selection of the synthetic peptides

Extensive sequence alignment of the pro-regions of various convertases has shown that there exists limited amino acid conservation between each convertase. Indeed, for example, the similarity of sequences between the pro-region of furin and those of the other family members ranges from 29 % for PC7 to a maximum of 51 % for PC5/6 [35]. This similarity is much lower than observed within the catalytic domain, which ranges from a minimum of 54 % for PC2 up to 70 % for PC4. However, such an analytical procedure might not indicate the real story because, if one allows the introduction of gaps within each pro-region, a different picture emerges. In addition, conservation in PCs of certain amino acid residues can be seen as, for example, in the case of a Gly-Gly-Xaa₃-Ala-Xaa₃-Ala-Xaa₃-Gly motif predicted to be a helical structure based upon Chou/Fasman and Garnier/Robson algorithms; this segment lies between positions 13 to 28 in proPC1/3. Similarly, it can be seen that in the C-terminal region of all proconvertases and hence preceding the proposed zymogen-activation site, a Val-Xaa-Trp-Xaa-Xaa-Gln-Xaa₄-Arg-Xaa-Lys-Arg↓ motif is strongly conserved, with the exception of the Trp being replaced in PC2. Also noteworthy is the presence of a His-Xaa-Xaa-His motif (where the Xaas are often aromatic and hydrophobic residues) near a putative secondary convertase cleavage site, Lys/Arg-Arg↓, occupying positions 50 and 51 in proPC1/3. Hence, in the present study, we have decided to focus on the last two above-mentioned motifs. This particular choice was further justified by the fact, as illustrated in Figure 1(A), that both lie within surface-accessible and highly hydrophilic segments of the proPC1/3 region and by earlier studies of furin and PC7 [18,21,22]. Hence, peptides encompassing either of the two regions (Figure 1B) were synthesized, namely peptides I–III, representing the region from residues 39 to 62, and peptides V–VII, representing residues 55–83. In addition, a longer peptide from residues 50 to 83 (peptide IV) was prepared together with three additional peptides spanning the first region as well as that found in the profurin region (peptides VIII–X). Moreover, two analogous mutant peptides, namely peptides IA and IIIA, were prepared by substituting the His residues for Ala to evaluate the contribution of the His residues. The rationale behind such mutation is based on our earlier observation that His-rich human salivary peptides that bear significant homology, including the conservation of all His residues of the prosegments, are inhibitors of PCs such as furin and PC7 [36].

Synthetic peptides derived from the proPC1/3 or profurin residues 39–62

The important feature of region 39–62 is that it contains solely the secondary processing region, which is not considered as the cognitive putative site responsible for activation of the enzyme. As shown in Table 1, these peptides, five in all with two His-to-Ala mutants, proved to be competitive inhibitors for either mPC1/3 or hfurin when assayed in the presence of the pGlu-Arg-Thr-Lys-Arg-MCA fluorogenic substrate. When examined against mPC1/3, all three synthetic peptides in the region 39–62, namely 55–62, 50–62 and 39–62, exhibited very close K_i values, ranging

Figure 1 (A) Hydrophilicity and surface-probability plots of the amino acid sequence encompassing mPC1/3 (1–90) and (B) schematic representation of the synthetic peptides used in this study with respect to mPC1/3 (1–90)

The complete propeptide region of mPC1/3 (1–90) corresponds to residues 1–83.

from 15 to 20 μM ; substitution of all His residues in peptides I and III by Ala residues had a similar effect by slightly increasing the K_i values. In the case of related profurin peptides in the same region, a slight increase in K_i values was observed, with values ranging from 10 to 70 μM . However, when examined against the enzymic activity of hfurin, the results were quite different, inasmuch as much lower K_i values were obtained with both proPC1/3 and profurin-related peptides. Indeed, as can be seen in the case of the longer peptide (Table 1), values in the nanomolar range were obtained. Thus for example, using pGlu-Arg-Thr-Lys-Arg-MCA as the substrate, promPC1/3 (39–62) displayed for hfurin a K_i value of 700 nM, which is 22-fold more potent than that for mPC1/3 (Figure 2A). These data indicated a lack of specificity for inhibition of cognate enzyme by its own prodomain derived synthetic peptides. Similar observation was also noted previously [21,22]. However, the same peptide inhibited furin activity more strongly ($K_i = 350$ nM, Figure 2B) when measured against a pro-PTH-derived intramolecularly quenched fluorogenic substrate, QFS-8 [34], which unlike the peptidyl-MCA substrate contains both P and P' residues. QFS-8 is a much better substrate ($K_m = 3$ μM) than pGlu-Arg-Thr-Lys-Arg-MCA ($K_m = 20$ μM).

Interestingly, substitution of the His residues by Ala had only marginal effect on the inhibition of enzymic activity of furin (Table 1), suggesting that at least in these peptides His residues do not seem to play any critical role towards their furin inhibitory property.

Synthetic peptides related to the proPC1/3 residues 64–83

The peptides derived from the region 64–83 all contain the important primary processing site [with P-residues (Scheeter and Berger's nomenclature) alone] responsible for release of the active enzyme. These peptides, numbered V–VII, exhibited upon incubation with mPC1/3 at least a 10-fold reduction in K_i values when compared with other peptides mentioned above, yielding values in the 1–2 μM range (Table 1). Interestingly, even the shortest peptide, VII, when assayed with hfurin exhibited nearly a 25-fold increase in K_i value compared with that against mPC1/3. This observation is noteworthy because of the fact that there is a high degree of sequence similarity between proPC1/3 and profurin in this region, with six residues out of 10 being identical. However, given the fact that the

Table 1 Apparent inhibition constant (K_i) of various proPC1/3- and profurin-related peptides

The basic residues at the primary and secondary processing sites are indicated in bold and underlined respectively. The His-to-Ala mutations are indicated in italics. ND, not determined.

Sequence position (peptide)	Amino acid sequence	K_i (μM)	
		mPC1/3	hfurin
ProPC1/3-related peptides			
55–62 (I)	SALHITKR	18.6*	31.6*
55–62/A (IA)	SALAITKR	22.1	13.0
50–62 (II)	RRSRRSALHITKR	18.2	2.3
39–62 (III)	NHYLFKHKSHPRRSRSALHITKR	15.4	0.7
39–62/A (IIIA)	NAYLFKAKSAPRRSRRSALAITKR	43.2	1.2
50–83 (IV)	RRSRRSALHITKRLSDDDRVTWAEQQYEKERS KR	0.7	4.8
55–83 (V)	SALHITKRLSDDDRVTWAEQQYEKERS KR	0.3	ND
(mixed)		2.4‡	
64–83 (VI)	SDDDRVTWAEQQYEKERS KR	0.6	ND
(mixed)		11.0‡	
74–83 (VII) (competitive)	QQYEKERS KR	0.89	47.5
Profurin-related peptides			
54–62 (VIII)	SPHRPRHSR	73.5*	23.0*
48–62 (IX)	VT K RSLSPHRPRHSR	36.0*	2.8*
39–62 (X)	DYYHFWHRGVTKRSLSPHRPRHSR	10.2*	0.9*
60–83 (XI)	HSRLQREPQVQWLWQQVAKRRT KR	ND	40§
74–83 (XII)	QQVAKRRT KR	ND	35§

* These values were computed from the IC_{50} values determined with $[S] = 3 \times K_m$ being 50 and 15 μM pGlu-Arg-Thr-Lys-Arg-MCA for baculovirus-infected mPC1/3 and vaccinia virus-infected hfurin respectively.

† Determined using the internally quenched fluorogenic substrate QFS-8 derived from the pro-PTH sequence [34] (see the Materials and methods section).

‡ K_i' values for mixed-type inhibition.

§ Values were taken from our previous study [22].

Figure 2 Representative Dixon and double-reciprocal Lineweaver-Burk plots for inhibition of furin activity by mPC1/3 (39–62) peptide as measured against the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA (A) and QFS-8 (B)

Substrate concentrations are indicated on the Figure. The enzymic activity was followed by fluorescence measurements as described in the Materials and methods section.

furin substrate-recognition cleft has been proposed to contain a very high number of acidic residues [37,38], the presence of two unfavourable Glu residues occupying positions P7 and P5 (Schechter and Berger's nomenclature of substrate positions) in peptide VII may explain this significant fall in potency for furin inhibition.

It was also noted that pro-mPC1/3 (55–83) is a more potent PC1/3 inhibitor ($K_i = 0.3 \mu\text{M}$) than pro-mPC1/3 (64–83) ($K_i = 0.6 \mu\text{M}$), which is again a stronger inhibitor than pro-mPC1/3 (74–83) ($K_i = 0.89 \mu\text{M}$; Table 1). This seems to suggest that elongation of the peptide chain towards the N-terminus direction from the putative site leads to more potent inhibition of PC1/3. However, further elongation, as noticed with peptide IV, led to a small decrease but a different mode of inhibition (see the following section) in the potency of PC1/3 inhibition, suggesting an optimum sequence for best interaction.

Type of enzyme inhibition

A previous study reported that a polypeptide containing the entire prosegment sequence of PC1/3 plus an additional 14 amino acid residues beyond the zymogen-activation site, i.e. segment 1–98, exhibited extremely potent but slow tight-binding inhibition with $K_i = 6 \pm 0.6 \text{ nM}$ against mPC1 and $10 \pm 1.0 \text{ nM}$ against hfurin [21]. Whereas none of the synthetic peptides exhibited enzyme inhibition potency as strong as this 98-amino-acid peptide, promPC1/3 (39–62) and profurin (39–62), both of which contain only the secondary processing site, were however the two most potent furin inhibitors in this study. Both these synthetic peptides, like most others examined, exhibited truly competitive inhibition. This is demonstrated by the linearity of the progress curves, as shown in Figure 3, as a model example for the inhibition of mPC1/3 activity by promPC1/3 (74–83) (peptide VII). This

Figure 3 Progress curves of PC1/3 activity in the presence of pro-mPC1/3 (74-83) peptide

Progress curves were obtained using 100 μ M pGlu-Arg-Thr-Lys-Arg-MCA in the presence of increasing concentrations of the purified peptide, as indicated, from 0 (top curve) to 6.6 μ M (bottom curve). The amount of peptide used was measured following amino acid analysis using norleucine as an internal standard and the peptide was added to the enzyme solution at the same time as the fluorogenic substrate in a total volume of 100 μ l in a 96-well plate.

Figure 4 Representative Dixon (A) and Woolf or Hanes (B) plots for inhibition of PC1/3 activity by pro-mPC1/3 (74-83)

Inhibition was measured against two different concentrations, S1 (15 μ M) and S2 (45 μ M), of pGlu-Arg-Thr-Lys-Arg-MCA.

conclusion is further reinforced by examining the Dixon and Woolf or Hanes plots, shown in Figure 4, for the inhibition of mPC1/3 activity by promPC1/3 (74-83) peptide. The converging linear curves observed in Dixon plot (Figure 4A) and the near parallel lines seen in Woolf or Hanes plot (Figure 4B) are typical for true competitive inhibition. However, not all inhibitions are of an identical nature, as exemplified by deviations from the above

observations (results not shown). For example, whereas the short C-terminal peptide pro-mPC1/3 (74-83) is a fully competitive inhibitor of PC1, the two longer pro-mPC1 (64-83) and pro-mPC1/3 (55-83) peptides were both mixed-type inhibitors of PC1/3 (results not shown). This indicates that, unlike the short peptide which interacts only at the catalytic site of PC1/3, the two larger peptides provide additional interactions at sites remote from the active site of the enzyme. Interestingly, the even larger pro-mPC1/3 (50-83) peptide behaves as a non-competitive inhibitor. Thus besides interacting with the catalytic residues, some peptide inhibitors can also interact with the non-catalytic domain of the enzyme thereby modifying its activity. In fact the prodomains of several convertases such as kexin and PC7 were shown to behave as mixed-type inhibitors of the cognate enzymes [20,22,36].

A comparative analysis of the inhibitory profile towards the proteolytic activity of mPC1 of three synthetic pro-mPC1/3-derived peptides, namely peptides IV (50-83), V (55-83) and VI (64-83), seems to suggest that the maximum PC1-inhibitory property is located within the 50-83 segment of the promPC1/3 sequence. It may be pointed out that despite the presence of potential PC-cleavage sites in some of these peptides, such as peptides IV-VII, none of them in fact were cleaved to any significant extent (within a detection limit of 2-3%) by the proteolytic action of either mPC1/3 or hfurin under the experimental conditions employed for the inhibition studies. This observation confirms the notion that these synthetic peptides behave mostly as true inhibitors and not as competitive substrates.

Carboxypeptidase E treatment of promPC1/3 (50-83) and its effect on the PC inhibitory property

CD studies of peptide IV [promPC1/3 (50-83)], one of the most potent PC1/3 inhibitors in this study, and some related peptides demonstrated important differences in their secondary structures, e.g. α -helix, β -strand and turn contents (results not shown). This and previous observations [21,22] prompted us to investigate the role of the two (P1-P2) C-terminal basic residues (Lys⁸²-Arg⁸³) in enzyme-inhibitory property exemplified by the peptides containing such residues, e.g. peptide IV [pro-mPC1/3 (50-83)]. In order to accomplish this objective, peptide IV was treated with carboxypeptidase E as described in the Materials and methods section. The resulting peptide digest was purified by RP-HPLC (Figure 5) and then subjected to mass-spectral analysis. As shown in Figure 6, the MALDI-TOF MS of purified carboxypeptidase E-treated peptide IV peak eluted at R_t = 34.8 min (Figure 5B) exhibited a major peak at m/z 3975, compared with the peak at m/z 4258 displayed by the parent peptide peak eluted at R_t = 35.5 min (Figure 5A). This represented a decrease of 284 mass units corresponding to the two C-terminal Lys-Arg residues, suggesting that upon treatment with carboxypeptidase E the peptide IV loses its C-terminal pair of basic residues (Lys-Arg), yielding the peptide pro-mPC1 (50-81). This was further confirmed by amino acid analysis (see the Materials and methods section). The above two peptides were then tested in parallel for their mPC1/3 inhibitory activity. Interestingly, as shown in Figure 7, the carboxypeptidase E-treated peptide, pro-mPC1/3 (50-81), lost significantly its ability to inactivate mPC1/3 compared with the parent peptide pro-mPC1/3 (50-83). This demonstrated clearly a critical role in enzyme inhibition of the terminal basic residues Lys⁸²-Arg⁸³ that incidentally represent the primary site (P1-P2) of activation for the enzyme. Thus while a concentration of 5.8 μ M pro-mPC1 (50-83) peptide inhibited $62 \pm 2\%$ of mPC1 activity, the C-terminal des-Lys-Arg peptide, pro-mPC1 (50-81), under

Figure 5 RP-HPLC chromatograms of pro-mPC1/3 (50–83) (A) and its product resulting from carboxypeptidase E digestion (B) on a C_{18} analytical column

See the Materials and methods section for details.

Figure 6 MALDI-TOF mass-spectral analyses of (A) purified pro-mPC1/3 (50–83) (peak eluted at $R_t = 35.5$ min) and (B) pro-mPC1/3 (50–81) (peak eluted at $R_t = 34.8$ min)

Pro-mPC1/3 (50–81) was obtained following carboxypeptidase E digestion of pro-mPC1/3 (50–83) and HPLC as shown in Figure 5.

Figure 7 Progress curves for PC1 activity in the presence of pro-mPC1/3 (50–83) and its C-terminal des-Lys-Arg peptide, pro-mPC1/3 (50–81)

Progress curves were obtained using $100 \mu\text{M}$ pGlu-Arg-Thr-Lys-Arg-MCA in the absence or presence of $5.8 \mu\text{M}$ of either pro-mPC1 (50–83) or pro-mPC1/3 (50–81). The latter peptide was obtained following carboxypeptidase E treatment of promPC1/3 (50–83) and purification by RP-HPLC as described in the Materials and methods section. The slope of each curve is represented by the accompanying dotted line. The two nearly overlapping curves at the top represent the progress curves for PC1/3 activity in the presence (black) and absence (grey) of peptide as indicated. The loss of PC1/3-inhibitory activity of the des-Lys-Arg peptide is clearly noticeable. The exact concentration of each peptide used was evaluated by amino acid analysis.

similar conditions inhibited only $0.8 \pm 0.25\%$ of the activity, indicating a difference of at least of 80-fold in mPC1/3-inhibitory property between the two peptides (Table 2).

DISCUSSION

The present study revealed that certain regions of prosegments of both mPC1 and hFurin possess modest to high degrees of enzyme-inhibitory ability against their cognate enzymes, mPC1/3 and hFurin. Based on studies involving a number of peptides located at different regions of the propeptide, the highest mPC1/3 inhibitory property was located within segment 50–83 of the promPC1/3 sequence, representing the N-terminal sequence preceding the primary activation site Lys-Glu-Arg-Ser-Lys-Arg⁸³↓. This suggests a highest degree of interaction of this segment with the various subsites representing the catalytic site of the enzyme. Interestingly, this region is characterized by the presence of α -helix at the N-terminus followed immediately by an exposed hydrophobic β -strand structure [39]. The presence of this structural motif may be crucial for the observed inhibition of PC1/3-activity. On the contrary, the most potent hFurin-inhibitory property in this study was located within the peptide sequence 39–62 of both promPC1/3 and proHfurin, which contains the secondary processing site alone ($K_i = 700$ – 900 nM) and characterized by the presence of a highly exposed β -strand structure at the N-terminus followed by a loop structure [39]. However, in an earlier study [22] we have already shown that hFurin (74–83) (referred as hFur-C10; QQVAKRRTR↓) and (60–83) (referred as hFur-C24; HSRLQREPQVQWLWQQVAKRRTR↓), both of which contain only the zymogen-activation site, inhibited furin with a much higher degree of potency (K_i ranging from 35 to 40 nM), confirming again the importance of a primary activation site in furin inhibition as well. This and other studies [21,22,26] support the notion that the presence of primary and/or secondary

Table 2 Comparison of inhibition of mPC1/3 activity by pro-mPC1/3 (50–83) and its C-terminal des-Lys-Arg-derived pro-mPC1/3 (50–81) at 5.8 μ M

Sequence position in mPC1/3	Amino acid sequence	Molecular mass (Da)	Inhibition (%)
50–83	RRSRRSALHITKRLSDDDRVTWAEQQYKERSKR	4259 (obs)/4258.77 (cal)	62 $\pm$ 2
50–81	RRSRRSALHITKRLSDDDRVTWAEQQYKERS	3975 (obs)/3974.25 (cal)	0.8 $\pm$ 0.25

processing sites in the prosegment-derived peptide enhances significantly the protease-inhibitory property. Furthermore, our data also revealed that contrary to the entire prosegment polypeptide [21,22], the small-sized synthetic peptides did not show any significant selectivity in enzyme inhibition within various members of the PC family tested so far. However, it is necessary to examine the behaviours of these peptides against the activity of all members of the PC families before such a conclusion can be established firmly.

The dramatic loss of the mPC1/3-inhibitory property of promPC1/3 (50–83) peptide following treatment with carboxypeptidase E indicated clearly the critical role in enzyme inhibition of Lys⁸²-Arg⁸³ residues located at the primary cleavage site. Previously the critical role of P1 Arg in inhibition of convertase activity had been demonstrated by mutational studies [21,22]. Our data with mutant peptides (Ala for His) did not reveal any significant roles of His residues in enzyme inhibition by these peptides. The study confirmed further that small-to-medium-sized synthetic peptides derived from prosegments of PCs are able to display modest to relatively potent PC-inhibitory ability, mostly in a competitive manner. However, none of the synthetic peptides reported in this study exhibited a slow tight-binding inhibition as displayed by the full-length propeptides of furin, PC1/3 and PC7 [21,22]. This suggested that the various subsites of the active enzymes are large enough to interact with the whole propeptide in a more effective manner compared with the relatively small peptides. This is particularly valid in light of the reported theoretical modelling structures of furin [37,38] and PC1/3 [40]. Further studies, including mutagenesis and secondary structure analysis using NMR and CD techniques that are currently in progress, are required to better understand molecular mechanisms involved in the pro-region binding to their cognate enzymes and to determine more precisely the nature of the residues involved in the inhibition process.

We thank Alain Boudreault (Aegera Therapeutics, Montréal, Canada), Dany Gauthier and Diane Savaria for the preparation of recombinant mPC1/3 and hfurin and for expert technical assistance, and Dr Brian Wilkes (IRCM, Montréal, Canada) for helping in modelling of peptides. We acknowledge the support of Dr Nabil G. Seidah (IRCM) and Dr Michel Chrétien (OHRI, Ottawa, Canada) during the course of this work. This work is supported by a grant from the Canadian Institute of Health Research (CIHR; MT-14766) to C. L., and Canadian Foundation of Innovation (CFI #2097) to A.B.

REFERENCES

- Ellis, R. J. (1998) Steric chaperones. *Trends Biochem. Sci.* **23**, 43–45
- Gallagher, T., Gilliland, G., Wang, L. and Bryan, P. (1995) The prosegment-subtilisin BPNV complex: crystal structure of a specific 'foldase'. *Structure* **3**, 907–914
- Cygler, M., Sivaraman, J., Grochulski, P., Coulombe, R., Storer, A. and Mort, J. S. (1996) Structure of rat procathepsin B: model for inhibition of cysteine protease activity by the proregion. *Structure* **4**, 405–416
- Carmona, E., Dufour, E., Plouffe, C., Takebe, S., Mason, P., Mort, J. S. and Ménard, R. (1996) Potency and selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. *Biochemistry* **35**, 8149–8157
- Taylor, M. A. J., Baker, K. C., Briggs, G. S., Connerton, I. F., Cummings, N. J., Pratt, K. A., Revell, D. F., Freedman, R. D. and Goodenough, P. W. (1995) Expression of the pro-regions of papain and papaya proteinase IV in *Escherichia coli* and their inhibition of mature cysteine proteinases. *Protein Eng.* **8**, 59–62
- Chagas, J. R., Ferrerdimartino, M., Gauthier, F. and Lalmanch, G. (1996) Inhibition of cathepsin B by its propeptide: use of overlapping peptides to identify a critical segment. *FEBS Lett.* **392**, 233–236
- Shinde, U. P., Liu, J. J. and Inouye, M. (1997) Protein memory through altered folding mediated by intramolecular chaperones. *Nature (London)* **389**, 520–522
- Mach, L., Mort, J. S. and Gloszl, J. (1994) Maturation of human procathepsin B. Proenzyme activation and proteolytic processing of the precursor to the mature proteinase, *in vitro*, are primarily unimolecular processes. *J. Biol. Chem.* **269**, 13030–13035
- Becker, D., Silen, J. L. and Agard, D. A. (1992) Protease pro region required for folding is a potent inhibitor of the mature enzyme. *Proteins* **12**, 329–344
- Seidah, N. G., Mbikay, M., Marcinkewicz, M. and Chrétien, M. (1998) The mammalian precursor convertases: paralogs of the subtilisin/kexin family of calcium-dependent serine proteinases. In *Proteolytic and Cellular Mechanisms in Prohormone and Proprotein Processings* (Hook, V. Y., ed.), pp. 49–76. R. G. Randes, Co., Austin, TX
- Rouillé, Y., Duguay, S. J., Lund, K., Furuta, M., Gong, Q. M., Lipkind, G., Oliva, Jr, A. A., Chan, S. J. and Steiner, D. F. (1995) Proteolytic processing mechanisms in the biosynthesis of neuroendocrine peptides: the subtilisin-like proprotein convertases. *Front. Neuroendocrinol.* **16**, 322–361
- Inouye, M. (1991) Intramolecular chaperone: the role of the pro-peptide in protein folding. *Enzyme (Basel)* **45**, 314–321
- Gutheil, W. G., Kettner, C. A. and Bachovchin, W. W. (1994) KinSq: a program for fitting kinetics data with numerically integrated rate equations and its application to the analysis of slow, tight-binding inhibition data. *Anal. Biochem.* **223**, 13–20
- Lesage, G., Prat, A., Lacombe, J., Thomas, D. Y., Seidah, N. G. and Boileau, G. (2000) The Kex2p proregion is essential for the biosynthesis of an active enzyme and requires a C-terminal basic residue for its function. *Mol. Biol. Cell.* **11**, 1947–1957
- Creemers, J. W. M., Vey, M., Schafer, W., Ayoubi, T. A. Y., Roebroek, A. J. M., Klenk, H. D., Garten, W. and Van de Ven, W. J. M. (1995) Endoproteolytic cleavage of its propeptide is a prerequisite for efficient transport of furin out of the endoplasmic reticulum. *J. Biol. Chem.* **270**, 2695–2702
- Muller, L., Cameron, A., Fortenberry, Y., Apletalina, E. V. and Lindberg, I. (2000) Processing and sorting of the prohormone convertase 2 propeptide. *J. Biol. Chem.* **275**, 39213–39222
- Zhou, A., Paquet, L. and Mains, R. E. (1995) Structural elements that direct specific processing of different mammalian subtilisin-like prohormone convertases. *J. Biol. Chem.* **270**, 21509–21516
- Anderson, E. D., Van Slyke, J. K., Thulin, C. D., Jean, F. and Thomas, G. (1997) Activation of the furin endoprotease is a multiple-step process: requirements for acidification and internal propeptide cleavage. *EMBO J.* **16**, 1508–1518
- Powner, D. and Davey, J. (1998) Activation of the kexin from *Schizosaccharomyces pombe* requires internal cleavage of its initially cleaved prosequence. *Mol. Cell. Biol.* **18**, 400–408
- Lesage, G., Tremblay, M., Guimond, J. and Boileau, G. (2001) Mechanism of Kex2p inhibition by its proregion. *FEBS Lett.* **508**, 332–336
- Boudreault, A., Gauthier, D. and Lazure, C. (1998) Proprotein convertase PC1/3-related peptides are potent slow tight-binding inhibitors of murine PC1/3 and Hfurin. *J. Biol. Chem.* **273**, 31574–31580
- Zhong, M., Munzer, J. S., Basak, A., Benjannet, S., Mowla, S. J., Decroly, E., Chrétien, M. and Seidah, N. G. (1999) The prosegments of furin and PC7 as potent inhibitors of proprotein convertases. *In vitro and ex vivo* assessment of their efficacy and selectivity. *J. Biol. Chem.* **274**, 33913–33920
- Jutras, I., Seidah, N. G., Reudelhuber, T. L. and Brechler, V. (1997) Two activation states of the prohormone convertase PC1 in the secretory pathway. *J. Biol. Chem.* **272**, 15184–15188
- Bhattacharjya, S., Xu, P., Xiang, H., Chrétien, M., Seidah, N. G. and Ni, F. (2001) pH-induced conformational transitions of a molten-globule-like state of the inhibitory prodomain of furin: implications for zymogen activation. *Protein Sci.* **10**, 934–42

- 25 Fugere, M., Limperis, P. C., Beaulieu-Audy, V., Gagnon, F., Lavigne, P., Klarskov, K., Leduc, R. and Day, R. (2002) Inhibitory potency and specificity of subtilase-like pro-protein convertase (SPC) prodomains. *J. Biol. Chem.* **277**, 7648–7656
- 26 Basak, A., Gauthier, D., Seidah, N. G. and Lazure, C. (1998) Synthetic proregion-related peptides of proprotein convertases, PC1 and furin represent potent inhibitors of each protease. *Peptides, Proceedings of the XVth American Peptide Symposium* (Tam, J. P. and Koumaya, P. T. P., eds.), pp. 676–677, Kluwer Academic Publishers, Dordrecht
- 27 Lazure, C. (2002) The peptidase zymogen proregions: nature's way of preventing undesired activation and proteolysis. *Curr. Pharm. Des.* **8**, 511–531
- 28 Fugere, M. and Day, R. (2002) Inhibitors of the subtilase-like pro-protein convertases (SPCs). *Curr. Pharm. Des.* **8**, 549–562
- 29 Basak, A., Schmidt, C., Ismail, A. A., Seidah, N. G., Chrétien, M. and Lazure, C. (1995) Peptidyl substrates containing unnatural amino acid at the P1 position are potent inhibitors of prohormone convertases. *Int. J. Pept. Protein Res.* **46**, 228–237
- 30 Qian, Y., Varlamov, O. and Fricker, L. D. (1999) Glu300 of rat carboxypeptidase E is essential for enzymatic activity but not substrate binding or routing to the regulated secretory pathway. *J. Biol. Chem.* **274**, 11582–11586
- 31 Jean, F., Basak, A., Rondeau, N., Benjannet, S., Hendy, G. N., Seidah, N. G., Chrétien, M. and Lazure, C. (1993) Enzymic characterization of murine and human prohormone convertase-1 (mPC1 and hPC1) expressed in mammalian GH4C1 cells. *Biochem. J.* **292**, 891–896
- 32 Boudreault, A., Gauthier, D., Rondeau, N., Savaria, D., Seidah, N. G., Chrétien, M. and Lazure, C. (1998) Molecular characterization, enzymatic analysis, and purification of murine proprotein convertase-1/3 (PC1/PC3) secreted from recombinant baculovirus-infected insect cells. *Protein Expr. Purif.* **14**, 353–366
- 33 Hatsuzawa, K., Murakami, K. and Nakayama, K. (1992) Molecular and enzymatic properties of furin, a Kex2-like endoprotease involved in precursor cleavage at Arg-X-Lys/Arg-Arg sites. *J. Biochem. (Tokyo)* **111**, 296–301
- 34 Lazure, C., Gauthier, D., Jean, F., Boudreault, A., Seidah, N. G., Bennett, H. P. J. and Hendy, G. N. (1998) *In vitro* cleavage of internally quenched fluorogenic human parathyroid hormone and parathyroid-related peptide substrates by furin, generation of a potent inhibitor. *J. Biol. Chem.* **273**, 8572–8580
- 35 Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem. J.* **327**, 625–635
- 36 Basak, A., Blair, E., Brewer, D., Seidah, N. G., Munzer, J. S., Lazure, C. and Lajoie, G. A. (1997) Histidine-rich human salivary peptides are inhibitors of proprotein convertases furin and PC7 but act as substrates for PC1. *J. Peptide Res.* **49**, 596–603
- 37 Siezen, R. J., Creemers, J. W. M. and Van de Ven, W. J. M. (1994) Homology modelling of the catalytic domain of human furin. A model for the eukaryotic subtilisin-like proprotein convertases. *Eur. J. Biochem.* **222**, 255–266
- 38 Siezen, R. J. and Leunissen, J. A. M. (1997) Subtilases: the super-family of subtilisin-like serine proteases. *Protein Sci.* **6**, 501–523
- 39 Tangrea, M. A., Bryan, P. N., Sari, N. and Orban, J. (2002) Solution structure of the pro-hormone convertase 1 pro-domain from *Mus musculus*. *J. Mol. Biol.* **320**, 801–812
- 40 Lipkind, G., Gong, Q. and Steiner, D. (1995) Molecular modeling of the substrate specificity of prohormone convertases SPC2 and SPC3. *J. Biol. Chem.* **270**, 13277–13284

Received 14 January 2003/25 March 2003; accepted 28 March 2003

Published as BJ Immediate Publication 28 March 2003, DOI 10.1042/BJ20030120