Barley Serine Proteinase Inhibitor 2-Derived Cyclic Peptides as Potent and Selective Inhibitors of Convertases PC1/3 and Furin†

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ABSTRACT: Proprotein convertases (PCs) are serine proteases containing a subtilisin-like catalytic domain that are involved in the conversion of hormone precursors into their active form. This study aims at designing small cyclic peptides that would specifically inhibit two members of this family of enzymes, namely, the neuroendocrine PC1/3 and the ubiquitously expressed furin. We studied peptide sequences related to the 18-residue loop identified as the active site of the 83 amino acid barley serine protease inhibitor 2 (BSPI-2). Peptides incorporating mutations at various positions in the sequence were synthesized on solid phase and purified by HPLC. Cyclization was achieved by the introduction of a disulfide bridge between the two Cys residues located at both the N- and C-terminal extremities. Peptides VIIA and VIIB incorporating P4Arg, P2Lys, P1Arg, and P2′Lys were the most potent inhibitors with K_i around 4 μM for furin and around 0.5 μM for PC1/3. Whereas peptide VIIB behaved as a competitive inhibitor of furin, peptide VIIA acted as a noncompetitive one. However, all peptides were eventually cleaved after variable incubation times by PC1/3 or furin. To avoid this problem, we incorporated at the identified cleavage site a nonscissile aminomethylene bond (ψ[CH_2−NH]). Those pseudopeptides, in particular peptide VIID, were shown not to be cleaved and to inhibit potently furin. Conversely, they were not able to inhibit PC1/3 at all. Those results show the validity of this approach in designing new effective PC inhibitors showing a certain level of discrimination between PC1/3 and furin.

Tailoring and/or modifying existing protein structures in order to elicit novel functions, modes of actions, or physicochemical properties has long been a much sought after objective of protein chemists. Through evolution, nature has also resorted to this approach, some of the structural changes being deleterious to existing proteins but most of the time giving rise to refined properties. A good example is the diversity in mode of action and specificity of proregion or propeptide associated with peptidases as, using a common structural scaffold, changes introduced in the structure enabled the proregion to keep the peptidase dormant until needed (reviewed in ref *1*).

Numerous efforts have been devoted to the isolation through natural and endogenous sources or to the synthesis of inhibitors of a closely related group of peptidases collectively known as members of the kexin/furin family of eukaryotic processing proteases, or subtilisin-like proprotein convertases. This family, whose members all shared a similar propensity to cleave following basic amino acids, comprises seven members, namely, furin, PC1/3, PC2, PC4, PC5/6, PACE4, and LPC/PC7/PC8 (reviewed in ref *2*). Numerous approaches have also been used in order to examine the mode of functioning and to define as precisely as possible their specificity toward a variety of protein substrates. This, in turn, led to the preparation and isolation of numerous inhibitors (reviewed in ref *3*), some of them exquisitely potent but often lacking in specificity. In many instances this is due to the fact that, though each convertase exhibits a tendency to cleave some preferred substrates at least in vitro, members of the family also share a preference for substrates exhibiting basic amino acids occupying P1, P2, P4, and even P6 or P8 positions. Furthermore, as no crystal structure of any convertase and/or convertase catalytic domain is yet available, refining the structure of inhibitors or substrates is complicated. Thus, one approach to the development of more specific inhibitors resides in the generation of a structural mutant of existing natural inhibitors. This has been accomplished on a variety of molecules, namely, α_1-antitrypsin (*4*), α_2-macroglobulin (*5*), eglin C (*6*), and turkey ovomucoid third domain (*7*). In all cases, substitutions of amino acids residing in the bait or the reactive loop of the inhibitor by sequences incorporating basic amino acids at preferred positions have yielded often potent inhibitors, but not

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necessarily selective ones. For example, α_1-antitrypsin Portland (α_1-PDX), incorporating the minimal furin substrate recognition and processing sequence Arg-Xaa-Xaa-Arg, not only is a potent (low nanomolar) inhibitor but is remarkably still able to function as a classical serpin-type inhibitor by forming a stable protease−inhibitor complex (*8*, *9*). However, it can nevertheless either behave as a competitive inhibitor toward other convertases albeit with lesser potency or, more interestingly, keep its mode of inhibition, namely, forming a stable complex with PACE4 (*10*, *11*). It is worth noting that further introduction of residues in the reactive loop of α_1-PDX can efficiently modulate the selectivity (*11*). Similarly, the mode of action of α_2-macroglobulin through entrapment of the peptidase was not modified by introducing in its bait region the furin recognition sequence of von Willebrand factor (*5*). This cannot be taken for granted in all cases as was shown, for example, for various engineered variants of eglin C where enzyme-specific interactions, by modifying the reactive loop conformation, can transform an inhibitor into a substrate (*6*). In addition to those engineered inhibitors, a naturally occurring human proteinase inhibitor (PI8) containing the minimal furin recognition sequence was shown to inhibit recombinant furin (*12*); moreover, and similarly to the above-described variants, modification of residues within its reactive loop can turn this trypsin-like protease inhibitor into a chymotrypsin inhibitor while also keeping the same inhibition mechanism (*13*).

Though differing largely in terms of substrate specificities, degradative subtilisin and members of the convertase family share a significant structural resemblance in their catalytic domain. Hence, bioengineering recognized subtilisin inhibitors may pave the way to novel ones. Furthermore, contrary to the above-mentioned proteins, such inhibitors could be made into small, easily obtainable, bioavailable and exoprotease-resistant compounds by encompassing only the reactive loop in a cyclic form. This approach has led to the development of a nine-residue disulfide-bridged peptide keeping the inhibitory potential as well as the specificity of the full larger Bowman−Birk proteinase inhibitor (*14*−*16*). More closely related to the present study, the interaction at the molecular level between barley serine proteinase inhibitor 2 (BSPI-2;[1] *17*, *18*), often improperly referred to as chymotrypsin inhibitor 2 (CI-2), was studied by solving the crystal structure of the inhibitor itself (*19*) and of the resulting complex formed with subtilisin−NOVO (*20*) and subtilisin−BPN′ (*21*). The structural information thus gathered was largely confirmed through the solution structure of BSPI-2 (*22*, *23*). Such structural data revealed that the interactions between the enzyme and the inhibitor solely reside in an 18-residue loop protruding from the inhibitor molecule able to behave in an almost autonomous fashion from the rest of the molecule. Using this information, Leatherbarrow and Salacinski (*24*) have prepared a synthetic replicate incorporating the 18-residue loop encompassed between two disulfide-linked cysteine residues. In doing so, they demonstrated that the inhibitory potential of the peptide toward subtilisin BPN′ was identical to the full-length 83-residue native inhibitor. We, herein, report the preparation of five variants of this loop, two of them incorporating an isostere bond between the P1 and P1′ positions and targeted at convertases. The introduced mutations, in addition to those already made in the original analogue (*24*), include incorporation of basic residues at positions P1, P2, P4, and P2′ in an effort to reconstruct the preferred substrate-recognizing ability of two proprotein convertases, namely, furin and PC1/3.

MATERIALS AND METHODS

Reagents. All Fmoc- and Boc-protected amino acid derivatives (L-form) for peptide synthesis were purchased from Chem-Impex International (Wood Dale, IL). The coupling reagents (HOBt and HBTU) as well as the Wang resin were obtained from Quantum Biotechnologies (Montréal, Quebec, Canada). For peptide synthesis using the Boc chemistry, the chloromethyl resin Bio-Beads S-X1 was purchased from Bio-Rad (Hercules, CA), and the BOP coupling reagent was obtained from Albatros Chem. Inc. (Montréal, Quebec, Canada). The fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA was purchased from Peptides International (Louisville, KY).

Peptide Synthesis. Synthesis of all peptides except for peptides VC, VD, VIIC, and VIID (Table 1) was performed on a manual multireactor peptide synthesizer using the HBTU/HOBt-mediated Fmoc chemistry. The following side-chain protecting groups were used: Boc for Lys; Pbf for Arg; *t*-Bu for Ser, Thr, Glu, Asp, and Tyr; and Trt for Cys. The first amino acid was anchored to the resin as described previously (*25*) and the peptide chain elongated using a 3-fold excess of amino acid derivatives in DMF in the presence of DIEA (5 equiv; Aldrich Chemical, Oakville, Ontario, Canada). Following a 30 min reaction, coupling was monitored with a ninhydrin test. If needed, that step was repeated using a 1.5-fold amino acid derivative. At each step, the N-terminal Fmoc-protecting group was removed using 20% (v/v) piperidine in DMF. Following completion of the synthesis, the resin was extensively washed with DMF, *tert*-amyl alcohol, acetic acid, methanol, and finally ether prior to being dried under vacuum. The bound peptides were then cleaved from the resin and fully deprotected by treatment for 2 h at room temperature with a solution containing 2.5% (v/v) 1,2-ethanedithiol, 2.5% (v/v) water, and 2% (w/v) phenol in trifluoroacetic acid.

To introduce the aminomethylene bond, $\psi[CH_2NH]$, between P1Arg−P1′Glu (peptides VC, VD, VIIC, and VIID; Table 1), an argininal derivative was first synthesized starting from Boc-$(Z)_2$-Arg (Chem-Impex International, Wood Dale, IL) as previously described (*26*). Progress of the reaction first into the dimethylhydroxamate, Boc-$(Z)_2$-Arg-N(Me)-OMe, and then into the desired aldehyde form through

[1] Abbreviations: Acm, acetamidomethyl; AMC, 7-amino-4-methylcoumarin; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate; BSPI-2, barley serine proteinase inhibitor 2; Bzl, benzyl; CMe, carboxymethyl; DCM, dichloromethane (methylene chloride); DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HCCA, α-cyano-4-hydroxycinnamic acid; HPLC, high-performance liquid chromatography; hPC1/3 or mPC1/3, human or murine proprotein convertase 1/3; hfurin, human furin; vv:hfurin, vaccinia virus recombinant human furin; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluophosphate; HOBt, *N*-hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MCA, 4-methylcoumaryl-7-amide; NMR, nuclear magnetic resonance; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Pbf, pentafluorophenyl; pMeBzl, *p*-methylbenzyl; TFA, trifluoroacetic acid; *t*-Bu, *tert*-butyl; Tos, tosyl (4-toluenesulfonyl); Trt, trityl (triphenylmethyl); Z, benzyloxycarbonyl; 2Cl-Z, 2-chlorobenzyloxycarbonyl.

Table 1: Amino Acid Sequence of BSPI-2 Analogues[a]

peptide	sequence
I	H-Val$_{53}$-Gly$_{54}$-Thr$_{55}$-Ile$_{56}$-Val$_{57}$-Thr$_{58}$-Met$_{59}$-Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-Val$_{66}$-Arg$_{67}$-Leu$_{68}$-Phe$_{69}$-Val$_{70}$-OH
II	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-Ile$_{56}$-Val$_{57}$-Thr$_{58}$-Met$_{59}$-Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
IIIA	H-**Cys(Acm)**$_{53}$-Gly$_{54}$-Thr$_{55}$-Ile$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(Acm)**$_{70}$-OH
IIIB	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-Ile$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
IVA	H-**Cys(Acm)**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(Acm)**$_{70}$-OH
IVB	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
IVC	H-**Cys(Acm)**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$$\psi$**[CH$_2$NH]**Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(Acm)**$_{70}$-OH
IVD	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$$\psi$**[CH$_2$NH]**Glu$_{60}$-Tyr$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
VA	H-**Cys(CMe)**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(CMe)**$_{70}$-OH
VB	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH**Cys**$_{70}$-OH
VC	H-**Cys(CMe)**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$$\psi$**[CH$_2$NH]**Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH**Cys(CMe)**$_{70}$-OH
VD	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$$\psi$**[CH$_2$NH]**Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
VIA	H-**Cys(CMe)**$_{53}$-Gly$_{54}$-Thr$_{55}$-Ile$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(CMe)**$_{70}$-OH
VIB	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-Ile$_{56}$-Val$_{57}$-Thr$_{58}$-**Arg**$_{59}$-Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
VIIA	H-**Cys(CMe)**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-**Lys**$_{58}$-**Arg**$_{59}$-Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(CMe)**$_{70}$-OH
VIIB	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-**Lys**$_{58}$-**Arg**$_{59}$-Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH
VIIC	H-**Cys(CMe)**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-**Lys**$_{58}$-**Arg**$_{59}$$\psi$**[CH$_2$NH]**Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys(CMe)**$_{70}$-OH
VIID	H-**Cys**$_{53}$-Gly$_{54}$-Thr$_{55}$-**Arg**$_{56}$-Val$_{57}$-**Lys**$_{58}$-**Arg**$_{59}$$\psi$**[CH$_2$NH]**Glu$_{60}$-**Lys**$_{61}$-Arg$_{62}$-Ile$_{63}$-Asp$_{64}$-Arg$_{65}$-**Thr**$_{66}$-Arg$_{67}$-**Ser**$_{68}$-Phe$_{69}$-**Cys**$_{70}$-OH

[a] Peptide numbering corresponds to positions 53−70 constituting the inhibitory loop in the native BSPI-2 molecule (peptide I; *17*, *18*). Peptide II corresponds to the inhibitory loop synthesized by Leatherbarrow and Salacinski (*24*). Substitutions of amino acids from peptide I are indicated in bold characters.

reduction with lithium aluminum hydride ($LiAlH_4$) was monitored using thin-layer chromatography on silica gel plates in an ethyl acetate−hexane (2:3) solvent system. The final product (obtained in a 58% yield) as well as the intermediate form were characterized by NMR using a Varian VXR-400S instrument.

Synthesis of peptides VC, VD, VIIC, and VIID (Table 1) was performed on a manual multireactor peptide synthesizer using BOP coupling reagent in DMF and using a 3.5-fold excess of amino acid derivatives. The following side-chain protecting groups were used: 2Cl-Z for Lys; Tos for Arg; Bzl for Ser, Thr, Glu, and Asp; and pMeBzl for Cys, respectively. The first amino acid was anchored to the resin through the cesium salt procedure (*27*) whereas the protected argininal derivative was incorporated through reductive alkylation (*28*) using sodium cyanoborohydride (*29*). After Boc removal with 40% (v/v) TFA in DCM, the peptide chain was elongated using a 45 min coupling step (repeated if needed) by means of a 3-fold excess of amino acid derivatives in DMF containing DIEA. The bound peptides were then cleaved from the resin and fully deprotected by a 1 h treatment at 0 °C with liquid hydrofluoric acid (HF; 10 mL/g of resin) in the presence of *m*-cresol (10%) and dimethyl sulfide (10%). Deprotected and cleaved peptides were solubilized in TFA. The solution was evaporated, and the peptide was precipitated with ether.

Peptide Purification and Chemical Characterization. The crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a semipreparative Delta Pak C_{18} (30 × 1.5 cm, Waters Associates, Mississauga, Ontario, Canada) and a Waters Associates Prep LC500A chromatography system. The aqueous system consisted of 0.06% (v/v) TFA in water solution while the organic phase was acetonitrile, also containing 0.06% (v/v) TFA. The elution was carried out by using a linear gradient from 5% to 55% organic phase in 120 min; the flow rate was adjusted to 40 mL/min. The separation was monitored by UV absorbency at 230 nm, and collected fractions were analyzed by MALDI-TOF spectroscopy. In all cases, the identified peptides were further purified using a Jupiter C_{18} analytical column (25 × 0.46 cm; Phenomenex, Torrance, CA) on a Beckman HPLC coupled to a diode array detector (Beckman, Mississauga, Ontario, Canada). The elution was carried out with a 20% to 60% linear gradient of organic phase in 20 min and at a flow rate of 1.5 mL/min. The isostere bond-containing peptides were purified using an identical solvent system, first using a semipreparative Jupiter C_{18} column (21 × 2.5 cm) with a linear gradient from 0% to 25% organic phase at a flow rate of 20 mL/min and then by a final purification step on an analytical Vydac C_{18} column (The Separation Group, Hesperia, CA) using a linear gradient from 10% to 50% organic phase in 30 min at a flow rate of 1 mL/min. In the latter case, purification was accomplished using a Varian 9010/9050 chromatography system.

The peptide purity and concentration were determined by quantitative amino acid analysis following 18−24 h hydrolysis in the presence of 5.7 N HCl in vacuo at 110 °C on a Beckman autoanalyzer, model 6300, with a postcolumn ninhydrin detection system coupled to a Varian DS604 integrator/plotter. The identity of each purified peptide was confirmed by mass spectral analysis using a Voyager DE-Pro MALDI-TOF instrument (PerSeptive Biosystems, Cambridge, MA). For MS analysis, the peptides in 0.1% TFA were mixed with a freshly prepared solution containing α-cyano-4-hydroxycinnamic acid (10 mg/mL) in 50% (v/v) acetonitrile−0.3% TFA and left to dry as a droplet on the sample plate. Alternatively, when particularly small concentrations of peptides were to be detected, peptide solution was mixed with HCCA (40 mg/mL in acetone), nitrocellulose

(Aldrich, 20 mg/mL), and 2-propanol in a 2:1:1 ratio (*30*).

Peptide Cyclization and Reduction/Alkylation. To quantitatively transform the linear form into the cyclic form, it was found necessary to incubate the peptide mixture for 30 min at 30 °C in ammonium hydroxide (pH 9.7). Conversely, to obtain quantitatively the linear form, it was found necessary to first reduce the cystine residue by incubation for 30 min using 10 mM dithiothreitol in 0.4 M Tris-HCl, pH 8.4, in the presence of 6 M freshly dissolved urea and then to fully alkylate the thiol using 40 mM iodoacetic acid. The resulting carboxymethylated peptides were purified by RP-HPLC as above described using an analytical CSC-Exsil C_{18} column (25 × 0.46 cm; Chromatographic Sciences Co., St. Laurent, Quebec, Canada) and a 5 min isocratic step at 100% aqueous phase at 1.0 mL/min followed by a linear gradient from 0% to 60% organic phase in 60 min.

Enzymatic Assays and Kinetic Analysis. All enzymatic assays were performed using either initial rate assays and/or stopped-time assays at room temperature in a final volume of 100 μL in 96-well flat bottom white plates (Dynex Technologies, Chantilly, VA). For hfurin, the final assay conditions were 50 mM sodium acetate or 0.1 M Tris-HCl buffer, pH 7.0, in the presence of 1 mM $CaCl_2$, whereas for mPC1/3, the conditions were 50 mM sodium acetate, pH 6.0, in the presence of 10 mM $CaCl_2$. Unless stated otherwise, all assays were performed using a final concentration of 50−100 μM pGlu-Arg-Thr-Lys-Arg-MCA substrate. The release of the fluorescent AMC was quantitated on-line with a Perkin-Elmer model LS50B spectrofluorometer (Beaconsfield, Buckinghamshire, U.K.) using an excitation and an emission wavelength of 370 ± 5 nm and 460 ± 3 nm, respectively. For the determination of rate in the absence and in the presence of inhibitor, emitted fluorescence measurements were made at five to ten time points in at least duplicate fashion. For measurement of both K_i (inhibition constant) and IC_{50} (concentration necessary to achieve 50% inhibition of enzymatic 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 the program Enzfitter (Elsevier-Biosoft, Cambridge, U.K.) or using the Enzyme Kinetic V1.0 module (SigmaPlot 2000 for Windows V6.1; SPSS Inc., Chicago, IL). In the case of K_i being derived from the IC_{50} value, the following equation, applicable for reversible and competitive inhibitor, was used: $K_i = IC_{50}/(1 + [S]/K_M)$, where [S] represents the substrate concentration and K_M is the Michaelis−Menten constant (*31*). The K_M values used were 8.0 μM for mPC1/3 (*32*, *33*) and 5.9 μM for hfurin (*34*), respectively.

All peptides examined (Table 1) contained possible sites of cleavage (Arg-Xaa-Lys/Arg-Arg) within their sequences and as such may be cleaved by PC1/3 and furin. To investigate this possibility, peptides (20 μg) were incubated at room temperature with enzymatically active hfurin or mPC1/3 as previously mentioned. Aliquots were withdrawn at different intervals, and the enzymatic digestion was stopped with glacial acetic acid. Samples containing the peptide fragments were cleaned using disposable ZipTip C_{18} (Millipore Corp., Bedford, MA) prior to their being mixed with an HCCA solution (40 mg/mL in acetonitrile) and identification by MALDI-TOF analysis.

Recombinant Enzymes. Initially, the recombinant hPC1/3 and hfurin endoproteases used were obtained from the medium of GH_4C_1 cell line following infection with the respective vaccinia viruses and partially purified through anion-exchange chromatography prior to use as described (*35*). In the later part of the study, the purified 71 kDa mPC1/3 form obtained from the medium of Sf9 insect cells following infection with full-length mPC1/3 encoding baculovirus was used (*32*), and recombinant hfurin was obtained from the medium of either Sf9 insect cells (*33*) or Schneider 2 cells (*36*). The enzymatic activity of each recombinant convertase was monitored by fluorometric assays using the fluorogenic substrate (*35*).

Model Building of the Catalytic Domain of PC1/3 and BSPI-2. The model of the catalytic domain of mPC1/3 was built upon the molecular coordinates of the furin model kindly given by Dr. R. J. Siezen (NIZO, The Netherlands) (*37*). The furin model was subjected to conjugate gradient minimization prior to manipulations. On the basis of the extensive amino acid sequence alignment previously reported (*37*, *38*), the furin sequence was mutated into the PC1/3 catalytic domain one amino acid at a time. The backbone dihedral angles and the side chains of each amino acid were adjusted until an acceptable conformation was obtained. All calculations have been carried out using SYBYL (Tripos Associates, St. Louis, MO) on a Silicon Graphics Indigo^2 Extreme workstation as described previously (*39*). Computation of the three-dimensional structure of the BSPI-2 cyclic loop was done as reported previously on the basis of the crystallographic data (*40*).

RESULTS

Preliminary Data Using Peptides Containing P1Arg and P1/P4Arg Substitutions. Initial studies were accomplished using the structure labeled as peptide I by Leatherbarrow and Salacinski (*24*) which, in addition to the proposed Val_{66} to Thr and Leu_{68} to Ser substitutions suggested for improved solubility, was terminated at both ends by Cys residues, occupying positions 53 and 70, enabling cyclization. Tridimensional structure computation and minimization studies using both solution and crystal structures of BSPI-2 (*19*−*23*) enabled us to confirm that substitutions at other positions not involved in maintaining the integrity of the loop structure were possible (*40*). Thus, Met_{59} and Ile_{56} were replaced by an Arg residue, leading to the structures shown as peptide IIIA and IVA, respectively. Peptide IVA was shown to behave as a substrate for both furin and PC1/3, but peptide IIIA was almost completely left intact by either. Furthermore, despite the presence of numerous basic residues in the linear peptides, the only site of cleavage observed was C-terminal to Arg_{59}; hence this cleavage was done in the context of the Arg-Xaa-Xaa-Arg sequence, in agreement with our previous data with smaller synthetic substrates (*41*). However, attempts at cyclizing these Trt-protected peptides directly on the resin after treatment with reagent K (for Trt-protected Cys) according to ref *42* led to the formation of multimers whereas treatment of purified Trt-deprotected peptides with methanolic iodine led to very low yields. Similar treatment first by TFA−phenol−water (for ACM-protected Cys) followed by methanolic iodine oxidation led to formation of the desired products as shown by MS and amino acid analysis (*40*; data not shown). Despite the small amount of the cyclic peptides

obtained, we showed that PC1/3 was inhibited, albeit weakly, by peptide IIIB but not by peptide IIIA nor by peptide IVB (*40*).

Subsequently, we attempted the synthesis of peptide IVC incorporating an aminomethylene bond between positions 59 and 60, but the synthesis proved difficult, yielding numerous internally truncated peptides (A. Fournier and C. Lazure, unpublished data). Nevertheless, small amounts of purified peptide IVC were obtained, and the peptide was shown to behave as a fully competitive inhibitor with a determined K_i of 4.1 ± 1.4 μM for furin and of 107 ± 16 μM for PC1/3, a 26-fold difference between the two enzymes. On the other hand, peptide IVD behaved as a competitive inhibitor against furin with a computed K_i of 12.3 ± 1.6 μM, but the low available amount precluded assaying with PC1/3 (data not shown). All of these data hinted that small octadecapeptides, as shown with subtilisin BPN′ (*24*), could be made as inhibitors of two members of the convertase family.

Design, Synthesis, and Purification of Peptides Containing P1Arg/P2′Lys, P1/P4Arg/P2′Lys, and P1/P4Arg/P2/P2′Lys Substitutions. Whereas peptides IIIA and IIIB were used as controls, three other peptides were prepared, all containing a P2′ substitution of Tyr_{61} by Lys in addition to previously mentioned substitutions (Table 1). Peptides VIIA, VIIB, VIIC, and VIID also contained a P2 substitution replacing Thr_{58} by Lys in order to reconstitute the widely occurring pair of basic residues encountered in natural convertase substrates. Incorporation of a P2′ Lys was justified by numerous factors. First, we have shown in PTH-related peptidyl sequences that a peptide [FS-10 (*43*)] containing this substitution was a mixed-type inhibitor of furin though it behaves as a substrate for PC1/3. Second, we prepared numerous dodecapeptides on the basis of this study, incorporating this residue, indicating that it was beneficial in terms of inhibiting potency (H. Bennett and C. Lazure, unpublished data). Third, as discussed in ref *43*, it is noteworthy that no P2′ Lys-containing potential substrates was ever shown to be cleaved by furin. This observation must also be confronted with the fact that, on the basis of modeling of the catalytic domain of furin (*37*, *38*), an Asp residue, occupying position 303 and absent in PC1/3 and PC2 but present in PC4, PC5/6, PC7, and PACE-4, resides in the S2′ site where it could interact with the amine function of the Lys residue. Last, it was shown in the case of cyclic peptides encompassing the reactive site loop of the Bowman−Birk proteinase inhibitor that positively charged residues were tolerated and the resulting peptides displayed low K_i values upon assaying with trypsin, an enzyme cleaving after basic residues (*44*).

Following solid-phase synthesis of all peptides and removal of all protecting groups, we noticed during purification of all four peptides (belonging to the III, V, VI, and VII series) a marked tendency to undergo spontaneous cyclization as evidenced by the appearance upon chromatography of two fractions as exemplified with peptide of the III series (Figure 1a). The early eluting one corresponds to the cyclic form (R_t, 37.3 min; MW_{obs}, 2176.3 as determined by MALDI-TOF) whereas the later eluting one is the linear form (R_t, 38.1 min; MW_{obs}, 2178.3). The identity of each peptide form was further confirmed following complete conversion into the cyclic form (Figure 1b) or following treatment using 10 mM DTT (Figure 1c). One notes that, even using a large excess of reducing agent, a nonnegligible amount of the cyclic form is still present in the sample, rendering mandatory the treatment with an alkylating agent following reduction. This chromatographic behavior was observed in our preliminary studies (*40*) as well as having been reported by Bolin et al. (*45*).

On the basis of initial inhibition screening (see below), two peptides displaying interesting characteristics were chosen and synthesized again, this time using the Boc chemistry in order to allow insertion of an aminomethylene bond between Arg_{59} and Glu_{60}. Incorporation of this bond was accomplished first through synthesis of the *O*,*N*-dimethylhydroxamate derivative of Boc-$(Z)_2$-Arg according to Guichard et al. (*26*), which was obtained in 60% yield and whose identity was fully confirmed by NMR analysis (data not shown). Conversion into the Boc-$(Z)_2$-argininal by reduction was much less successful as analyzed by thin-layer chromatography where, in addition to the desired product, unreacted starting material as well as likely degradation products could be seen. Nevertheless, enough aldehyde derivative was recovered as to prepare in limited amount peptides VC, VD, VIIC, and VIID. The presence of the isostere bond was confirmed through amino acid analysis, whereupon one less Arg and the complete absence of a Glu residue were seen, and also MALDI-TOF analysis, which yielded MW_{obs} of 2164 (MW_{calc} 2166) and 2191 (MW_{calc} 2193) for peptides VC and VIIC prior to alkylation with iodoacetic acid, respectively (data not shown).

Peptides Containing P1Arg/P2′Lys, P1/P4Arg/P2′Lys, and P1/P4Arg/P2/P2′Lys Substitutions Are Transient Inhibitors Being Cleaved by Both Enzymes. The peptides that were synthesized without the isostere bond were shown, in both their linear and cyclic forms, to be temporary inhibitors of furin and PC1/3. Indeed, after a certain amount of incubation time, varying from 40 to 60 min with PC1/3 up to 240 min with furin, we observed a significant increase in the slope of fluorescence intensity, meaning that the enzyme was gradually recovering its normal activity. An example of such behavior is given in Figure 2 for the peptide VA incubated with PC1/3 in the presence of 100 μM competing substrate. We hypothesized that the preferential cleavage site of the peptides would be at the P1−P1′ bond (Arg_{59}−Glu_{60}). To verify this, 20 μg of each peptide was incubated in the presence of active furin or PC1/3 in the appropriate conditions and in the absence of any other substrate. Small aliquots of the incubation media at different intervals of time were taken up, the acidified samples cleaned by passage through a Zip-Tip, and the eluted peptides analyzed using MALDI-TOF mass spectrometry. Figure 3 shows the example with peptide VA, from which a fragment of MW 1469.2 (expected 1468.6) was generated by furin after a 360 s incubation. The 849.92 counterpart fragment was not detected in this instance. However, we easily detected the two peptide fragments from VB, after treatment with β-mercaptoethanol which corresponded to MW 792.9 and 1411.3 (expected 791.9 and 1410.6; data not shown). Finally, for all other peptides, the fragments generated with both enzymes furin and PC1/3 were identified by MALDI-TOF analysis and corresponded to the sequences N- and C-terminal to the P1−P1′ bond (data not shown). However, it should be mentioned that, upon much longer incubation times, some other sites of cleavage are used to a limited extent; nevertheless, cleavage of the P1−

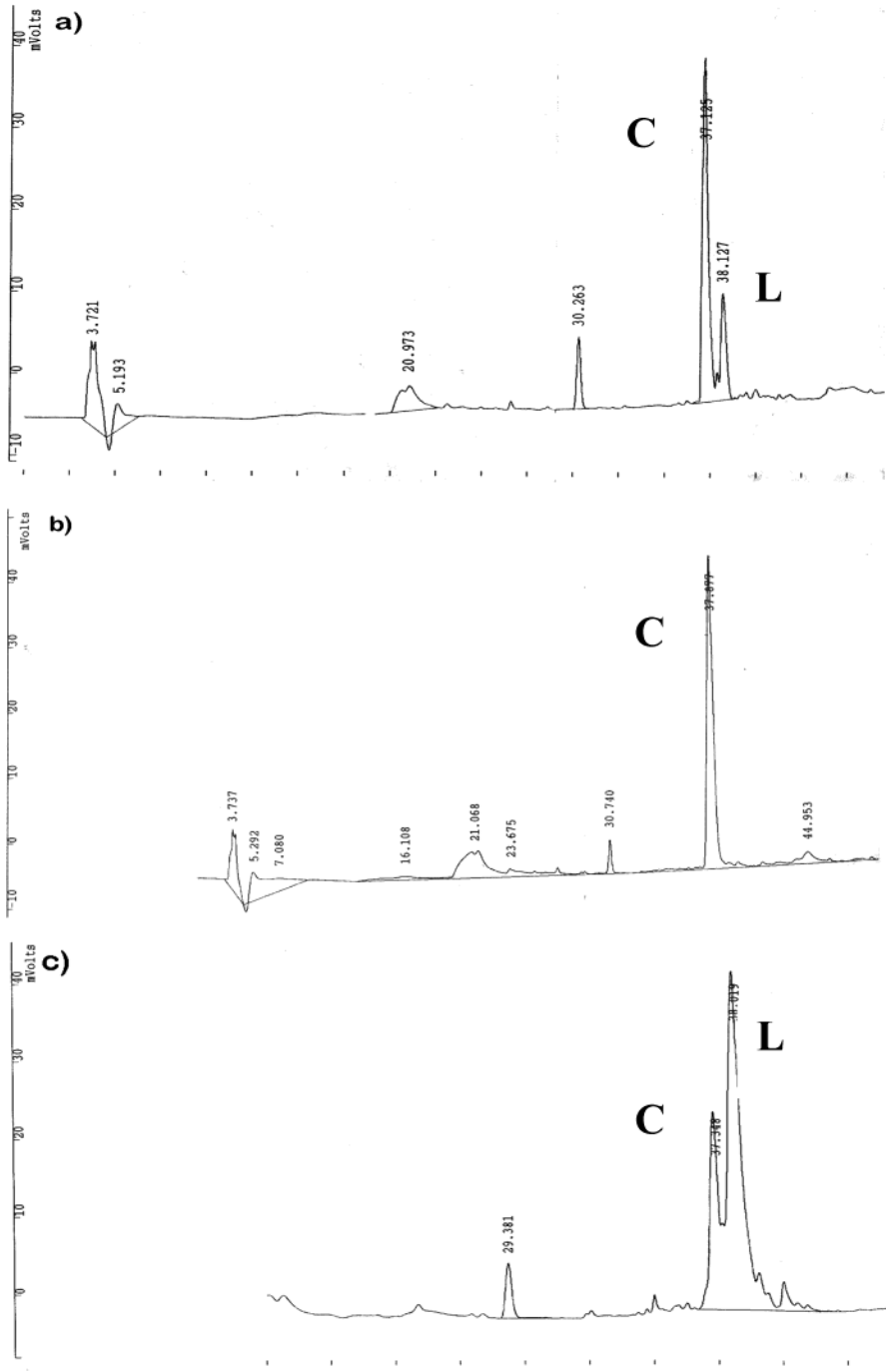

FIGURE 1: HPLC separation of the various forms of the P1Arg-containing peptide: (a) immediately following synthesis and subsequent purification, (b) following incubation, yielding peptide IIIB at pH 9.7 at 40 °C for 30 min, and (c) following incubation in the presence of 10 mM DTT for 30 min. The cyclic form corresponds to the early eluting peak labeled C whereas the linear form is the later eluting one labeled L.

P1′ bond is always the first to occur and hence the preferred one.

Peptides Containing P1Arg/P2′Lys, P1/P4Arg/P2′Lys, and P1/P4Arg/P2/P2′Lys Substitutions Display Different Inhibition Mechanisms. Though cleavage of these peptides complicated greatly the acquisition of inhibition parameters, it was nevertheless possible to analyze them. To do so, conditions were chosen so as to render negligible, based upon MALDI-TOF analysis, degradation of the inhibitors. As expected, peptides IIIA and IIIB, despite the fact that they contain four Arg residues, proved to be unrecognized and/or remained uncleaved by either enzyme. On the other hand, including an Arg at position P4 led in both cases to significant inhibition as can be seen in Table 2. Interestingly, addition of a P2′ Lys, in the absence of the P4 Arg, as done in peptides VIA and VIB, was able to confer inhibition properties to the control sequence represented by peptides IIIA and IIIB. This could be explained either by the fact that this Lys is able to occupy the S2′ site as previously discussed, hence improving recognition of the peptide, and/or by the fact that insertion of the Lys creates an Arg-Glu-Lys-Arg sequence in the peptide. As previously mentioned, this sequence represents an ideal one for members of the convertase family. However, we did not observe any cleavage

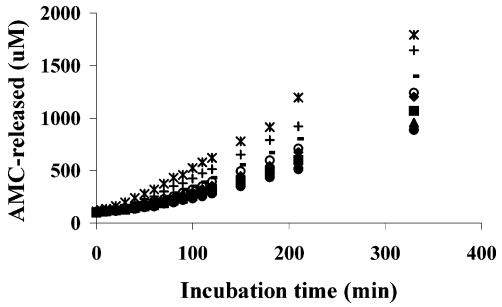

FIGURE 2: Release of AMC by PC1/3 incubated with 100 μM pERTKR-MCA fluorogenic substrate and different concentrations of peptide VA: ×, 0 μM; +, 0.3 μM; −, 0.6 μM; ○, 0.9 μM; ◆, 1.2 μM; ■, 1.5 μM; ▲, 1.8 μM; ●, 2 μM.

of the peptide upon long-term incubation, which we should have observed if this sequence was now preferentially recognized. This could be explained by the fact that Arg_{62} is itself involved in stabilization of the inhibitor loop by forming an hydrogen bond with the side chain of Asp_{64} (*24*) and hence would not be available to interact with the active site. Alternatively, the residue C-terminal to the Arg_{64} residue is an Ile and would correspond to the P1′ position. In the latter case, it has been reported that the presence of a hydrophobic residue with an aliphatic side chain is not favored at this position (*41*, *46*). Interestingly, peptides VA, VB, VIA, VIB, VIIA, and VIIB exhibited identical modes of inhibition for PC1/3 and were much more potent (as confirmed by their increased cleavage during long inhibition) than in the case of hfurin. The behavior of the same peptides toward furin was quite different, as can be seen in Table 2 and shown, for example, for peptides VIIA and VIIB, in Figure 4. Indeed, peptides VA, VB, VIA, and VIB behaved as mixed inhibitors, with peptides VA and VB being better likely by virtue of possessing a P4 Arg residue. In the case of peptide VIIA (K_i of 4.5 ± 0.5 μM), it clearly exhibits a noncompetitive behavior toward furin as demonstrated with Dixon (Figure 4, top left) and Cornish−Bowden representations (Figure 4, top right) whereas peptide VIIB (K_i of 3.9 ± 0.4 μM) appears to behave much more like a competitive or mixed-type inhibitor using identical representations (Figure 4, bottom left and bottom right). As previously mentioned, numerous synthetic peptides have been observed to behave

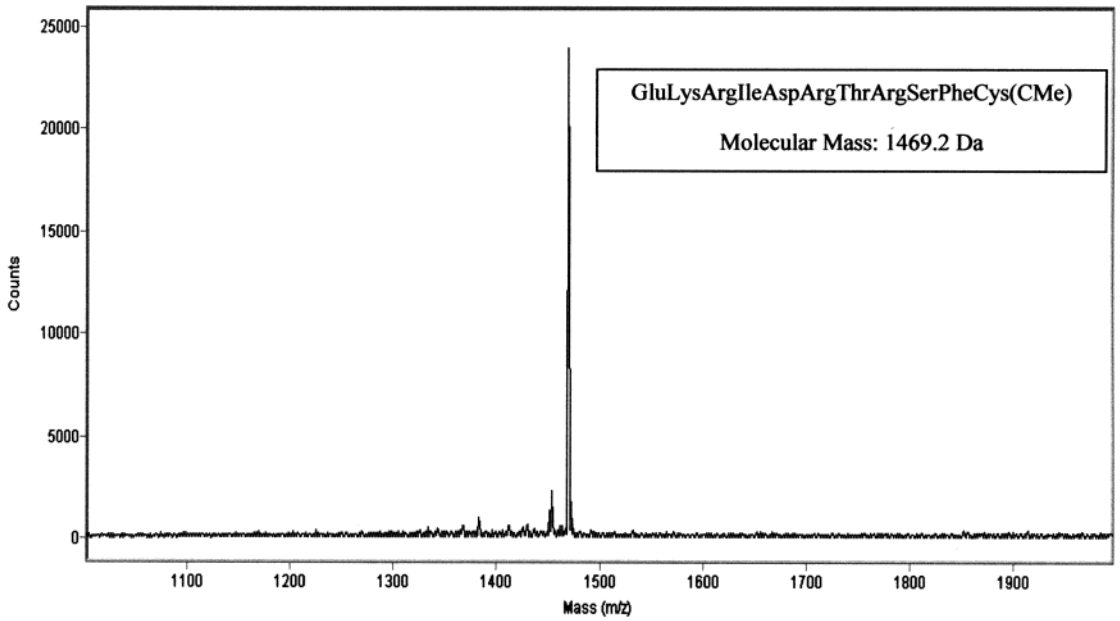

FIGURE 3: Mass spectrum of the C-terminal fragment of peptide VA following cleavage by hfurin. Incubation was done at room temperature in 50 mM sodium acetate buffer containing 1 mM $CaCl_2$ at pH 7.0. This sample was removed after 360 s, cleaned on a small C18 column, and mixed with HCCA for MALDI-TOF analysis. The sequence of the fragment is indicated, and the molecular mass includes the weight of one of the carboxymethyl groups that was added to the extremities of the linear peptide.

Table 2: Inhibition Constants (K_i) of BSPI-2 Analogues toward hfurin and mPC1/3

	hfurin				mPC1			
peptide	IC_{50} (μM)	K_i (μM)	K_i' (μM)	mode of inhibition	IC_{50} (μM)	K_i (μM)	K_i' (μM)	mode of inhibition
VA		9.2	39.5	mixed inhibitor	1.3	0.4		competitive[a]
VB		14.4	40.7	mixed inhibitor	1.7	0.5		competitive[a]
VIA		35.3	44.6	mixed inhibitor	0.8	0.2		competitive[a]
VIB		66.0	87	mixed inhibitor	3.0	0.9		competitive[a]
VIIA		4.0		noncompetitive	3.2	0.6		competitive[a]
VIIB		3.9		competitive	1.1	0.3		competitive[a]
VC		23.0		competitive				
VD		17.0		competitive				
VIIC		3.2		competitive				
VIID		0.8		competitive				

[a] In these cases, the mode of inhibition was considered strictly competitive as determined using peptides VIIA and VIIB, and the indicated K_i values were computed from the IC_{50} as described in Materials and Methods.

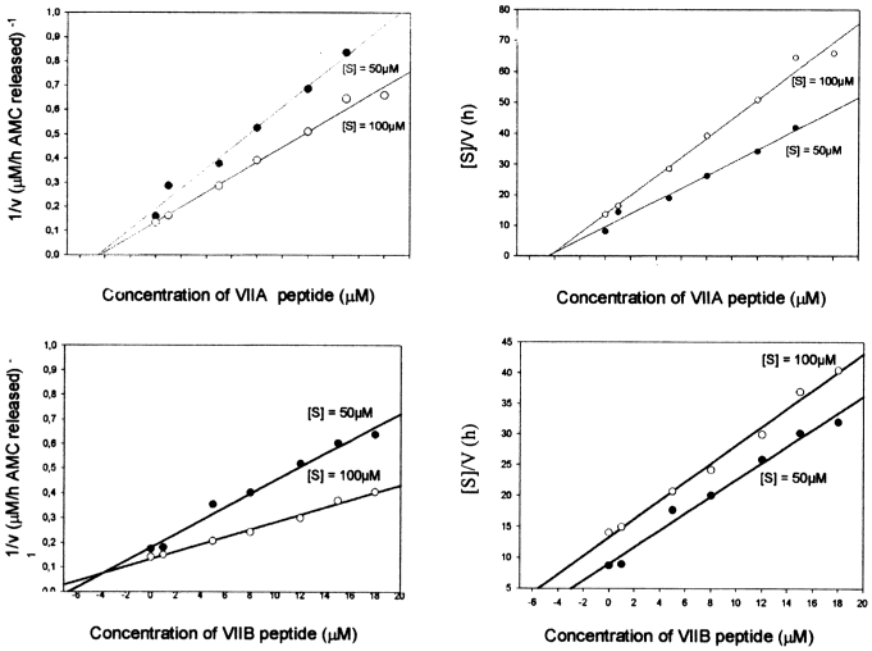

FIGURE 4: Inhibition of hfurin by peptide VIIA (top) and VIIB (bottom) in the presence of the fluorogenic substrate pERTKR-MCA using Dixon (left part) and Cornish−Bowden (right part) representations. *V* represents the rate of the reaction, and [S] is the concentration of the fluorogenic substrate.

as mixed-type inhibitors and/or to contribute to inhibition at high substrate concentration, implying that possibly a second binding site could be present in furin (*43*, *47*).

Peptides Containing an Isostere P1−P1′ Bond Are Selective and Potent Inhibitors of Furin. The two most potent inhibitors observed in this study were synthesized again, and an isostere bond of the aminomethylene type was inserted at the site of cleavage observed upon long-term incubation. Insertion of this bond completely abolished cleavage by PC1/3 and furin, and the resulting peptides proved to be stable upon both short or long incubation (data not shown). Furthermore, as expected, peptides VIIC and VIID in this modified form were also much better inhibitors than peptides VC and VD, yet again confirming that inclusion of the P2 Lys was beneficial. Finally, it was possible to determine the inhibition values for furin as seen in Table 2. As shown, insertion of the isostere bond did not appreciably change the K_i value obtained with peptide VIIA though peptide VIIC **(**(K_i of 3.2 ± 0.5 μM) exhibited now a strictly competitive mode of inhibition. On the other hand, peptide VIID (K_i of 0.8 ± 0.5 μM) proved to be much more potent. In the same conditions, both isostere-containing peptides proved to be totally unreactive up to 30 μM toward PC1/3, although these peptides, prior to introduction of the isostere, were much more potent toward this enzyme than toward furin (Table 2).

DISCUSSION

In this study, the inhibitory properties of small peptides derived from the barley serine proteinase inhibitor 2 loop were assessed with respect to the proprotein convertases PC1/3 and furin. The approach was based on the fact that PCs possess a subtilisin-like catalytic domain and hence could prove reactive toward the known subtilisin inhibitor BSPI-2 after suitable modifications. Furthermore, preliminary results showed that such peptides (IIIA, IIIB, IVA, and IVB; see Table 1), presenting specific mutations corresponding to the PC substrate affinity, could behave as potent inhibitors of PC1/3 and furin (*40*, *42*). Here some new sequences were prepared, incorporating changes at the P4, P2, and P2′ positions (V, VI, and VII peptide series in Table 1), and these peptides, in their linear or cyclic forms, displayed large differences in their inhibiting properties, varying in both their inhibiting constants and modes of inhibition toward the two enzymes. Peptides VA, VB, VIIA, and VIIB proved to be the most potent inhibitors though they were all found to be only temporary ones as they were slowly cleaved over long-term incubation with PC1/3 and furin. We thus synthesized again these peptides, this time replacing the P1−P1′ amide bond, identified as the scissile bond, by a nonpeptidic aminomethylene ψ[CH_2−NH] bond. The latter proved to be quite potent inhibitors of furin without being cleaved, as expected, but PC1/3 was not at all inhibited by any at the same concentrations.

In the original 18 amino acid peptide first synthesized by Leatherbarrow and Salacinski (*24*) on the basis of the structure of the BSPI-2 active site loop, some residues, especially Arg_{65}, Arg_{67}, and Phe_{69}, were conserved because they were considered primordial for the structural integrity of the loop. However, Val_{66} and Leu_{68} were substituted by Thr and Ser, respectively, to overcome solubility problems. In addition, Val_{53} and Val_{70}, being in close proximity to each other, were replaced by Cys residues to permit easy cyclization of the peptide through disulfide bridge formation. While taking this sequence as a starting point for the synthesis of our own peptides, we conserved those convenient substitutions but made other changes at some strategic positions in order to incorporate the substrate specificity of the PCs (Table 1). Following synthesis, it was observed during the purification steps that peptides tend to naturally cyclize upon air oxidation. This observation demonstrated that the added modifications made to the original sequence were not

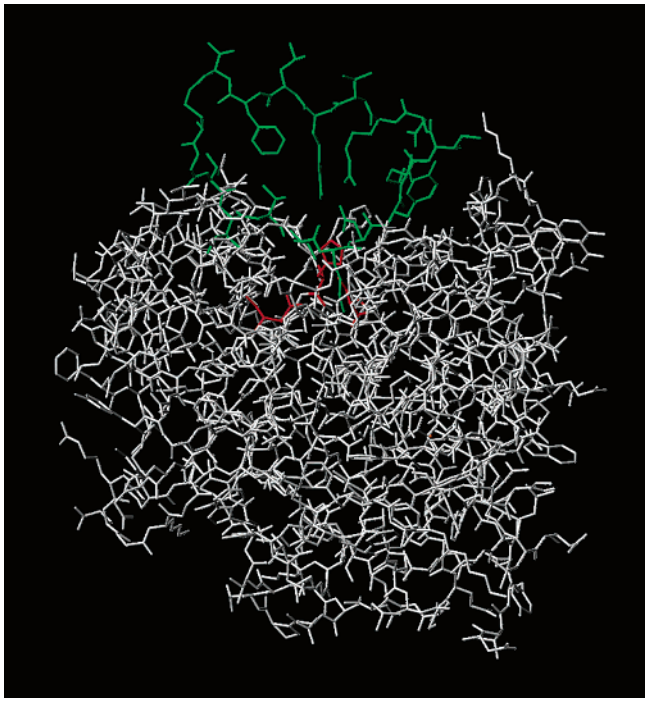

FIGURE 5: Model structure of the BSPI-2 loop inserted into the active site of PC1/3. All calculations and the final structure were obtained using the Sybyl software (see Materials and Methods). The inhibitor loop is shown in green and the enzyme in white with the exception of the residues in red composing the catalytic triad.

detrimental to the closing of the loop as we had originally noted during *in silico* modeling (*40*).

First, introduction of an Arg residue in the P1 position (Arg_{59}) in all peptides was essential for PC recognition. We also inserted a Lys in P2′ (Lys_{61}) in the V, VI, and VII peptide series which, when compared to peptide IIIA or IIIB, was able to confer on its own some inhibiting properties. The presence of a hydrophobic pocket at the S2′ subsite (*38*) is predicted and is also probable in the active site of PCs. Therefore, the long aliphatic side chain of Lys could insert itself in this pocket while its charged amino group could possibly interact through electrostatic interaction with one of the negatively charged residues situated in the bottom of the pocket (possibly Asp_{303} or Glu_{224} in the furin sequence). Among these three peptides, the V and VII peptide series containing Arg in P4 (Arg_{56}) were better inhibitors than VIA or VIB which had an Ile residue in that position, as seen in Table 2. This observation is largely confirmed by the demonstrated preference of PC1/3 and furin for an Arg-Xaa-Lys/Arg-Arg sequence. Finally, the Lys at the P2 site (Lys_{58}) contributed favorably in terms of inhibition of furin but much less so in the case of PC1/3. It is well established that PCs require at least one basic amino acid residue at the P2, P4, or P6 positions for optimal recognition of the substrate. The results obtained herein suggest that there could be an additive effect, at least for the P2 and P4 sites, when both are occupied by basic residues.

Cyclization of the peptides, in addition to increasing their resistance to eventual exopeptidase degradation, also contributes in maintaining the proper orientation of the residues for recognition and binding by the enzyme since it preserves the loop conformation adopted by the native inhibitor. This aspect is illustrated in Figure 5, which represents one possible mode of interaction between a model structure of PC1/3 and the structure of the BSPI-2 related loop. As a matter of fact, the crystallographic structure of subtilisin BPN′ with BSPI-2 (*21*) showed that inhibition occurs through high-affinity interaction that does not involve significant conformational changes in either partners whatsoever, though the relatively flexible loop becomes more ordered within the complex. In that respect, however, the results we obtained were not entirely those envisioned, as analysis of all the inhibition data showed no consistent trend in the differences between the inhibiting properties of the cyclic peptides relative to their carboxymethylated linear analogues. Depending on the sequence, it is quite clear at this stage that either counterpart could be the better candidate, hinting that these structures do not as yet exhibit the optimum interaction between the loop structure and the enzyme. Indeed, VA and VIA had smaller inhibition constants than their cyclic counterparts toward furin and PC1/3, whereas VIIB was a more potent inhibitor than its linear form of both enzymes. To establish a correlation between these results, we must also consider another factor, namely, the mechanism of inhibition. The most striking example is peptides VIIA and VIIB, whereby it behaved as a mixed or competitive inhibitor of furin in its cyclic form (VIIB) and as a noncompetitive one in its linear form (VIIA). However, both forms act in a competitive fashion with PC1/3. Hence, with the exception of peptide VIIB and both isostere-containing peptides, the constraint imposed by the cyclization does not contribute as yet in a positive manner to the inhibition properties exhibited by the more flexible linear counterparts.

The peptides incorporating the isostere bond between residues Arg_{59} and Glu_{60} proved to be considerably more potent with furin than with PC1/3. It is quite surprising and, at this stage, difficult to explain that the presence of the isostere bond would allow such obvious discrimination. This is especially true when one considers the relatively small effects of changes made in the sequence or through cyclization of the peptides. At this time, any explanation of this observation would be largely speculative. Inasmuch as the aminomethylene bond does not contain the carbonyl function of the normal peptide bond, it can always be surmised that there exist structural differences in the active site of furin and PC1/3 and that one of these differences could be the ability of forming an hydrogen bond with this carbonyl group. Another possibility could be that the increased rotational liberty around the aminomethylene bond as compared to the normally occurring peptide bond allows better contacts in the active site of furin. Once again, this can be taken as evidence of structural differences in the active site of PC1/3 and furin though these two members of the PC family were often shown to have similar enzymatic specificities. It can also be proposed that one way to improve on the specificity of peptide inhibitors for furin would be to include the use of an isostere bond. However, the type of bond to be used may represent a determinant factor. There is a wide range of backbone modifications that can replace the amide peptide backbone, for which the enzyme recognition process imposes various constraints, namely, the ψ, ϕ, χ, and ω torsion angles that can be adopted, the charge distribution between N- and C-termini, and the donor/acceptor abilities of the bond. A recent study conducted by Gupta and Payne (*48*) compared all of these parameters for a large selection of isostere bonds. In that list, the amino-

methylene $\psi[CH_2-NH]$ isostere bond is said not to be the ideal choice since it can exhibit torsional angles often occurring out of the range of a typical bond conformation. In addition, these different backbone torsions have an influence on the length of the bond, and a high percentage of the conformers would be too short to allow optimal stabilizing interactions with the residues inside the active site. It nevertheless keeps its hydrogen bond donor ability, although not perfectly oriented. In this study, we chose this bond mainly for practical purposes, especially its easier synthesis, but in future perspectives it would be interesting to try another type of isostere, one which would be closer to the standard conformational state and would thus possibly increase the binding affinity with no harm to specificity.

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