Single Amino Acid Substitution in the PC1/3 Propeptide Can Induce Significant Modifications of Its Inhibitory Profile toward Its Cognate Enzyme*

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The proprotein convertase PC1/3 is synthesized as a large precursor that undergoes proteolytic processing of the signal peptide, the propeptide and ultimately the COOH-terminal tail, to generate the mature form. The propeptide is essential for protease folding, and, although cleaved by an autocalytic process, it remains associated with the mature form acting as an auto-inhibitor of PC1/3. To further assess the role of certain residues in its interaction with its cognate enzyme, we performed an alanine scan on two PC1/3 propeptide potential cleavable sites (50RRSRR54 and 61KR62) and an acidic region 66DDD67 conserved among species. Upon incubation with PC1/3, the ensuing peptides exhibit equal inhibitory potency, lower potency, or higher potency than the wild-type propeptide. The Kᵢ values calculated varied between 0.15 and 16.5 nM. All but one mutant exhibited a tight binding behavior. To examine the specificity of mutants, we studied their reactivity toward furin, a closely related convertase. The mutation of certain residues also affects the inhibition behavior toward furin yielding propeptides exhibiting Kᵢ ranging from 0.2 to 24 nM. Mutant propeptides exhibited against each enzyme either different mode of inhibition, enhanced selectivity in the order of 40-fold for one enzyme, or high potency with no discrimination. Hence, we demonstrate through single amino acid substitution that it is feasible to modify the inhibitory behavior of propeptides toward convertases in such a way as to increase or decrease their potency, modify their inhibitory mechanisms, as well as increase their selectivity.

One of the most common methods used by cells to diversify the pool of their biologically active molecules is protein processing. Indeed, numerous secreted proteins are synthesized first as an inactive precursor, which is rendered biologically active upon cleavage at clusters of basic residues. Members of a family of proteins named proprotein convertases (PCs)⁵ primarily perform this cleavage. To date, seven members were described: furin, PC1/3, PC2, PACE4, PC4, PC5/6, and PC7/PC8/lymphoma proprotein convertase. Some of them are ubiquitously expressed such as furin, PACE4, and PC7, whereas others exhibit a more restricted expression pattern such as PC1/3 and PC2, which are solely present in endocrine and neuroendocrine tissues, and PC4, which is expressed only in germ cells. However, all of them belong to the larger family of serine proteases and are structurally related to bacterial subtilisins and yeast kexins (reviewed in Ref. 1). In terms of biological activities, numerous transfection experiments using recombinant enzymes and substrates, generation of knock-out animals as well as human cases of convertase deficiency pointed out the importance of convertases in crucial biological processes such as patterning during embryogenesis, angiogenesis, prohormone processing, tissue remodeling, and complement activation (2). Furthermore, some members are also implicated in many disease states, because they are able to activate various bacterial toxins and to process viral envelope glycoproteins needed for cell penetration (3). For all these reasons convertases represent attractive therapeutic targets.

Structurally, they share common features such as the presence of (i) a signal peptide guiding the protein to the secretory pathway, (ii) a propeptide (alternatively called prosegment, prodomain, or proregion), implicated in enzyme folding and inhibition, (iii) a catalytic domain that possesses the classic catalytic triad Asp, His, and Ser residues conserved among serine proteases, (iv) a P domain, which appears to regulate Ca²⁺ and pH dependence of the enzyme, and finally, (v) a COOH-terminal tail often defining the localization in organelles and cells. Such a topological distribution of structural regions makes a convertase an efficient self-controlled molecule whereby activation of its zymogen into its active form requires sequential removal of particular segments. This characteristic is best exemplified by the complex activation of PC1/3, which requires removal of the signal peptide, removal of the propeptide, and further cleavage of the COOH-terminal tail.

Among these excised regions, the propeptide plays a pivotal role in enabling efficient functional expression of convertases. Indeed, not only does it prevent undue and untimely activation, but also it is proposed to act as an intramolecular chaperone. Well documented with degradative subtilisins, this feat relies on intimate protein-protein interactions between the propeptide and its cognate enzyme. Hence, concomitantly with the interaction of the COOH terminus of the propeptide where the primary cleavage site is located and the catalytic site, the propeptide could provide the enzyme with a template for acquiring the correct active conformation. However, this role though firmly established with subtilisins has not been widely documented in the case of convertases with the exception of results obtained from expression of chimeric enzymes (4, 5) and mutagenesis of some furin and PC2 propeptide residues (6, 7). According to Fu et al. (8), the convertase propeptides are best classified as type I propeptides through their role in folding and inhibition of the enzyme. Following the correct folding of the active site,
an autocatalytic process leads to cleavage of the propeptide, which can
remain associated with the mature form, thus acting as an auto-inhibi-
tor (9, 10). Based upon the structural relatedness of proconvertases
and prosubtilins, it is predicted that a secondary cleavage site might
exist within the propeptide. Indeed, such a secondary cleavage is present
in the case of profurin (11), prokexin (12), pro-PC1/3 (13), and pro-PC2
(7). Proteolytic cleavage at this secondary site is responsible for disas-
sembly of the enzyme-inhibitor complex and for preventing further
inhibition of the cognate enzyme by an otherwise intact propeptide. In
most instances, these cleavages are sufficient to generate fully active
enzyme, an exception being the activation of pro-PC1/3, which requires
further cleavage in the COOH-terminal region.

Propeptides in the various convertases represent the first 80–100
residues following the signal peptide. Unlike the very well conserved
catalytic and P domains, they show very little sequence conservation
except in their COOH-terminal portion. They contain multiple basic
residues gathered in two regions namely, the region between positions
45 and 55 and the extreme COOH terminus (Fig. 1).4 The COOH-
terminally located basic cluster is strongly conserved and represents
the site of the first autocatalytic cleavage. In all other convertases, with
the exception of PC7, basic residues containing regions represent the site
of the second cleavage leading to the degradation of the propeptide and
the release of the fully enzymatically active enzyme. In vitro and overexpres-
sion studies showed that mutations or removal of the COOH-terminal
basic residues abolish the production of mature enzyme and prevent
inhibition of the active protease by the propeptide. Studies conducted
by various groups revealed that peptides derived from the middle por-
tion (near the second cleavage site) and the NH2-terminal domain of the
propeptide were weak inhibitors, whereas peptides derived from the
first cleavage site are very potent competitive inhibitors, often in the
nanomolar range (11, 14–16). Moreover, it was also shown that the
longer the COOH-terminally derived peptides the better was their
inhibitory capacity (11, 16, 17). Fugère and colleagues (18, 19) reported
similar results upon investigating the inhibitory potency of all conver-
tases propeptides against furin, PC5/6, and PC7. All these studies taken
jointly indicate that the COOH-terminal portion of the propeptides
confers a strong inhibitory potency but appears not to be very discrimi-
native toward its cognate enzyme. Conversely, the remaining NH2-
terminal part of the propeptide appears to be responsible for improved
selectivity as well as ensuring the tight fit necessary to form the
propeptide-enzyme complex as seen with furin and pro-PC1/3.

Herein we investigate the ability of certain residues within the
propeptide of PC1/3 to confer specificity for its cognate protease. To do
so, we performed individual alanine substitution of residues contained
within two potential PC1/3 convertase-cleavable clusters as well as sub-
stitution of three negatively charged residues, two being specific to
PC1/3. We tested the generated mutants in inhibitory assays against
PC1/3 and furin. Our results show that the generated mutants can be
subdivided in three categories, the first one exhibiting identical inhibi-
tion properties to the wild-type propeptide, the second having a dimin-
ished inhibition, and finally the third one exhibiting increased inhibi-
tion, with KI values ranging from 0.15 to 24 nM. Some of these mutations
appear to be advantageous in increasing the inhibitory potency of the
propeptide whereas some others clearly can affect the mechanism of
inhibition thus conferring selectivity. Finally, we confirm through
mutagenesis the location of the second cleavage site in the PC1/3
propeptide.

* * *

4 The numbering used herein corresponds to the pro-PC1/3 complete sequence devoid
of the signal peptide. Thus, the 83-residue propeptide corresponds to residues 1–83.
The same applies to the numbering used with other convertases.

**MATERIALS AND METHODS**

**Expression and Purification of Recombinant mPC1/3 and Human Furin**—Recombinant murine PC1/3 is produced using the baculovirus
expression system in Sf9 insect cells and whole larva as recently
described (20). Modifications to the original recombinant virus
described in Boudreault et al. (21) include substitution of the signal
peptide of mPC1/3 by the one of the viral glycoprotein 67 (gp67) to
enhance the secretion of the recombinant enzyme into the medium of
Spodoptera frugiperda cells. Once expressed, the enzyme is recovered
and purified as previously described (20, 21). The recombinant soluble
(COOH terminus truncated) human furin is obtained from the medium
of Sf9 insect cells (13). The enzymatic activity of each recombinant
convertase is assayed routinely by fluorometric assays using a fluoro-
genic substrate (22).

**Cloning, Mutagenesis, Expression, and Purification of Recombinant
mPC1/3 Propeptides**—All the enzymes used for cloning as well as all
oligonucleotide primers (Table 1) used in mutagenesis were purchased
from Invitrogen. The bacterial expression vector PET24b (+) (Novagen,
WI) was modified as previously described (23). The cDNA correspond-
ing to the wild-type (WT) mPC1/3 propeptide was amplified by PCR
using the NAD000 and NAD001 primer nucleotides (listed in Table 1)
and ligated into the vector between the BamH1 and NotI sites. Similarly,
the propeptide mutants were generated by site-directed mutagenesis
using the Stratagene QuickChange site-directed mutagenesis kit, as
described in the manufacturer’s protocol. All propeptide cDNA
sequences were verified by DNA sequencing. In addition to the 83 resi-
dues of mPC1/3 propeptide, the expression construct contains two
extensions at the NH2 and COOH termini, MASMTGGQMGDRP
and SVQMAALEHHHHHH, respectively, to facilitate cloning and
purification. Each mPC1/3 propeptide, was expressed as a 112-residue
His-tagged polypeptide in Escherichia coli strain BL21(DE3) (Novagen)
following induction by the addition of 1 mm isopropyl-1-thio-
D-galactopyranoside for 4 h at 37 °C. Following this period, the cells were
harvested through centrifugation.

**Propeptide Purification and Chemical Characterization**—The Bacter-
ial cells were lysed by repeated sonications in the presence of 100 µg/ml
lysozyme, and the resulting suspension was loaded unto a Ni2+-Sephar-
rose column (Amersham Biosciences). Following extensive washings of
the column, the propeptide was eluted using 1 m imidazole. The elute
was dialyzed against 0.1% acetic acid, and the proteins were subse-
sequently further purified on an analytical Vydac-C4 RP-HPLC column
(25 × 0.46 cm, The Separation Group, Hesperia, CA) using a Varian
9010/9050 chromatography system. The aqueous phase consisted of
0.1% trifluoroacetic acid (v/v) in water, and the elution was carried out
first isocratically at 10% organic phase (acetonitrile containing 0.1% tri-
fluoroacetic acid) followed by a 1%/min linear gradient of organic phase
to 65% with a flow rate of 1 ml/min. The elution was monitored by
measuring absorbency at 225 nm. Typically, all propeptides eluted
between 40 and 43% of the organic phase. A specific polyclonal antibody
was obtained in rabbits following repeated immunizations using the
complete WT-mPC1/3 propeptide (as described above) bacterially pro-
duced and purified; according to procedures developed at the Sheldon
Biotechnology Center (McGill, Montreal, Canada). Hence, RP-HPLC
fractions were analyzed for propeptide content using this antibody
against dot blotting. The content of individual immunoreactive frac-
tions was analyzed by SDS-PAGE followed by coloration and Western
blotting and subsequently pooled and kept at −20 °C. Routinely, start-
ing with a 1-liter culture, ~5–10 mg of each propeptide can be recov-
ered following purification.
The peptide purity and concentration were determined for each mutant 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 NH₂-terminal amino acid sequence, corresponding to ASPMTGGGQMQGRDPKROFVNE-W(A)AAE (the underlined sequence indicates the NH₂-terminus of the mature mPC1/3) was determined through automated Edman degradation using an Applied Biosystems Procise 949cLC sequencer (Foster City, CA). Molecular mass determination and mass spectral analysis were done on a Voyager DE-Pro matrix-assisted laser desorption ionization time-of-flight instrument (PerSeptive Biosystems, Cambridge, MA); the propeptide in 0.1% trifluoroacetic acid was mixed with a freshly prepared solution of α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% (v/v) acetonitrile-0.3% trifluoroacetic acid and 1 μl deposited on the sample plate. Alternatively, a sample corresponding to 0.3 μg of each propeptide was directly injected unto a Zorbax SB-C18 column (0.3 × 250 mm, Phenomenex, Torrance, CA), connected to a μ-Liquid chromatograph coupled to a QSTAR-XL hybrid LC/MS/MS Mass spectrometer (Applied Biosystems, Foster City, CA). The data generated were analyzed with the Analyst 1.5.2-LS Q5 V1.1 software (Applied Biosystems/MDS-Sciex).

**Table 1**

| Name* | Mutation | Oligonucleotide sequence (5’ to 3’) |
|-------|----------|-----------------------------------|
| NAD000 | R50A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| NAD001 | R51A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO1A | S52A | GGCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO3A | R53A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO4A | R54A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO5A | K61A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO6A | R62A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO7A | D65A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO8A | D66A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |
| PRO9A | D67A | GCCATCGGCGCAATTTGGACAGCGTTGAAATGC |

* S denotes the sense strand, whereas A denotes the antisense strand.

**Enzymatic Assays and Kinetic Analysis**—All enzymatic assays of recombinant mPC1/3 and human furin were performed using initial rate determinations at room temperature on a SpectraMax Gemini EM spectrofluorimeter ( Molecular Devices, Sunnyvale, CA). The assays were done in a final volume of 100 μl in black 96-well flat bottomed plates (Corning Life Sciences, Acton, MA) using 100 μM of the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA (Peptides International, Louisville, KY, USA). For mPC1/3, the buffer consisted of 100 mM sodium acetate at pH 6.0 containing 10 mM CaCl₂. Prior to use, the freshly prepared solution of 370 and 460 nm, respectively. All the assays were started by the addition of the enzyme (corresponding to an activity measured as 15 μM AMC-released/h), and the data points were collected every 30 s. The evaluation of the various inhibition parameters was done as previously described (24). Briefly, the progress curves obtained for the inhibition of mPC1/3 by its propeptide has been shown to follow a tight binding character and, hence, can be defined by the equation (25), \[ P = v_t + \left( v_i - v_t \right) / k_i \] , where \( P \) is the product formed (AMC released), \( v_t \) is the initial velocity, \( v_i \) is the steady-state velocity, and \( k \) is the apparent rate constant for inhibition. Progress curves were submitted to non-linear regression curve analysis using the software Graf 4 (Erithacus Software, Horley, Surrey, UK), which allows the determination of the individual parameters \( v_i \), \( v_t \), and \( k \) for each curve. The obtained \( k \) values were then plotted against inhibitor concentrations. However, competitive tight binding inhibitors can display two different behaviors. The first one corresponds to a single-step process (Mechanism A) whereby the enzyme and the inhibitor combine to form a stable complex. In this case the plot \( k \text{ versus } [I] \) is linear and best described by Reaction 1.

\[
k = k_1 [I] + k_2 \text{ representative of } E + I \rightarrow E \cdot I
\]

**Reaction 1**

where \( K = k_2 / k_1 \). The second one (Mechanism B) is a two-step process whereby the enzyme-inhibitor complex (EI) undergoes a conformational change. In this case, the plot \( k \text{ versus } [I] \) saturates with increased amounts of inhibitor deviating from a straight line, and the \( K \) value can be determined following non-linear regression analysis through curve-fitting using Reaction 2.

\[
k = k_3 [I] / (1 + K_i) + k_4, \text{ indicative of}
\]

\[
k_1 \quad k_2 \quad k_3 \quad k_4
\]

**Reaction 2**

In the case of classic competitive inhibition, the various kinetic parameters are evaluated using the Enzyme Kinetic V1.0 module (SigmaPlot 2000 for Windows V6.1, SPSS Inc., Chicago, IL). In most cases, the
computed results were in close agreement with the equations exhibiting overall fit exceeding 0.990.

Propeptide iodination and Cleavage by Recombinant mPC1/3 and Human Furin—The purified mutant and WT propeptides were chemically labeled with radioactive iodine. For this purpose, 2.5 μg of each propeptide was dried then resuspended in 0.05 M sodium phosphate buffer, pH 7.4. After the addition of 250 μCi of [Na\(^{125}\)I] (Amersham Biosciences), the reaction was started by the addition of 50 μg of chloramine-T in phosphate buffer. The reaction was stopped with 100 μg of sodium metabisulfite. The volume of the reaction was made up to 1 ml with 0.1% trifluoroacetic acid (v/v) in water, and the sample passed through a Sep-Pak C\(_{18}\) cartridge (Millipore, Billerica, MA) as described in the manufacturer’s protocol. The iodinated peptide was recovered by elution with 60% acetonitrile (v/v) in 0.1% trifluoroacetic acid-water, and the radioactivity present in the elution fractions was determined using an automatic Gamma Counter (LKB-Wallac model 1272). The cleavage reaction was carried out in a total volume of 100 μl containing 2.5 × 10\(^{10}\) cpm of radiolabeled propeptide (~2 nM, based on protein content), the sodium acetate buffer specific for each enzyme as above described and the respective enzyme preparation (50 μl of each). After a 30-min incubation period, the reaction was stopped with 10 μl of glacial acetic acid. The sample was dried, reconstituted, and loaded on a Laemmli buffer, and its content was analyzed by electrophoresis on a 15% SDS-polyacrylamide gel. The separated peptides were subsequently electro-transferred overnight unto an Immobilon-P membrane (Millipore). The radioactivity on the membrane was measured using a Storm model 860 Imaging system (Amersham Biosciences) with PhosphorImager capability and using the ImageQuaNT TL software.

Model Building and Modeling of mPC1/3, Human Furin, and the Various mPC1/3 Propeptides—The model of the catalytic domain of mPC1/3 was built upon the atomic coordinates obtained from the crystal structure of the proprotein convertase furin (26) available using the accession code 1P8 in the Brookhaven Protein Data Bank (PDB). Based on the extensive amino acid sequence alignment previously reported (27, 28), the furin sequence was mutated into the mPC1/3 sequence one amino acid at a time. The backbone dihedral angles and the side chains of each amino acid were adjusted until an acceptable low energy conformation was obtained. Similarly, each mutant propeptide was modeled using the atomic coordinates derived from the NMR solution structure of the mPC1/3 (29, 30) also deposited in the PDB under the accession number 1NK6. The variation of Gibbs free energy was computed following 1000 steps of structure minimization. All calculations were carried out using SYBYL version 6.91 software (Tripos Associates, St. Louis, MO) on an IBM-PC platform as described previously (31).

RESULTS

Production of Recombinant Propeptides—Because the major caveat in inhibiting convertases by their propeptides points to the lack of specificity due to the redundancy of the inhibitory COOH-terminal region, we tried to identify some other residues that might confer increasing specificity without modification of the inhibition properties. Furthermore, we and others have previously shown that synthetic peptides of various lengths did not exhibit any significant selectivity (14, 17, 19) nor did the isolated propeptides when assayed against a variety of convertases (19). However, single amino acid substitutions were shown to exhibit a profound effect on both inhibition and activation of convertases. Indeed, single amino acid substitution in the furin propeptide rendered the activation process inoperative and thus yielded no enzymatically active furin (6). However, the propeptide being presented in a cis fashion, i.e. being part of thezymogen, this study could not address the specificity aspect between convertases. The sequence alignment of the convertases propeptides shows very weak sequence similarities between the seven members of the family, except at the extreme COOH-terminal portion (Fig. 1A). Nevertheless, the similarity of certain key residues in the propeptide of mPC1/3 and prosubtilisin, the secondary structure might be conserved. Analysis of the secondary structures of the propeptides of PC by CD measurements appears to agree with such an assumption (19). We therefore speculated that certain structural features of the propeptide could be modified as to confer increased specificity. To test our hypothesis, we carried out site-directed mutagenesis of 10 individual residues located in two convertase potential cleavable sites (66RRSRR69 and 61KR62) and in an acidic region (65DDD67) uniquely conserved among species thus disrupting the a2 helix (Fig. 1B). Alanine residues, chosen because of their least structurally disrupting effect (32), replaced these residues and the resultant mutant propeptides expressed in E. coli BL21 cells, because the propeptide structure does not reveal any potential sites of glycosylation or sulfation. In addition, as mentioned under “Materials and Methods,” our propeptide was elongated at both the NH\(_2\) and the COOH termini to help in cloning and purification. The added sequence had no observable detrimental effect in inhibition properties. The propeptides were first purified from bacterial extracts using classical His-affinity chromatography and then further purified by HPLC and immunoreactive fractions were pooled together (representative data are shown in Fig. 2). Each propeptide was analyzed by mass spectrometry, and its molecular mass was found to be within 1 Da of the computed mass: for example, the mass of WT propeptide is 12,733.34 (average). This expression system allows us to obtain ~5–10 mg of each purified propeptide mutant per liter of culture.

Effect of the Propeptide Mutations upon mPC1/3 and Human Furin Activity—We assessed the inhibitory potency and selectivity of the generated mutants through processing of a small fluorogenic substrate pERTKR-MCA by recombinant mPC1/3 and human furin. We determined the IC\(_{50}\) value of the inhibition of the WT propeptide against mPC1/3 and human furin as being ~20 nM (data not shown). Using this concentration, each mutant, numbered M1 to M10, was assayed against enzymatically active mPC1/3 (Fig. 3A) and human furin (Fig. 3B). The results indicate that, irrespective of the enzymes, the generated mutants belong to three categories namely, mutants exhibiting lower, higher, or equal inhibitory potency to the WT propeptide (Fig. 3).

In the case of mPC1/3, it is readily seen that M1, M2, M5, and M10 are better inhibitors than the WT (Fig. 3A). Interestingly, the first three mutants correspond to the substitution of arginine residues in the RRSSR sequence and hence were expected to play a role in the interaction with the enzyme. On the other hand, the last one contains a mutation of an aspartate residue at position 67 not likely to interact either with the active site or with the enzyme. The computed K\(_{i}\) values (see below) also confirm this result, as they are 6–30 times more potent than the WT (Table 2). Even more intriguing is the fact that the M9 mutation corresponding to an Ala for Asp at position 66 results in a significant diminution of inhibition contrary to an identical substitution at position 65 in M8. The mutation of the Ser\(^{52}\) and Arg\(^{53}\) (M3 and M4), present in the second potential cleavable site, to alanine also had opposite effects, in terms of inhibition, but much less than the other substitutions in this cluster. Finally the substitution at the other potential cleavable site Lys\(^{61}\) and Arg\(^{62}\) (M6 and M7) had very little effect on the inhibition of the active enzyme. It is noteworthy to mention that again, substitutions in the a2 helix where the three aspartate residues are located, led to the most significant changes in inhibition properties hinting that this region, while not likely to interact with the active site, does play an
important role in the interaction of the propeptide with the enzyme (see "Discussion").

When we tested the same mutants on a baculovirus preparation of human furin, a similar but different scenario appeared. First, the mutations induce either an increase or a decrease in the inhibitory potency of the PC1/3 propeptide toward human furin (Fig. 3B). In marked contrast to mPC1/3, most of the mutants generated are more potent than the WT. Thus, all substitutions in the second cleavage site, namely mutants M1, M2, M3, M5, and to a much lesser extent, M4, proved beneficial in terms of human furin inhibition (Table 2). It is worth noting in Fig. 3B that the inhibition afforded by M3, although competitive (see below), appears to diminish with time. Indeed, human furin appears to recover enzymatic activity hinting that M3 is rapidly cleaved by the enzyme thus neutralizing its inhibition. Substitution of the two amino acids occupying the third putative cleavage site led to mixed results, as M6 (K61A) appeared to have minimal effect whereas M7 (R62A) yielded a much improved inhibitor. Finally, similarly to what we observed with mPC1/3, mutations of the three aspartate residues led to significant changes in inhibition profile. Indeed, substitution at position 66 (M9), diminishes significantly the inhibitory potency of this mutant. On the other hand, M10, also a very potent inhibitor of either enzyme, displays no selectivity whatsoever as it inhibits human furin and mPC1/3 with the same $K_i$.

In an attempt to relate the effect of the mutation upon the structure of each propeptide, we decided to model each mutant on the solution structure of pro-PC1/3 as previously determined (29, 30). This was accomplished by computing the change in free energy resulting from each mutation. As presented in Table 2, no clear correlation is readily apparent. However, those mutants found to have the strongest effect (as seen by an increase in free energy) are M7, M8, and M9, mutants that displayed significant modifications in inhibition properties as compared with the WT. Interestingly, these mutants correspond to substitution at amino acids not residing in the major cleavage sites nor thought to interact with the active site.

**Mechanism of Inhibition of PC1/3 Propeptide Mutants toward mPC1/3 and Human Furin**—Synthetic inhibitory peptides against convertases, engineered from the COOH-terminal part, presented a competitive pattern of inhibition supporting the fact that this portion of the propeptide interacts directly with the active site (14, 19). Upon elongating these peptides toward the NH$_2$ terminus, a change in inhibitory behavior was observed, because the initial competitive inhibition changed with the length of the peptide to mixed-inhibition and even...
non-competitive inhibition (14). In the case of the propeptide of the closely related Kex2p enzyme, the propeptide behaved as a mixed inhibitor (33). When we tested the complete propeptides against various convertases, we and others found that most of them behaved as tight binding inhibitors of their cognate enzyme (7, 13). Overall, these results indicate that sites other than the extreme COOH terminus on the propeptide play important roles in regulating enzyme activity by interacting with the enzyme outside of the active site cleft.

To better document the nature of the inhibition mechanism, we performed on-line assays using fixed concentrations of enzyme and substrates but varying amounts of each mutant. Mutants M3 and M9 displayed inhibition curves typical of purely competitive behavior (Fig. 4A), confirmed by further analysis through linear regression of data plotted using classic representation (data not shown). On the other hand, all the other mutants exhibited inhibition curves more characteristic of slow tight binding inhibition and identical to the curve observed with the WT-propeptide (13). However, slow tight binding inhibition can fit a one- or a two-step mechanism depending upon the pathway used to form the stable inhibitory complex (24). In the single-step process herein referred to as mechanism A, the inhibitor binds tightly to the enzyme without inducing any conformational change. By contrast, mechanism B is best explained by first initial tight binding to the enzyme (step 1) followed by a conformational change (step 2) leading to enhancement of the stability of enzyme-inhibitor complex. To differentiate between these two mechanisms, it is best to measure observed $K_{\text{obs}}$ at different inhibitor concentrations. Using the progress curves shown in Fig. 4 (B and D), a plot of $K_{\text{obs}}$ as a function of inhibitor concentrations yields a straight line in the case of a single-step inhibition mechanism (Fig. 4C) or an hyperbolic curve for two-step inhibition (Fig. 4E). Illustrated for the mutant M10, similar curves are obtained for the other mutants with the exception of mutant M2 whose inhibition followed the two-step mechanism. As indicated in Table 2, the computed $K_i$ values ranged from 150 pM up to 16.5 nM when assayed against mPC1/3. The computed $K_i$ for the WT-propeptide (4.4 nM) was in good agreement with the one measured originally (6 nM) (13).

In the present study, the slow tight binding kinetic though still present was much less apparent though the tight binding characteristic of the various propeptides with the exception of M9 was clearly observed. A $K_i$ value for inhibition of furin by WT-PC1/3 propeptide was computed as $1.1 \pm 0.3 \text{ nM}$, a value well in agreement with the value previously reported of $1.6 \text{ nM}$ (19). Indeed as shown in Fig. 5A for the mutant M3, the velocity does not decrease in linear fashion with the inhibitor concentration as would be expected if the enzymatic activity is nullified by the formation of an inactive inhibitor-enzyme complex. This behav-

FIGURE 2. Representative purification and characterization of bacterially produced M2- pro-PC1/3. A, reversed-phase HPLC purification of the material recovered following extraction from bacteria and purification on a Ni$_2$-Sepharose column. B, Western blot analysis of an RP-HPLC-purified aliquot following separation by SDS-PAGE. C, mass spectrum of the RP-HPLC-purified material following electro-spray ionization. Following deconvolution of the spectrum showing the multiple charged ions, the average mass of the propeptide shown was 12,648.29 Da in complete agreement with the theoretical mass.
Mutagenesis Study of the PC1/3 Propeptide

A progress curve obtained following incubation of PC1/3 with 100 μM fluorogenic substrate (pERTKR-MCA) in the presence of a constant amount of each mutant (20 μM), an amount equivalent to the IC_{50} of the WT propeptide. Each data point is the mean value derived from assays done in duplicate of three separate experiments.

The PC1/3 WT-propeptide is cleaved by human furin in an identical fashion to what we observed with PC1/3 yielding a single band of apparent molecular mass of 7.5 kDa. However, in certain samples, we observed an additional band exhibiting an apparent molecular mass of 8.5 kDa as indicated in Fig. 6B. Interestingly, mutations of the Arg^{51} as well as the Arg^{54} yields exclusively the band of 8.5 kDa indicating that furin is not able to cleave the identical motif formed by Arg^{50} and Arg^{53}.

In this situation, human furin prefers to cleave at the other possible site namely the 61KR62 yielding a fragment of computed mass of 8387 Da (−13 to 54). However, as seen in Fig. 6A, all mutations of the arginine residues occupying position P1 and P4,5 mutation at any position within that sequence would be expected to perturb the cleavage. This confirms our prior identification of the identity of the PC1/3 propeptide internal cleavage site as being 60RRSRR64 in agreement with the convertase recognition motif RXRR. Furthermore, it seems that the mutations found in M2 and M5 have a more pronounced effect on inhibition than M1 and M4 hinting that the former might be the favored motif. On the other hand, cleavage at the other potential site, namely 61KR62, could not be detected contrary to what has been observed with a similarly located pair of basic residues in the mPC2 propeptide sequence cleaved by mPC2 (7).

The PC1/3 WT-propeptide is cleaved by human furin in an identical fashion to what we observed with PC1/3 yielding a single band of apparent molecular mass of 7.5 kDa. However, in certain samples, we observed an additional band exhibiting an apparent molecular mass of 8.5 kDa as indicated in Fig. 6B. Interestingly, mutations of the Arg^{51} as well as the Arg^{54} yields exclusively the band of 8.5 kDa indicating that furin is not able to cleave the identical motif formed by Arg^{50} and Arg^{53}.

In this situation, human furin prefers to cleave at the other possible site namely the 61KR62 yielding a fragment of computed mass of 8387 Da (−13 to 62). Indeed, whenever the sequence 53RXRR65 is present, one can see the appearance of this band though in the case of the WT and M3 it is faint. As mentioned previously in conjunction with Fig. 3B, M3 though a potent inhibitor appears to be very rapidly cleaved possibly explaining the absence of the 8.5-kDa band. More surprising, however, is the observation that mutating either Lys^{61} or Arg^{62} fails to completely abolish cleavage at that site, although it can be seen clearly impaired. The latter mutation, if it does not strongly influence cleavage, does lead to a 2.6-fold increase in Ki when compared with M6 and more importantly to a considerable increase in specificity over PC1/3.

**DISCUSSION**

The activation of subtilisin and subtilisin-like serine proteases is a multistep process requiring that the propeptides play a dual role. First, as an intramolecular chaperone, it is assisting the folding of the catalytic domain. This feat is accomplished by lowering the transition state energy allowing the conversion of a collapsed metastable intermediate to a native enzyme (38). The importance of this role was first revealed for prosubtilisin and pro-α-lytic protease (reviewed in Ref. 10), and further results led to proposal of the concept of "protein memory" (39).

In this context, an identical protein sequence can give rise to different conformations through the folding with a mutated chaperone. Application of this concept through propeptide engineering led to the production of new proteases exhibiting altered stability, substrate specificity, and activity (40). Second, as an inhibitor of its cognate enzyme, the propeptide prevents undue activation both in terms of location and time.

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5 The designation of the cleavage site follows the nomenclature introduced by Schechter and Berger (37) defining the cleavage site as the bond between P_{-1} and P_{+1}. Conversely, the enzyme subsites are referred to as S_{-1} and S_{+1}.
in such a way as to allow full enzymatic activity until the active enzyme is needed and properly located. However, it is worth noting that these two functions are not absolutely linked as in the case of subtilisin E (41). The latter study actually prompted us to initiate the present study. Indeed, the introduction of point mutations in the peptidase-propeptide interface seriously compromised the propeptide inhibition potential when added in trans without affecting its folding capacity of the propeptide. Hence, using the pro-PC1/3 as a model, we wanted to know

### TABLE 2

| Mutant | Mutation | Free energy \( \Delta E \) kcal | \( \Delta E \) kcal | mPC1/3 \( K_i \) | Internal cleavage | Human furin \( K_i \) | Internal cleavage | \( K_i (\text{mPC1/3})/K_i (\text{human furin}) \) ratio |
|--------|----------|----------------------------------|------------------|-----------------|------------------|------------------|------------------|----------------------------------|
| WT     | None     | 464.9                            | 0.0              | 4.4 ± 0.1       | Yes              | 1.1 ± 0.3        | Yes              | 4.0                              |
| M1     | R50A     | 478.4                            | 13.5             | 0.36 ± 0.01     | No               | 0.42 ± 0.08      | No               | 1.25                             |
| M2     | R51A     | 471.0                            | -6.1             | 0.74 ± 0.08     | No               | 0.59 ± 0.09      | No               | 7.4                              |
| M3     | S52A     | 463.1                            | +1.8             | 4.00 ± 0.15d    | Yes              | 0.5 ± 0.2        | Yes              | 7.4                              |
| M4     | R53A     | 483.5                            | -18.6            | 5.4 ± 0.1       | No               | 1.1 ± 0.3        | Yes              | 4.9                              |
| M5     | R54A     | 484.6                            | -19.7            | 0.20 ± 0.06     | No               | 0.7 ± 0.1        | No               | 0.3                              |
| M6     | K61A     | 449.6                            | +15.3            | 10.0 ± 0.9      | Yes              | 1.1 ± 0.1        | Yes              | 9.3                              |
| M7     | R62A     | 420.2                            | +44.7            | 16.5 ± 0.2      | Yes              | 0.4 ± 0.1        | Yes              | 39.3                             |
| M8     | D65A     | 432.2                            | +32.7            | 10.0 ± 0.15     | Yes              | 0.26 ± 0.09      | Yes              | 38.5                             |
| M9     | D66A     | 422.5                            | +42.4            | 8.6 ± 0.6^c     | Yes              | 24 ± 2          | Yes              | 0.36                             |
| M10    | D67A     | 462.6                            | +2.3             | 0.15 ± 0.08     | Yes              | 0.20 ± 0.05      | Yes              | 0.75                             |

\(^{a}\) This value was obtained following energy minimization as described under “Materials and Methods.”

\(^{b}\) The site 2 is hereby defined as corresponding to the 50RRSRR54, whereas the site 3 is defined as being the 61KR62 pair.

\(^{c}\) These \( K_i \) values were obtained using a fully competitive model, whereas the others for PC1/3 were based upon a slow tight binding model and those for human furin were based upon a tight-binding model.

**FIGURE 4. Differences in inhibition behavior of propeptide mutants M2, M9, and M10 toward mPC1/3.** A, progress curves obtained from the inhibition of mPC1/3 by the mutant M9, the slope of the each curve was used to derive the \( K_i \) by Dixon’s representation. B, progress curve obtained using increasing concentrations from 0 to 100 nM of mutant M10. C, graphic representation of \( K_{\text{obs}} \) versus M10 concentrations yielding a straight line with a correlation coefficient of 0.9808. D, progress curve obtained using increasing concentrations from 0 to 100 nM of mutant M2. E, graphic representation of \( K_{\text{obs}} \) versus M2 concentrations yielding a hyperbolic curve with a correlation coefficient of 0.9130. In all cases, data used for computation and curve fitting represent the mean value derived from duplicate assays of three separate experiments.
Mutagenesis Study of the PC1/3 Propeptide

whether by substituting certain amino acids, we could modify the inhibitory potency, the inhibitory mechanism, and the selectivity of the propeptide.

This objective also resulted from the previously reported difficulties in developing synthetic peptides of various sizes based upon the propeptide sequences, which could prove potent and selective. Thus, small peptides mimicking the COOH-terminal portion of the propeptide were synthesized, and their properties were assayed. Even though these peptides could be made into very potent inhibitors in the nanomolar range, they are mostly competitive and nonselective, which restrict their use to structural characterization of the enzymes. Such peptides have been synthesized and assayed with numerous PCs, including furin, PC1/3, PC5/6, and PC7 (14, 19, 42). As mentioned above, there exists in the propeptide sequence a second potential cleavable site known to be important for the activation and the secretion of PC5/6, PC2, and furin. In PC1/3, mutations in the second cleavable region (50RSRR53 to 51SSGR54) had no effect on PC1/3 processing in X. laevis egg system (43). When synthetic peptides derived from the second cleavable site were tested against PC1/3, PC7, and furin, they were found to be either non-inhibitory or generally weak inhibitors, exhibiting $K_i$ in the micromolar range. In any case, no synthetic peptide was able to duplicate mechanistically the inhibition generated by the complete propeptide when presented in trans. Indeed, the inhibition afforded by the propeptide leads to the formation of a stable complex as previously shown with various convertases. This is explained by important interactions between the propeptide and sites close or remote of the convertase active site as visualized in the crystal structure of the propeptide of subtilisin BPN', $E$, and $\alpha$-lytic protease in complex with their cognate enzyme (44–46). Similar though distinct contacts were also inferred in the case of the convertase propeptides with their cognate enzymes following the determination of the NMR solution structure of pro-PC1/3 (30). Nevertheless, the entire propeptides, independent of the way they are presented to the enzyme be it in cis or in trans, lack selectivity, because many convertase propeptides inhibit their own protease and other related convertases with variable potency. Conversely, swapping of the propeptide has been shown, in certain cases (for example, PC1/3 and furin), to yield enzymatically active proteinases (5). Hence, it can be proposed that selectivity might not be derived from the basic architecture of the propeptide, which is reported to be composed of four-stranded antiparallel $\beta$-sheets and two $\alpha$-helices, but more so by the localized interactions of certain residues at the interface of the propeptide-enzyme complex. Thus we tried herein by site-directed mutagenesis to explore the possibility of modulating the extent of the interactions between the propeptide and its enzyme. To do so, ten mutants were generated, and their interactions with two enzymatically active convertases were determined.

In summary, this study demonstrates that modifying a single amino acid within the structure of propeptide can have profound effect on its reactivity. Indeed, when compared with the native propeptide (WT), single residue mutation yielded propeptides (i) with different inhibition mechanism depending on the enzyme assayed (M3); (ii) that are weaker and displaying different mechanisms with both enzymes (M9); (iii) with identical inhibition mechanism but with enhanced selectivity (M7, M8); and (iv) that are very potent but not at all selective (M1, M2, M5, and M10).

The mutation of the arginine Arg50 (M1), Arg51 (M2), or Arg54 (M5) in the RRSRR sequence all led to very potent inhibitors with $K_i$ in the low picomolar range for both enzymes. This validates the previous assumption that this sequence represents the site of the secondary cleavage of the propeptide for PC1/3. This site contains two possible RXXR motifs, 50RRSR53 and 51RRSR54. As illustrated in Fig. 6, mutations at positions 50, 51, 53, and 54 severely impair the cleavage by PC1/3 hinting that PC1/3 can use either of the recognition sites. On the other hand, furin appears more selective as only the mutations at position 51 or 54 have an effect on cleavage. This obviously confirms the reported strong preference for substrates having both P$_1$ and P$_2$ residues as basic amino acids. However, in the context of the PC1/3 propeptide sequence, it thus seems that furin prefers much more the 50RRSR53 site than the 50RRSR54 even though both display the minimal furin recognition motif namely RXXR. Interestingly, the crystal structure of furin revealed that, in contrast to the yeast convertase Kex2, the former does not exhibit the strong requirement of the latter for a basic amino acid at the P$_2$ position (27, 47, 48). Nevertheless, our results using either enzyme favor a
sequence containing basic residues at positions P3, P2, and P4. This observation is also consistent with our previous results using Barley serine proteinase inhibitor 2-derived cyclic peptides. In that study, we noticed an additive favorable effect in terms of substrate recognition when basic residues occupy positions P3 and P4 (31). The mutation of these residues, especially the arginine occupying position 54, severely impairs the cleavage of the propeptide and thus could contribute to an increase in inhibitor potency. This observation is especially true with the M5 mutant, because M5 exhibits a Kic of 0.2 nM, and this could reasonably be explained by the fact that the enzyme, due to lack of cleavage, cannot dissociate from the propeptide. In the case of furin, the consequence of mutations in this region is not as significant, and this might be due to the presence of another furin-sensitive site in the propeptide (see below). Finally, the M1 mutant (Arg50) if one considers the Arg54 as the P1 position corresponds to a P3 substitution and is a very potent inhibitor of both enzymes, but only furin can cleave it efficiently. The S5 site in furin offers a negatively charged environment (Glu233/Asp235) incomplete agreement with its preference for positively charged P5 side chain; substitution by a small neutral residue such as alanine thus appears not detrimental. On the other hand, substitutions by acidic residues are much less tolerated at that position (49, 50). The inability of PC1/3 to cleave this mutant is difficult to rationalize, because the S5 site in PC1/3 is less negatively charged than found otherwise in other convertases such as furin, PACE4, PC5/6, and PC7. Hence, substituting the arginine at this position by an alanine should not be expected to prevent the cleavage by PC1/3 unless the arginine side chain plays an as yet undetermined role in the propeptide structure or in the propeptide-enzyme complex. However, it could also be that, in this particular instance, the arginine residue is essential for the interaction with Glu233 and with Asn244, both residues being present in PC1/3 S5. In the solution structure of PC1/3 propeptide this residue resides in a solvent-accessible loop located between the third β-sheet segment and the second α-helix (30). Another plausible explanation might be that by introducing an alanine residue, one decreases the high concentration of charged residues in the loop and in turn, this prevents complete dissociation of the enzyme-inhibitor complex.

The mutation of the Ser52 (M3) rendered the propeptide purely competitive toward PC1/3, whereas it did not change inhibitor’s behavior against furin. This observation is interesting, because, in view of possible therapeutic applications, a tight binding inhibitor forming a stable complex is largely preferred to the high concentration of a competitive inhibitor required to abolish the activity. Essentially, this serine residue appears to play an important role in stabilizing or promoting the enzyme-propeptide complex. The replacement of this polar residue by an alanine could abolish some important weak interactions. Both enzymes cleave this mutant efficiently, and this is in agreement with biochemical and structural data reported for furin and PC1/3. Actually, the substrate-binding site S5 in furin is highly permissive, because the x-ray structure does not reveal a discrete binding pocket (47). However, we have shown that one way to affect substrate recognition and cleavage by furin is to introduce at P3 an acidic residue such as Glu (49). Based on the now available structure, this is explained by unfavorable contact with Glu235, which, though occupying the S5 cleft, is surface-located and can possibly interact with a P3 residue (48). In PC1/3, a neutral Asn residue replaces this Glu residue; hence, it is not surprising that the only substitution reported to compromise PC1/3 substrate cleavage efficiency at this position is a Pro residue (15, 49).

The basic amino acid pair, 61KR62, a possible convertase-cleavable site present in PC1/3 propeptide and containing P3 and P4 basic motifs, is not cleaved by PC1/3. However, substituting either one or the other leads to the propeptide being among the weakest in this series toward PC1/3. Being located in the second α-helix segment, their replacement by a small neutral residue could potentially have an impact on the integrity of this helical segment. As previously mentioned, a similarly located pair of basic residues in the pro-mPC2 sequence is recognized and cleaved by mPC2 (7). This pair of basic residues is also recognized as a substrate by furin in the context of the pro-PC1/3 sequence. Interestingly, in the profurin sequence, a typical furin recognition sequence, namely RRSR, is similarly located. Moreover, the substitution of the Arg62 residue leads to an increase in propeptide inhibitory force against furin suggesting at first sight that this residue is interacting elsewhere with the enzyme to emphasize the contact. Alternatively, the degradation of the propeptide by furin requires the cleavage at that site, in addition to the second cleavage. The ability of furin to cleave M7 (R62A) at two positions, namely at the RSRR and at the KA sites, complicates the interpretation. Indeed, one is left wondering whether furin itself can cleave on the COOH-terminal to a Lys residue. Obviously, looking at the results of the cleavage experiment of mutants M6 and M7, furin would be able to cleave after both Lys61 and Arg62, because either mutant yields two lower molecular weight peptides. Interestingly, as observed with M2 and M5 mutation that removes either of the arginines occupying P4 or P1, furin can utilize efficiently this basic pair to cleave the PC1/3 propeptide. Nevertheless, furin cleavage at a pair of basic residues flanked by a P4 hydrophobic residue instead of the usual basic...
Molecular modeling of mPC1/3 propeptide. A, a representation of one possible conformer of the mPC1/3 propeptide (in purple) docked upon the crystal structure of the catalytic chain (blue) and the P domain (green) of furin. The yellow region of the propeptide represents the COOH-terminal part of the propeptide interacting with the substrate-binding region. B, CPK (Corey-Pauling-Koltun) model of the mPC1/3 propeptide based on the NMR solution structure (29) indicating the deeply buried Asp66 (colored in blue). C and D, molecular interactions and hydrogen bonds formed by the mainchain and side-chain atoms of Asp66 with its immediate neighbors in the WT-propeptide (C) and following its replacement by an Ala residue (D). The mutation from Asp66 into an Ala residue completely abolishes numerous hydrogen bonds formed by the Asp sidechain atoms with the two neighboring basic residues, namely Arg62 and Arg68.

In conclusion, this study highlighted the fact that, using the PC1/3 propeptide as a model structure, one can introduce small changes in the primary sequence that will affect its global structure, its sensitivity to proteolysis, and its inhibitor potency as well as mechanism.

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Individual mutation of the three Asp residues located in the α2 helix exhibited significant and unique properties. A clear relationship between the levels of perturbation introduced in the propeptide structure by the various mutations was not established with the inhibitory potency (see Table 2). However, the three weakest inhibitors of PC1/3 correspond to M7, M8, and M9, and these mutations appear to be the most perturbing in terms of conformational stability. It is worth mentioning that these mutations were introduced at sites remote from the primary reactive site and unlikely to play a role in the formation of the enzyme-inhibitor complex. Similarly to the parallel drawn between the M2 and M5 mutants in terms of cleavage by PC1/3 or furin, a similar one can be identified here between M7 and M9, the latter exhibiting a more profound effect, because M9 is not able to form a tight complex with PC1/3. As shown in Fig. 7, examination of the pro-PC1/3 solution structure reveals that the Arg⁶² (mutated in M7) and the Asp⁶⁶ (mutated in M9) are not only deeply buried within the structure but more importantly can interact through hydrogen bonding. Indeed, it can be seen that the Asp backbone atoms as well as those in the side chain can participate in six hydrogen bonds. Hence, mutating the Arg⁶² leads to the loss of two of these bonds, but the Asp residue is still able to link with the Arg⁶⁶. However, mutating this Asp will remove all those interactions except those arising from the main-chain atom, a condition likely to lead to severe conformational perturbations. Interestingly, whereas this modification is detrimental in terms of inhibiting pro-PC1/3 (mechanistically as well as in potency), the M7 mutant is a better inhibitor of human furin and exhibiting an increased selectivity. Clearly, even buried deeply in the structure and hence unlikely to actively participate in the molecular interactions between the enzyme and the propeptide, the Asp⁶⁶ appears very important in terms of the global folding of the propeptide. The furin propeptide possesses the equivalent Arg⁶² and Asp⁶⁶ but not the Arg⁶⁶, and it would thus be expected considering that PC propeptides are proposed to share similar structure (19, 30) that mutations in furin propeptide at these positions could have significant effects. Considering that the side chain of Asp⁶⁶ is pointing inwards and is involved in hydrogen bonding, it can be safely assumed that it serves as an anchor point for this short helix, and hence the two other Asp are likely to point outwards and be solvent-accessible. Mutating these two residues is thus likely to perturb locally the α-helix but more importantly to decrease the negative surface potential. Interestingly, this does not have the same effect on PC1/3 and on furin, because M10 is the strongest inhibitor of either enzyme, whereas M8 is a much stronger inhibitor of human furin than of PC1/3.

In conclusion, this study highlighted the fact that, using the PC1/3 propeptide as a model structure, one can introduce small changes in the primary sequence that will affect its global structure, its sensitivity to proteolysis, and its inhibitor potency as well as mechanism.

FIGURE 7. Molecular modeling of mPC1/3 propeptide. A, a representation of one possible conformer of the mPC1/3 propeptide (in purple) docked upon the crystal structure of the catalytic chain (blue) and the P domain (green) of furin. The yellow region of the propeptide represents the COOH-terminal part of the propeptide interacting with the substrate-binding region. B, CPK (Corey-Pauling-Koltun) model of the mPC1/3 propeptide based on the NMR solution structure (29) indicating the deeply buried Asp⁶⁶ (colored in blue). C and D, molecular interactions and hydrogen bonds formed by the main-chain and side-chain atoms of Asp⁶⁶ with its immediate neighbors in the WT-propeptide (C) and following its replacement by an Ala residue (D). The mutation from Asp⁶⁶ into an Ala residue completely abolishes numerous hydrogen bonds formed by the Asp side-chain atoms with the two neighboring basic residues, namely Arg⁶² and Arg⁶⁸.
