

## The Proteolytic Maturation of Prohormone Convertase 2 (PC2) is a pH-Driven Process

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**Recombinant proPC2 purified from the medium of CHO cells overexpressing both the prohormone convertase (PC) precursor proPC2 and the 21-kDa amino terminal portion of the neuroendocrine protein 7B2 can spontaneously convert to an active species. In the present report, we have characterized the proPC2 zymogen conversion process. Sequencing of the mature 66 kDa enzyme revealed a single site of cleavage at the paired basic site amino terminal to the GYRDI sequence. In contrast to mature PC2 activity, proPC2 conversion was inhibited neither by the eukaryotic subtilisin inhibitor pCMS nor by the specific PC2 inhibitor, 7B2 CT peptide, suggesting significant differences between the proPC2 conversion reaction and the hydrolysis of synthetic substrates by mature PC2. In support of this idea, proPC2 conversion was not calcium dependent and was unaffected by 5 mM EDTA. The rate of conversion of proPC2 remained similar with a 10-fold difference in zymogen concentration, implicating an intramolecular rather than intermolecular mechanism of activation. Interestingly, the rate of proPC2 conversion was extremely pH dependent, occurring most extensively between pHs 4.0 and 4.9. Taken together, our results suggest that cellular proPC2 maturation occurs via an autocatalytic, intramolecular process controlled not by 7B2 inhibition nor by calcium levels, but by the decreasing pH gradient along the secretory pathway.** © 1999 Academic Press

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Prohormone maturation is mediated by a number of endoproteases that cleave at dibasic amino acid resi-

dues (reviewed in 1, 2). These enzymes represent a family of calcium-dependent, subtilisin-like enzymes. PC1 and PC2 are the only members of this family of proteases whose expression is largely limited to neuroendocrine tissues (1–3). Both enzymes exhibit optimal enzymatic activity at acid pH and are inhibited by pCMS,<sup>2</sup> which interacts with a thiol group near the active site, and by EDTA, which inactivates this family of enzymes by chelating Ca<sup>2+</sup> ions (4–9).

Like prosubtilisin (10), the proforms of subtilisin-like conversion enzymes mature through proteolytic cleavage at dibasic amino acid sequences, supporting the idea that activation of the eukaryotic subtilisin-like enzymes occurs via an autocatalytic mechanism. Site-directed mutagenesis of the catalytic triads of four of these enzymes, kexin, furin PC1, and LPC (PC7), has confirmed the notion of autocatalytic activation (11–16). The conversion of the zymogens of the prohormone convertases PC1 and PC2 has been less well studied than that of furin and kexin. *In vivo*, proPC1 conversion occurs very rapidly after synthesis (4, 17, 18), and PC1 isolated from the conditioned medium of overexpressing CHO cells consists solely of the mature 87-kDa enzyme. *In vitro*, oocyte-expressed proPC1 was shown to convert autocatalytically with a rapid half-life; in contrast, oocyte proPC2 conversion was very slow (19). The carboxyl terminus of 87-kDa PC1 is further slowly truncated intracellularly, resulting in 74- and 66-kDa active PC1 proteins (4, 20, 21). Carboxyl terminal truncation of mature PC2 does not appear to occur in many neuroendocrine cell lines (22–

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<sup>2</sup> Abbreviations used: AMC, aminomethyl coumarin; CT peptide, human 7B2<sub>155–185</sub>; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; ir, immunoreactive; PC, prohormone convertase; PC1 and PC2, prohormone convertases 1 and 2; pCMS, *p*-chloromercuriphenylsulfonic acid; TLCK, *N* $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine ketone.

24), although truncation of insulinoma granule PC2 has been reported by Hutton and co-workers (7).

PC2 is the only prohormone convertase known to require a neuroendocrine binding protein for intracellular maturation. Binding of 7B2 to proPC2 was first shown through coimmunoprecipitation experiments (25) and was later shown to represent a reaction specific to this convertase (26). Overexpression of mouse proPC2 cDNA in CHO cells resulted only in the production of catalytically inactive proPC2 which was incompetent for activation (23). However, supertransfection of proPC2-producing CHO cells with cDNA encoding the neuroendocrine 21-kDa protein 7B2 resulted in the secretion of proPC2 which was enzymatically competent for activation (9, 24). 7B2 is now known to represent a bifunctional protein; the amino terminal 21-kDa domain of this protein is responsible for the *in vivo* acquisition of enzymatic activity by recombinant proPC2, while the remaining carboxy terminal 31 amino acid portion of the molecule represents a potent inhibitor of PC2 activity, as does the parent 27-kDa 7B2 molecule containing this peptide (8, 27–29).

Aside from its requirement for 7B2, proPC2 distinguishes itself from other zymogens within this family by the extremely long intracellular half-life of the zymogen form. Radiolabeled proPC2 is present in cells even after 3 h of chase, and many neuroendocrine tissues and cell lines contain a reservoir of unprocessed proPC2 (7, 18, 22–24). The longer time frame for intracellular maturation of proPC2 may represent a regulatory feature of this particular convertase, which is thought to act upon prohormones late within the secretory pathway (1, 7, 18). Two cleavage sites for zymogen activation have been determined using mature PC2 isolated from insulinoma cell granules (30), but only one amino terminus was observed for mature PC2 prepared from chomaffin cell granules (31). Whether alternative site usage plays a role in activation is not clear. Indeed, the biochemistry of proPC2 activation is not well understood, and the role of 7B2 in the activation reaction has not yet been elucidated.

In the present study, we have studied the process of proPC2 conversion using recombinant proPC2 purified from the medium of CHO cells overexpressing proPC2 and 7B2. We here report that proPC2 maturation is unaffected by the presence of 21-kDa 7B2, EDTA, pCMS, and the potent PC2-specific inhibitor, 7B2 CT peptide, but is highly pH dependent, suggesting a role for pH in the regulation of proPC2 conversion *in vivo*.

## EXPERIMENTAL PROCEDURES

**Materials.** Cyanogen bromide-activated Sepharose 4B was obtained from Sigma Chemical Company (St. Louis, MO). Protein A-Sepharose was purchased from Pharmacia Biotech (Piscataway, NJ). pGlu-Arg-Thr-Lys-Arg-AMC was supplied by Peptides International, Inc. (Louisville, KY). Recombinant His-tagged rat 21-kDa 7B2

was prepared by bacterial expression as described by Lamango *et al.* (9). Human CT peptide 1–31 was synthesized by LSUMC Core Laboratories.

**Preparation of purified recombinant proPC2.** Recombinant proPC2 (75- and 71-kDa forms) were purified from the conditioned medium of CHO cells transfected with expression plasmids encoding cDNAs for rat 21-kDa 7B2 and mouse proPC2 as previously described (9). Alternatively, for some experiments, proPC2 was obtained from conditioned medium of the same CHO cells cultured in a Cellmax artificial capillary cell culture system (Cellco Inc., Germantown, MD) via purification on a 5 × 50-mm Protein-Pak anion-exchange column (Waters Chromatography, Milford, MA). Buffer A was 20 mM Bis-Tris, pH 6.5, 0.1% Brij, and buffer B was 1 M Na acetate, 20 mM Bis-Tris, 0.1% Brij, pH 6.5. A step gradient from 0 to 35% B in 175 min at a flow rate of 0.25 ml/min was followed by a further gradient to 100% B in 225 min at 0.5 ml/min. One-milliliter fractions were collected and were assessed for purity on 8.8% polyacrylamide gels using Coomassie blue staining. This method provided larger quantities of proPC2 (3–10 mg per purification) in more concentrated form, although also occasionally exhibited increased amounts of amino-terminally truncated forms.

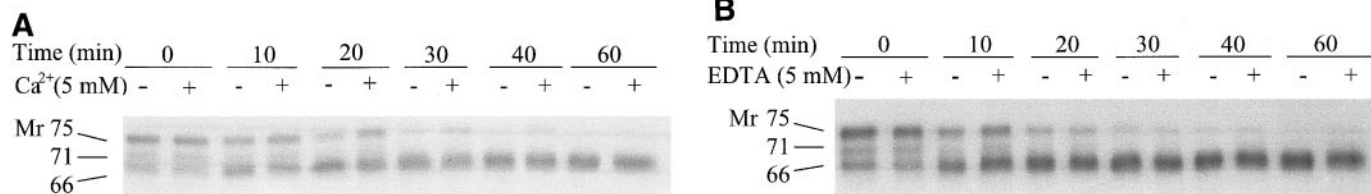
**Enzyme assay.** Routine enzymatic assays were performed in a 50- $\mu$ l incubation volume consisting of 100 mM sodium acetate buffer, pH 5.0, in the presence of 5 mM CaCl<sub>2</sub> and 0.1% Brij at 37°C with pGlu-Arg-Thr-Lys-Arg-AMC (200  $\mu$ M) as substrate. The enzymatic activity was determined by measuring the fluorescence of the released AMC in a microtiter plate fluorometer as previously described.

**pH dependence of proPC2 conversion.** Purified proPC2 (1.3  $\mu$ g) was incubated at 37°C for 10 min in 100 mM Bis-Tris/100 mM sodium acetate buffer, set at pH values varying between 4 and 7.4 and containing 0.1% Brij. SDS-PAGE sample buffer stock was then added to the incubation mixtures, and the samples were boiled and subjected to SDS-PAGE on an 8.8% gel and Coomassie blue staining. These experiments were repeated on two separate occasions with similar results.

**Effect of Ca<sup>2+</sup>, pCMS, CT peptide, and EDTA on proPC2 conversion.** To determine the effect of Ca<sup>2+</sup> on proPC2 conversion, purified proPC2 (0.58  $\mu$ g) was incubated at 37°C in 200 mM sodium acetate buffer, pH 5.0, containing 0.1% Brij either in the presence or in the absence of 5 mM CaCl<sub>2</sub>. The effect of pCMS on proPC2 conversion was determined following similar incubations conducted with or without 1 mM pCMS, all in the presence of 5 mM CaCl<sub>2</sub>. To study the effect of EDTA on the conversion process, incubations were carried out in the presence or absence of 5 mM EDTA (with no added CaCl<sub>2</sub>). The effect of CT peptide on the conversion process was determined by incubating purified proPC2 (0.58  $\mu$ g) at 37°C in 200 mM sodium acetate buffer, pH 5.0, containing 0.1% Brij and 5 mM CaCl<sub>2</sub> either in the presence or in the absence of 100  $\mu$ M 7B2 hCT peptide. At each time point, aliquots were frozen at -70°C for subsequent SDS-PAGE and Western blotting analysis. The experiments were repeated once with identical results.

**Effect of TLCK, TPCK, E-64, and pepstatin on proPC2 conversion.** Purified proPC2 (2  $\mu$ g) was incubated at 37°C for 32 min in 100 mM sodium acetate, pH 5.0, containing 0.1% Brij and 5 mM CaCl<sub>2</sub>, in the presence of 0.14 mM TLCK, 0.5 mM TPCK, 10  $\mu$ M E-64, or 1  $\mu$ M pepstatin (final concentrations). The control sample contained no inhibitor. Reactions were stopped by adding Laemmli sample solution and boiling. The samples were analyzed by SDS-electrophoresis and Coomassie staining.

**Effect of proPC2 concentration on the rate of proPC2 conversion.** To investigate whether proPC2 conversion is an intra- or an intermolecular reaction, the concentration dependence of purified proPC2 conversion was examined. ProPC2 was incubated at different concentrations at 37°C in 200 mM sodium acetate buffer, pH 5.0, containing 5 mM CaCl<sub>2</sub> and 0.1% Brij. Either 0.2  $\mu$ g (54 nM final



**FIG. 1.** ProPC2 conversion is neither calcium dependent, nor is it inhibited by EDTA. (A) Purified proPC2 (0.58  $\mu$ g) was incubated at 37°C in the absence (-) or presence (+) of 5 mM  $\text{CaCl}_2$ . At the indicated time points, one-third of each sample was then analyzed on an 8.8% SDS-PAGE gel by Western blotting using rabbit antiserum against PC2. (B) Purified proPC2 (0.58  $\mu$ g) was incubated at 37°C in the absence (-) or presence (+) of 5 mM EDTA. At the indicated time points, aliquots were frozen for analysis by Western blotting.

concentration) or 2.0  $\mu$ g (540 nM) of proPC2 was used per 50- $\mu$ l incubation volume. At varying time intervals, aliquots were frozen for subsequent analysis by SDS-PAGE and Western blotting. To ensure that equal amounts of protein were loaded onto the gel, samples containing the higher concentration of proPC2 were appropriately diluted with the incubation medium before boiling. Thirty microliters of the 54 nM sample and 30  $\mu$ l of a 10-fold dilution of the 540 nM sample were analyzed on an 8.8% SDS-PAGE gel followed by Western blotting with anti-PC2 antiserum. Similar results were obtained upon independent repetition of the experiment with another proPC2 preparation and using a 30-fold range of concentrations.

**SDS-PAGE and Western blotting.** Samples for SDS-PAGE analysis were boiled for 5 min after addition of 1/10 vol of 10 $\times$  sample buffer. After electrophoresis on 8.8% gels, the separated proteins were electroblotted onto nitrocellulose and subjected to Western blotting using PC2 antiserum directed against the last 13 amino acids of PC2 as described by Shen *et al.* (23).

**Immunoaffinity adsorbent preparation.** IgGs were purified from 5 ml of 7B2 antiserum (LS12, directed against residues 23-39) or from preimmune serum via protein A chromatography. Following overnight dialysis into coupling buffer (0.1 M borate buffer, pH 8.5), these IgGs (7-9 mg protein) were added to a resin cake prepared from 5 ml of a 50% slurry of Reacti-Gel (Pierce Chemical Co), which was dried by filtration just prior to use, and rocked overnight in the cold. The immunoadsorbents were washed twice with 40 ml of cold TBS, once with TBS containing 0.5 M NaCl, and finally with TBS containing 0.02% sodium azide and stored at 4°C. Prior to use 0.3 ml of each immunoadsorbent was washed with 3 ml of 100 mM glycine, pH 3.0, to remove any unbound material and quickly reequilibrated in 10 ml of TBS containing 0.05% Brij.

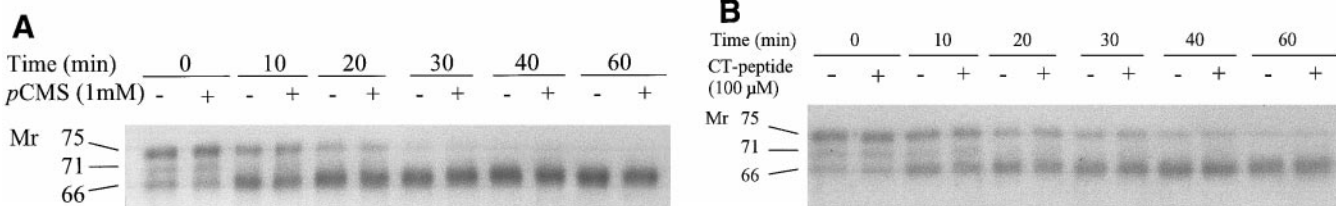
To examine the effect of 7B2 depletion on proPC2 activity, 10  $\mu$ g of purified recombinant proPC2 (a mixture of the 75- and 71-kDa forms) were added to 300  $\mu$ l of the packed, washed immunosorbent in a total volume of 1 ml of TBS, 0.05% Brij, and the mixtures were then rocked for 24-72 h at 4°C to allow binding to occur. The samples were then centrifuged for 2 min in a microfuge and the supernatant

removed (designated "supernatant"). The immunoadsorbents were then washed with 1 ml of TBS containing 0.1% Brij and centrifuged again (designated "wash"). 7B2 immunoreactivity was measured in the applied sample, the unbound material, the wash, and the glycine eluant of the immunoadsorbent ("resin") using a sensitive radioimmunoassay (24). PC2 activity was measured in the same samples by diluting 15  $\mu$ l of each sample into the 50- $\mu$ l PC2 enzyme reaction described above under "Enzyme assay"; the resin was used directly in the enzyme assay. Finally, to examine the effect of 7B2 depletion on proPC2 activation kinetics, 10- $\mu$ l aliquots of the supernatants from the 7B2 IgG and preimmune immunoadsorbents were activated by addition of 40  $\mu$ l of 100 mM Na acetate, 100 mM Bis-Tris, pH 5.5, 0.1% Brij for varying lengths of time. Eight microliters of 10 $\times$  sample buffer were then added and the samples were boiled for subsequent SDS-PAGE and Western blotting of 50  $\mu$ l aliquots using anti-PC2 antiserum LSU18. The entire experiment was repeated four times with qualitatively similar results; the last two experiments are presented.

**RESULTS**

*Biochemical Characterization of Recombinant Proteins*

To determine the identity of the recombinant proteins in the various enzyme preparations as well as the cleavage site of proPC2 to PC2, zymogens and the mature form were subjected to Edman degradation on an ABI Procise sequencer. The amino termini of the 75- and 71-kDa zymogen forms were ERPVF and SLHHK, respectively, in agreement with the 75-kDa proPC2 sequence previously reported using tritiated phenylalanine microsequencing of CHO cell PC2 (23). The amino terminus of the mature 66-kDa protein was GYRDI, consistent with the use of a single activation



**FIG. 2.** ProPC2 conversion is not inhibited by pCMS or the CT peptide. (A) Purified proPC2 (0.58  $\mu$ g) was incubated at 37°C in the absence (-) or presence (+) of 1 mM pCMS. At the indicated time points, aliquots were frozen for analysis by Western blotting. (B) Purified proPC2 (0.58  $\mu$ g) was incubated at 37°C in the presence (+) or absence (-) of 100  $\mu$ M CT peptide.

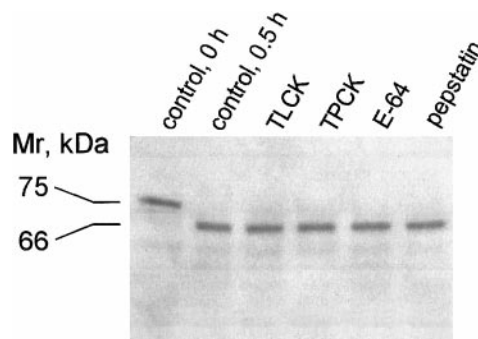
site for chromaffin granule proPC2 (31) and inconsistent with a previous finding of additional cleavage after the GYR sequence in insulinoma granules (30). Variable amounts of the amino-terminally truncated forms were recovered in different enzyme preparations, potentially due both to differences in propeptide cleavage in the bioreactor (a function of cell density, which affects pH), and to autocatalysis during ion exchange (performed at pH 6.5).

#### Effect of $Ca^{2+}$ and EDTA on proPC2 Conversion

The PCs are known to represent calcium-dependent enzymes; we therefore expected that activation of proPC2 might also require calcium. However, addition of 5 mM  $CaCl_2$  to the reaction did not increase the rate of proPC2 conversion relative to controls (Fig. 1A). Instead, a slight retardation of the conversion process in the presence of  $Ca^{2+}$  was observed; this retardation was reproduced in three independent experiments using three different enzyme preparations and suggests that calcium may have a stabilizing effect on the zymogen. No differential effects were observed on the effect of  $Ca^{2+}$  on the conversion of the 75- vs the 71-kDa forms. When incubations performed in the absence of  $Ca^{2+}$  included 5 mM EDTA, no difference in the rate of conversion from reactions lacking EDTA was apparent (Fig. 1B).

#### Effect of PC2 Inhibitors and Other Enzyme Inhibitors on proPC2 Conversion

Unlike mature PC2 activity, proPC2 conversion was not affected by 1 mM pCMS (Fig. 2A). Full-length 27-kDa 7B2 and 7B2 CT peptide represent potent inhibitors of vertebrate PC2 activity at nanomolar concentrations (8, 27). However, even at concentrations as high as 100  $\mu$ M, CT peptide did not affect proPC2 conversion (Fig. 2B). It should be noted that all of the PC2 generated in the presence of CT peptide was enzymatically inactive, indicating the effectiveness of the inhibitor. We also tested recombinant 27-kDa 7B2 as



**FIG. 3.** ProPC2 conversion is not affected by TLCK, TPCK, E-64, or pepstatin. Purified proPC2 was incubated in the presence of 0.14 mM TLCK, 0.5 mM TPCK, 10  $\mu$ M E-64, or 1  $\mu$ M pepstatin. The control sample was incubated without any inhibitor (second lane from left). The leftmost lane represents an unincubated control. The image depicts total Coomassie-stained protein.

an inhibitor of activation; even in the context of the entire 7B2 molecule, the CT peptide had no effect on activation (not shown), supporting the idea that it cannot access the active site of proPC2.

Figure 3 depicts the conversion of proPC2 (shown as a Coomassie-stained protein) at pH 5.0 in the presence of protease inhibitors such as TLCK, TPCK, E-64, and pepstatin. In several independent experiments, these inhibitors produced absolutely no effect on the kinetics of conversion of proPC2, supporting the notion that proPC2 conversion is not performed by a copurifying proteinase.

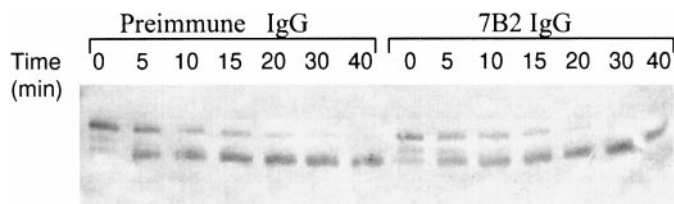
#### Effect of 7b2 on proPC2 Conversion

We have previously reported that recombinant His-tagged rat 21-kDa 7B2 stabilizes the activity of mature mouse PC2 (9). We included 100 nM 7B2 in the proPC2 conversion reaction to examine its effects on conversion; however, no apparent effect was observed (data not shown). We then examined the effect of removing 7B2 from the incubation reaction (using immunoaffinity chromatography) on proPC2 activation. Although

**TABLE I**  
Effect of Immunodepletion of 7B2-Immunoreactive Peptides from ProPC2 on Final Activity<sup>a</sup>

	Supernatant (%)		Wash (%)		Resin (%)	
	PC2 activity	7B2-ir	PC2 activity	7B2-ir	PC2 activity	7B2-ir
7B2 IgG	87 + 7	1.1 + 0.6	21 + 3	0.63 + 0.26	23 + 4.7	99 + 0.6
Preimmune IgG	80	81	22	17	15	2
IgG	95	100	26	21	15	0

<sup>a</sup> Results are expressed as percentages of total PC2 activity or total 7B2 immunoreactivity. For anti-7B2 IgG data, the results are the mean  $\pm$  SD of four proPC2 preparations (two independent experiments). For preimmune IgG data, the results shown represent two different proPC2 preparations. Approximately 10  $\mu$ g of proPC2, containing 1–2 pmol of 7B2, were applied to the immunoadsorbents in each case.

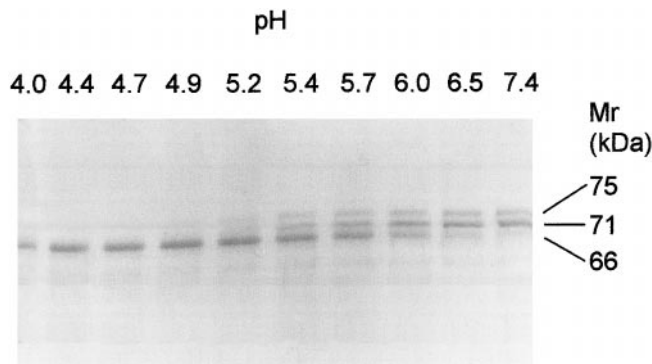


**FIG. 4.** ProPC2 conversion is not dependent on 7B2 concentration. Recombinant proPC2 was incubated with immunoaffinity resins containing either anti-7B2 IgG or preimmune IgG. ProPC2 recovered in the supernatants from these resins (which in the case of the anti-7B2 IgG was 99% depleted in 7B2; Table I) was activated by incubation at pH 5.5 for the times indicated. The image represents a Western blot.

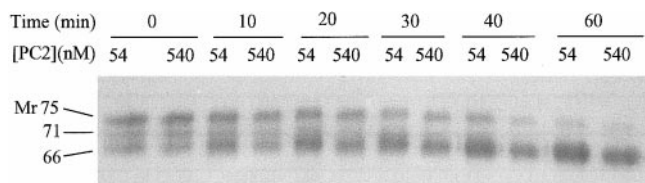
98–99% depletion of 7B2 from the proPC2 preparation was achieved with good recovery of PC2 activity (Table I), the rate of proPC2 activation in 7B2-depleted preparations was not affected (Fig. 4). The 21-kDa 7B2 which copurifies with recombinant proPC2 therefore does not appear to be directly involved in the proPC2 conversion reaction.

#### *pH Dependence of proPC2 Conversion*

The conversion of proPC2 to PC2 was examined by subjecting aliquots of proenzyme to incubation at different pH, separation by SDS-PAGE, and Coomassie staining (Fig. 5). Our data show that conversion reaction is strongly pH dependent. The rate of conversion increased from around neutral pH where it was virtually undetectable (i.e., the Coomassie stain of this batch of starting material corresponds to the profile seen in the pH 7.4 lane; data not shown) to extensive conversion below pH 5.2. Maximal conversion was observed at pHs between 4.0 and 4.9. By contrast, incubation of proPC2 at pH 3.5 did not result in conversion (data not shown), most likely due to protein denaturation. Note that Fig. 5 represents a Coomassie stain



**FIG. 5.** ProPC2 conversion is highly pH-dependent. Purified proPC2 was incubated at 37°C at the indicated pH for 10 min. Concentrated SDS-PAGE sample buffer was then added, and samples were subjected to SDS-PAGE and Coomassie blue staining.



**FIG. 6.** ProPC2 conversion is concentration independent. Purified proPC2 was incubated at 37°C at final proPC2 concentrations of 54 (–) or 540 nM (+). At the indicated time points, aliquots were frozen at –70°C for subsequent analysis. Samples with higher initial concentrations of proPC2 were appropriately diluted 10-fold with the incubation mixture. SDS-PAGE sample buffer stock was added, and equal amounts of protein from each sample were then analyzed on an 8.8% SDS-PAGE gel by Western blotting.

rather than a Western blot; the absence of bands other than proPC2/PC2 forms supports the purity of the preparation (with respect to other PC2-derived proteins). In this particular experiment some sequentiality of conversion of the 75- to the 71- to the 66-kDa species was observed; this sequentiality was however by no means a consistent phenomenon, and most time-dependent experiments, either pH or other parameters, did not exhibit sequentiality (see Figs. 1–4 and 6). Taken together, our results do not support the idea that the 71-kDa species, which represents proPC2 containing only a portion of the propeptide, is a required intermediate for the production of 66-kDa mature PC2.

#### *Concentration Dependence of proPC2 Conversion*

Incubation of proPC2 at 10-fold differing concentrations (54 and 540 nM) was performed in order to test the concentration dependence of conversion (Fig. 6). Quantitation of the bands yielded the following ratios of the 75- to the 66-kDa species (given for 54 and 540 nM): at time 0, 1.3 and 1.3; 10 min, 1.02 and 0.99; 20 min, 0.72 and 0.70; 30 min, 0.59 and 0.52; 40 min, 0.40 and 0.43; and 60 min, 0.28 and 0.29. These highly similar ratios of conversion for enzyme solutions 10-fold different in concentration support the lack of concentration dependence of the conversion process. These results were confirmed in a second experiment with a different proPC2 preparation and 30× concentration differences (not shown).

#### DISCUSSION

Site-directed mutagenesis studies have revealed that the proteolytic maturation of the precursor forms of subtilisin, kexin, furin, PC1, and LPC (PC7) occurs by an intramolecular autocatalytic process (10–16, 32). ProPC2 containing an active-site mutation and expressed in COS cells cannot become activated; however, this mutated proPC2 is also not secreted and accumulates in the ER (33). The lack of competence for ER exit implies that the active-site mutant may not be

properly folded; consequent inability to mature can therefore not be taken as evidence of autocatalytic maturation (33). An intermolecular reaction mechanism for activation has been suggested in the *Xenopus* egg extract expression system; for example, mutation of the catalytic Asp in proPC2 to Asn prevented its conversion to the 68-kDa form (19). This mutated proPC2 could be converted to a lower molecular weight species in the presence of wild-type PC2, leading to the conclusion that maturation occurred by an intermolecular autocatalytic mechanism (19). However, a direct demonstration of catalytic activity by mature oocyte PC2 generated solely by the intermolecular reaction was not provided in this work. Using proPC2 mutants, we have recently demonstrated that propeptide cleavage can be uncoupled from actual activation, when activation is defined as the assumption of a catalytically active species (34). We have termed this type of propeptide removal “unproductive.” The intermolecular reaction observed in oocytes when active PC2 is expressed *in trans* with active-site mutated PC2 might represent such an unproductive reaction; further work will be required to determine whether proPC2 can actually undergo a productive intermolecular activation reaction. It should be noted that the physical basis for the difference between productively and unproductively cleaved PC2 molecules remains obscure; both species migrate identically upon SDS–polyacrylamide electrophoresis.

Purified recombinant proPC2 contains nonstoichiometric quantities of associated 21-kDa 7B2 (9; this paper). We have tested the idea that this associated 7B2 is physically required for the proPC2 activation reaction by adding or removing 7B2 prior to activation; however, no facilitatory effects on activation were observed. These results support the idea that 7B2 is apparently not directly involved in the actual activation reaction but must act upon proPC2 during an intracellular maturation step to generate a species later competent for activation. We have recently shown using a cell-free reconstitution assay that proPC2 must reach the Golgi apparatus for the 7B2 facilitation effect to be manifest (35). The mechanism of this effect is currently under study.

Despite the fact that PC2—like other enzymes of the eukaryotic subtilisin family—requires calcium for activity, we found that proPC2 converted to PC2 at the same rate in the presence or absence of calcium. We have also observed conversion of proPC2 in the presence of EDTA with the cell-free Golgi assay mentioned above (35). These data showing lack of calcium dependence support the idea that the conversion reaction differs significantly from the reaction of mature enzyme with substrate. However, Shennan *et al.* (36) found a strong calcium requirement for conversion of proPC2 synthesized *in vitro* using oocyte egg extract

(but not of proPC1); the reason underlying this discrepancy is unclear, but may involve the fact that the oocyte experiments were performed in the absence of 7B2. It is of interest to note that oocyte PC2—which is secreted as the 71-kDa partially processed proenzyme (37)—exhibits a much higher calcium requirement for enzymatic activity than insulinoma granule PC2 (7) or recombinant mouse PC2 (9), indicating that oocyte-expressed PC2 differs significantly not only for the propeptide conversion process, but also for the active enzyme.

While calcium addition did not enhance the conversion of proPC2, a strong influence of pH was observed, with very rapid conversion at acid pH and virtually no conversion at neutral pH. The low pH requirement for zymogen conversion contrasts with the neutral pH conditions required for the conversion of prosubtilisin, prokexin, and proPC1 (10, 14, 36, 38), but is in general agreement with the low pH of the maturing secretory granules into which PC1 and PC2 are targeted (39) and is consistent with the experimental evidence that cellular proPC2 conversion actually occurs in these compartments (18, 22, 40). Indeed, we have recently shown that prevention of secretory granule acidification with bafilomycin completely blocks proPC2 maturation (35).

7B2 (27 kDa) and the 7B2 CT peptide are potent inhibitors of active PC2 (8, 27). The CT peptide can be hydrolyzed by active PC2, implying that this peptide binds to the active site in a manner similar to actual substrates (28). Our initial data using proPC2/PC2 immunopurified from conditioned medium of  $\beta$ TC3 cells indicated that the CT peptide could block the conversion reaction, albeit weakly (8). However, in the present experiments, using recombinant proPC2, even 100  $\mu$ M concentrations of CT peptide exerted absolutely no effect on proPC2 conversion. The CT peptide was similarly ineffective in blocking proPC2 maturation in the cell-free Golgi assay (35). We speculate that this discrepancy is due to significant differences in the enzyme preparation used in this work as opposed to the previous study: the immunopurified enzyme consists predominantly of active PC2 with only a small amount of zymogen forms, in the presence of immunoglobulins, while the recombinant enzyme preparation consists predominantly of zymogen species rather than active 66-kDa PC2. Immunopurified *Xenopus* proPC2 conversion was also slightly inhibited by forms of 7B2 containing the CT peptide; however, the majority of the enzyme was converted (41). The inability of the CT peptide—which totally inhibits the enzymatic activity of mature PC2 (8, 24)—to affect the conversion of purified recombinant proPC2 provides a strong argument against a major role for intermolecular activation, i.e., a mechanism in which one molecule of PC2 activates a molecule of proPC2. Taken together, these results suggest that—contrary to our previous ideas (8, 27)—

neither 27-kDa 7B2 nor CT peptide can access the zymogen active site and therefore most likely do not directly function to inhibit premature autocatalytic cleavage of proPC2.

The lack of inhibition of proPC2 conversion by the CT peptide and PCMS may indicate that the active site is not fully formed—or is already occupied—in proPC2, precluding the binding of inhibitors. The latter hypothesis is supported by the lack of inhibition of proPC2 conversion by pCMS, an inhibitor of this family of enzymes which is also thought to bind in the active site region. It is interesting to note that Ikemura and Inouye (10) have shown that the subtilisin inhibitor PMSF is unable to block the conversion of prosubtilisin to mature enzyme. These results were interpreted as being due to lack of access of PMSF to the active site, known to be occupied by the subtilisin propeptide. With regard to the eukaryotic subtilisin family, Anderson *et al.* (42) have recently shown that the furin propeptide remains associated with the enzyme and acts as a potent inhibitor of furin activity until arrival of the complex at the Golgi apparatus. Despite the disparate subcellular sites of propeptide cleavage between profurin (which undergoes propeptide removal in the ER (12)) and proPC2 (which undergoes propeptide removal in the TGN/secretory granule compartments (18, 22, 40)), it appears likely that for both enzymes, secretory pathway acidification may represent the primary controlling factor for actual activation. In the case of furin, acidification represents the means to remove associated, but cleaved propeptide, while for PC2, acidification is apparently also required for the actual initial cleavage reaction.

In summary, our data support the notion that proPC2 maturation occurs via an autocatalytic, intramolecular process with enzymatic properties dissimilar to those of the active enzyme. Our pH dependence studies further imply that cellular proPC2 maturation is controlled largely by the decreasing subcellular pH gradient along the secretory pathway, reaching maximal levels in the mature secretory granule compartment.

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