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Purification and Enzymatic Characterization of Recombinant Prohormone Convertase 2: Stabilization of Activity by 21 kDa 7B2[1]

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Although previous efforts to produce significant quantities of purified prohormone convertase 2 from either recombinant or natural sources have been unsuccessful, our recent finding that the neuroendocrine polypeptide 7B2 is necessary for the biosynthesis of enzymatically active prohormone convertase 2 (PC2) has enabled us to obtain active recombinant enzyme from the conditioned medium of PC2-producing CHO cells supertransfected with cDNA coding for 21 kDa 7B2. The recombinant enzyme was purified to apparent homogeneity, with a 40% recovery, in milligram quantities. Two protein bands of M_rs 71 and 75 kDa were observed after SDS–PAGE followed by either Coomassie staining or Western blotting with PC2 antiserum. Spontaneous conversion of the 71- and 75-kDa species to the 66-kDa form occurred during incubation at pH 5.0; the degree of conversion correlated with a dramatic increase in activity. K_ms of 124 and 131 μM and K_{cat}s of 0.49 and 0.81 s^{-1} were obtained for the substrates Cbz-Arg-Ser-Lys-Arg-AMC and Pyr-Arg-Thr-Lys-Arg-AMC, respectively. The pH optimum was 5.0, and the enzyme was inhibited by $h7B2_{155–185}$, *p*-CMS, and EDTA but not by other inhibitors tested. Interestingly, 21 kDa 7B2 was observed to copurify with the enzyme in a molar ratio of about 1:100 (7B2:PC2). Prior addition of recombinant 21 kDa 7B2 to activated 66 kDa PC2 provided significant protection against thermal denaturation. When coassociated 7B2 was mostly removed from activated PC2 through gel filtration, subsequent addition of recombinant 7B2 exerted a significant stabilizing effect on enzyme activity. Millimolar Ca^{2+} and pHs between 5 and 6 were required to observe this effect. Since these conditions resemble those thought to occur within secretory granules, and since 21 kDa 7B2 represents a stored secretory granule protein, our data suggest a physiological role for 21 kDa 7B2 in the stabilization of PC2 activity.

Peptide hormones and neuropeptide neurotransmitters, synthesized as parts of larger biologically inactive precursor proteins, are rendered biologically functional following a series of posttranslational modifications. Such modifications almost invariably involve endoproteolytic activity at restricted positions, most often occurring at pairs of basic amino acid residues (1). The enzymes responsible for these endoproteolytic actions are thought to belong to a new branch of the subtilisin family (reviewed in 2). The yeast enzyme kexin, the mammalian prohormone convertases PC1[3] (also known as PC3), PC2, PACE4, and PC5 (PC6), and the mammalian enzyme furin are collectively known as the kexin/prohormone convertase subfamily of serine proteases (2). These enzymes exhibit a high degree of homology in the catalytic domain, contain proregions of roughly similar lengths, and possess dissimilar carboxyl terminal domains (reviewed in 3). Furin has been shown to have pockets that bind positively charged amino acid side groups (4), thus accounting for its restricted speci-

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[3] Abbreviations used AMC, aminomethylcoumarin; BSA, bovine serum albumin; CHO, Chinese hamster ovary; POMC, proopiomelanocortin; PC1 and PC2, prohormone convertase 1 and 2; DMEM, Dulbecco's modified Eagle's medium; NP-40, Nonidet P-40; DTT, dithiothreitol; IDA, iminodiacetic acid; ir, immunoreactive; IMAC, immobilized metal ion affinity chromatography; PMSF, phenylmethylsulfonyl fluoride; *p*-CMS, *p*-chloromercuriphenylsulfonic acid; E-64, *trans*-epoxysuccinic acid; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; TLCK, *N*-tosyl-*l*-lysine chloromethyl ketone; 7B2 CT peptide, human $7B2_{155–185}$.

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ficity. The restricted specificity for basic residues exhibited by the subtilisin-like processing enzymes distinguishes them from other, structurally related members of the subtilisin family.

Like subtilisin, the prohormone convertases are synthesized with prosequences that must be cleaved to release enzymatic activity (reviewed in 3, 5). Through experiments involving site-directed mutagenesis of catalytic residues, the conversion of profurin to furin and prokexin to kexin has been shown to occur autocatalytically (6, 7). The evidence that the maturation of proPC1 also occurs by an autocatalytic action is also strong (1, 8–11). However, the mechanism by which proPC2 is converted to an active form is less well understood. While Matthews *et al.* (12) have provided evidence to indicate that autocatalytic conversion of proPC2 can in fact occur, we have been unable to observe any autocatalytic maturation of CHO cell-synthesized proPC2 (13).

We have recently demonstrated that the intracellular presence of the neuroendocrine protein 7B2 is required for the activation of proPC2 in CHO cells (14). This interesting bifunctional protein contains two domains involved in proPC2 maturation and regulation, an amino terminal 21-kDa domain responsible for the facilitation of proPC2 activation (14), and a carboxyl terminal inhibitory domain which acts as a potent inhibitor of active PC2 (15, 16). Interaction between 7B2 and PC2 forms occurs early in the secretory pathway (14, 17, 18), and it has been proposed, though not confirmed, that 27 kDa 7B2 may act to prevent premature activation of proPC2 during transport to the Golgi apparatus (17, 19). The precise role of 21 kDa 7B2 in the production of active PC2 is less clear. The fact that the 21-kDa portion of 7B2 is sufficient to facilitate the maturation of proPC2 and transport through the secretory pathway (14) indicates that 21 kDa 7B2 itself must possess affinity for PC2 and/or its proforms. The 21 kDa 7B2 is known to represent a stored proteolytic product of 7B2, probably produced by furin (20), and can be released upon stimulation of cells with secretagogue (21, 22). Coimmunoprecipitation studies investigating the question of whether 21 kDa 7B2 remains associated with mature PC2 have yielded conflicting results (17, 18). In the studies described below, we present data which address the interaction of 21 kDa 7B2 with PC2 forms.

The production of milligram quantities of enzymatically active, purified prohormone convertases represents a necessary step toward the biochemical characterization and eventual crystallization of these important processing enzymes. Although partially purified active PC1 and PC2 have been successfully prepared from insulinoma (23–26) and chromaffin (27) granules, in general the purification of large quantities of PC enzymes from natural sources has been difficult to achieve due to low expression levels and instability. Recombinant DNA technology has greatly facilitated the production of the subtilisin-like enzymes, resulting in the purification and characterization of recombinant kexin (28), furin (29–31), and PC1 (32–34). However, similar attempts at implementing such procedures for PC2 have met with little success, resulting in the production only of catalytically inactive proPC2 (13). Pursuant to our finding that 7B2 is required for the production of active PC2 in CHO cells (14), we have been able to produce large quantities of this prohormone convertase. We present below the purification and characterization of milligram quantities of active PC2, prepared from a CHO cell line initially amplified for the production of proPC2 by the dihydrofolate-coupled amplification method (13) and then supertransfected with cDNA coding for 21 kDa 7B2. Additionally, we provide evidence for an intragranular physiological role of 21 kDa 7B2 in the modulation of PC2 activity by (1) demonstrating the coassociation of 21 kDa 7B2 and mature PC2 during gel filtration; (2) showing that 21 kDa 7B2 can stabilize the activity of mature PC2 *in vitro;* and (3) providing evidence that 21 kDa 7B2 can protect PC2 from denaturation.

EXPERIMENTAL PROCEDURES

Materials. The 3.1-kDa human 7B2 C-terminal peptide (7B2 CT peptide) was synthesized by LSUMC Core Laboratories. Other proteinase inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Custom synthesis of Cbz-Arg-Ser-Lys-Arg-AMC was by Enzyme Systems Products (Dublin, CA). Other fluorogenic substrates were obtained either from Peptides International, Inc. (Louisville, KY) or from Peninsula Laboratories, Inc. (Belmont, CA).

Cell culture and construction of the CHO/PC2-7B2 cell line. We have previously reported the production of a dihydrofolate–reductase-coupled amplified CHO cell line, CHO/PC2, which expresses large quantities of recombinant (but inactive) mouse proPC2 (13). CHO/PC2 cells, grown in 10% dialyzed fetal bovine serum (FBS; Irvine Scientific) in Alpha minus medium (Gibco/BRL, Gaithersburg, MD) containing 50 μM methotrexate, were used as the starting material for transfection with a plasmid encoding a 21-kDa fragment of rat 7B2 (residues 1–155; (35)) subcloned into pCEP4 (Invitrogen) as previously reported (16). CHO/PC2 cells were transfected using the Lipofectin method (Gibco/BRL) and stable colonies were selected with 100 μg/ml hygromycin in Alpha minus medium containing 50 μM methotrexate and 10% FBS. Approximately 50 colonies were screened for 7B2-immunoreactive peptides (14) through radioimmunoassay of overnight conditioned medium (OptiMEM, Gibco) containing 100 μg/ml aprotinin (Miles Laboratories). Results obtained using radioimmunoassays were confirmed through enzyme assays of conditioned medium for PC2 activity, conducted with Cbz-Arg-Ser-Lys-Arg-AMC as substrate as described below. The highest-expressing line, clone 20, secreted approximately 400 fmol/ml of 7B2-immunoreactive peptide following overnight incubation of a 80% confluent 16-mm well in OptiMEM. Clone 20 cells were routinely cultured at 37°C in an atmosphere of 5% CO_2 and were subcultured by splitting 1:6 weekly. Enzyme purified and used for the gel filtration studies was obtained by supertransfecting clone 20 cells with 21 kDa 7B2 cDNA inserted into Rc/CMV. The specific activities of enzyme from both cell lines were similar.

Enzyme assays. PC2 activity was routinely determined by incubating triplicate samples with 200 μM Cbz-Arg-Ser-Lys-Arg-AMC in

100 mM sodium acetate buffer, pH 5.0, containing 5 mM $CaCl_2$, 0.1% Brij, and 0.5 μg bovine serum albumin (BSA) in a sealed polypropylene microtiter plate (50 μl total volume/reaction). Incubations conducted with nonpurified enzyme also routinely contained an inhibitor cocktail consisting of pepstatin (1 μM), tosylphenylalanyl chloromethyl ketone (TPCK, 0.28 mM), *trans*-epoxysuccinic acid (E-64, 1 μM), and tosyllysyl chloromethyl ketone (TLCK, 0.14 mM) (23). Unless stated otherwise, this inhibitor cocktail was not included in incubations with purified enzyme. All incubations were conducted at 37°C; released AMC was estimated in a Cambridge Technology microtiter plate fluorometer at multiple time points ranging from 5 min to several hours, and the amount of product was calculated by reference to a standard curve of free AMC.

Enzyme purification. Conditioned medium (100 ml, OptiMEM, Gibco) was collected from each confluent roller bottle every 24 h. Medium was centrifuged at low speed to remove floating cells and the supernatant was stored at −70°C until needed. A liter of this medium was thawed and pumped at 10 ml/min through a Pharmacia XK 16 column containing 10 ml of iminodiacetic acid (IDA) fast-flow chelating resin (Pharmacia Biotech, Inc., Piscataway, NJ) charged with $CuSO_4$ and previously equilibrated with the eluting and washing buffers. The IDA-Cu^{2+} resin with the bound enzyme was washed with 20 mM Bis–Tris, pH 6.5 (buffer A), for 5 min at a flow rate of 4 ml/min followed by a 2-min linear gradient to 0.5 M sodium acetate in the same buffer (buffer B). This composition was maintained for a further 18 min before a 5-min wash with buffer A. Elution was achieved by a linear increase of 0 to 20 mM L-histidine in 20 mM Bis–Tris, pH 6.5 (buffer C), in 15 min. This eluting buffer was maintained for an additional 55 min (at 4 ml/min). Aliquots of the 4-ml fractions collected were assayed for enzyme activity as described above. The peak enzyme-containing fractions were pooled and loaded onto a Waters Protein-Pak Q 8HR column (1 ml, Waters Chromatography Division, Milford, MA) that had been equilibrated in buffer A. After loading was complete, the column was washed with 2.5 ml of the loading buffer and then eluted with buffer D (1 M sodium acetate in buffer A) using two linear gradients, from 0 to 0.35 M in 175 min and from 0.35 to 1 M in 100 min, respectively. A flow rate of 0.5 ml/min was maintained throughout. Aliquots of the 2-ml fractions were assayed as described earlier. Elution was monitored throughout the purification by measuring the UV absorbance at 280 nm. The total protein present after each purification step was determined using the Bradford method (36). Samples from each purification step were concentrated on MicroCon-10 concentrators (Amicon, Inc., Beverly, MA) before the addition of SDS–PAGE sample buffer. Samples were then boiled for 4 min before electrophoresis on an 8.8% gel followed by Coomassie staining (see below).

SDS–PAGE and Western blotting. Aliquots of purified enzyme were frozen at −70°C immediately after the final ion exchange purification step for subsequent analysis. Sample buffer was added to the frozen samples, which were then boiled for 4 min before SDS–PAGE on 8.8% gels followed by Coomassie staining. Western blotting of nitrocellulose-transferred samples was achieved using anti-PC2 polyclonal antiserum (directed against the last 11 amino acids of mouse PC2) as described by Shen *et al.* (13).

pH dependence of the purified enzyme. The effect of varying pH on the purified enzyme (0.22 μg) was determined by measuring the rate of hydrolysis of 200 μM Cbz-Arg-Ser-Lys-Arg-AMC in 100 mM Bis–Tris/100 mM sodium acetate buffer set at pHs varying between 3.5 and 7.5 (adjusted with acetic acid) in the presence of 5 mM $CaCl_2$, 0.1% Brij, and 0.5 μg BSA (50 μl total volume).

Effect of calcium ion concentration on PC2 activity. The effect of calcium ion concentration on the activity of purified, preactivated enzyme (0.22 μg) was determined by measuring the rate of hydrolysis of 200 μM Cbz-Arg-Ser-Lys-Arg-AMC in 100 mM Bis–Tris/100 mM sodium acetate buffer at pH 5.0 in the presence of various $CaCl_2$ concentrations, 0.1% Brij, and 0.5 μg BSA (50 μl total volume). Results are presented as the mean ± SE of triplicate determinations.

Effect of proteinase inhibitors on PC2 activity. Various proteinase inhibitors were preincubated with purified PC2 (0.11 μg) in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM $CaCl_2$, 0.1% Brij at 22°C for 30 min. Substrate (Cbz-Arg-Ser-Lys-Arg-AMC, 200 μM final concentration) was then added followed by incubation at 37°C for an additional 30 min. The degree of hydrolysis was measured by fluorometry.

Active site titration. The amount of active PC2 in the enzyme preparation was determined by titration with a specific PC2 inhibitor, the carboxy-terminal fragment of human 7B2 (7B2 CT–peptide) (15). Preactivated, purified enzyme (1.23 μg; approximately 370 nM based upon a molecular weight of 66,000 kDa) was incubated with varying nanomolar concentrations of 7B2 CT-peptide in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM $CaCl_2$, 0.1% Brij for 60 min at 37°C. Cbz-Arg-Ser-Lys-Arg-AMC (200 μM final concentration) was then added, followed by further incubation at 37°C for 39 min and fluorometry to determine the amount of uninhibited activity. The experiment was repeated once with similar results.

Spontaneous cleavage of the 75- and 71-kDa forms of PC2. Purified enzyme (0.22 μg) was incubated at 37°C in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM $CaCl_2$, 0.1% Brij in duplicate. After each incubation period, samples were immediately frozen at −70°C for subsequent enzymatic and SDS–PAGE analysis. For Western blot analysis, SDS–PAGE sample buffer (37) was added to the samples, which were then thawed and boiled for 4 min prior to SDS–PAGE and Western blot analysis of a fifth of each sample. Parallel samples for enzymatic analysis were thawed and the initial rates of PC2 activity immediately determined using Cbz-Arg-Ser-Lys-Arg-AMC as substrate. A time course was also conducted under identical conditions except that 200 μM Cbz-Arg-Ser-Lys-Arg-AMC was included in the initial incubation mixture; fluorescence readings were obtained at multiple time points (as indicated in the figure legend). Continuous monitoring of the release of AMC was performed in order to detect potential changes in catalytic rate during long incubation periods.

Hydrolysis of purified rat proenkephalin. Recombinant rat proenkephalin (1 μg; (38)) purified by ion-exchange rather than by reverse-phase chromatography (Bui and Lindberg, unpublished results) was incubated with 0.22 μg of purified enzyme in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM $CaCl_2$ and 0.1% Brij (50 μl total volume). After the incubation periods indicated, samples were frozen for subsequent analysis by Western blotting of a fifth of the sample. SDS–PAGE sample buffer (1/10th vol of a 10× concentrated stock solution; (37)) was added to the frozen samples, which were immediately boiled for 4 min before SDS–PAGE on 10–20% gradient gels (Bio-Rad Laboratories, Inc.), transfer to nitrocellulose, and Western blotting using antiserum raised against peptide F (39). In one experiment, various inhibitors were included in the reaction mixture, as indicated in the figure legend, and in the other experiment, a time course of proenkephalin cleavage was performed in the presence or absence of 7B2 or 7B2 plus an inhibitor cocktail (0.14 mM TLCK, 1 μM pepstatin, and 1 μM E-64).

Size-exclusion chromatography of purified PC2. Purified PC2 (140 μg) was loaded onto a Superdex 75 HR 16/60 column (120 ml bed volume, 60 cm long, Pharmacia Biotech, Inc., Piscataway, NJ) and eluted with 20 mM Bis–Tris buffer, pH 5.5, containing 0.2 M sodium acetate at a flow rate of 1 ml/min. Fractions (2 ml) were analyzed for PC2 and 21 kDa 7B2 by assaying for enzymatic activity and radioimmunoassay (RIA) for 7B2 (14), respectively. Enzyme for size-exclusion experiments was preactivated to generate the 66-kDa form of PC2 by incubating purified enzyme in sodium acetate buffer, pH 5.0, at 37°C for 2 h.

Effect of 21 kDa 7B2 on the thermal stability of PC2. PC2 (20 ng, ~8 nM) obtained from size-exclusion chromatography was incubated at temperatures between 37 and 65°C for 5 min in 100 mM sodium acetate buffer containing 5 mM $CaCl_2$ and 0.2% Brij in the presence or absence of 100 nM 21 kDa 7B2. Time dependence of the 7B2

stabilization effect was conducted at 50 and at 52.5°C over a time range of 1 h. To determine the effect of pH, calcium ion and 21 kDa 7B2 concentration on the stability of PC2, incubations were conducted at 52.5°C over a period of 1 h in 20 mM Bis–Tris/20 mM sodium acetate set at different pHs or 21 kDa 7B2 concentrations depending on the parameter under investigation; all other factors remained constant. Aliquots of the various samples were kept on ice for subsequent determination of their residual PC2 enzymatic activity under standard conditions, in the presence or absence of added 21 kDa 7B2 (100 nM).

Preparation of rat recombinant 7B2. The QIAexpress system (Qiagen Inc., Chatsworth, CA) was used to express 21-kDa 7B2 protein in *Escherichia coli.* Two primers, 5′-CGGCCGGATCCTATAGTCCACGGACTCCT-3′, and 5′-CCGGCAAGCTTTTACTGTCCTCCCTTCATC-3′, were used in the PCR reaction. The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI- and *Hin*dIII-linearized QE30 plasmid. The ligation mixture was then used to transform *E. coli* XL-Blue (Promega, Madison, WI). The selection of 21 kDa 7B2-expressing clones and the large-scale production of this protein was performed similarly as in Ref. (19), using the guanidine–HCl denaturing method essentially according to the manufacturer's instructions for denatured proteins. A single band of purified protein was observed on a 15% SDS–PAGE gel. Radioimmunoassay for 7B2 was carried out as previously described (14). The cross-reaction of recombinant rat 21 kDa 7B2 in this assay is about 30%; results have not been corrected for cross-reaction.

RESULTS

Enzyme Purification

The use of IDA-Cu^{2+} chelating resin proved to be a valuable first step toward the purification of recombinant PC2 from the conditioned medium, resulting in rapid concentration of the enzyme. On all occasions, all of the activity bound to the resin while phenol red essentially flowed through during the loading phase. The enzyme remained firmly bound to the resin even during prolonged washes with 0.5 M sodium acetate-containing buffer until a stable baseline was obtained. When a gradient of L-histidine in 20 mM Bis–Tris, pH 6.5, was applied to the column, a single UV absorbing peak was observed. This UV peak coincided with the peak of activity when the fractions were assayed with Cbz-Arg-Ser-Lys-Arg-AMC as substrate (Fig. 1). The total activity recovered (34% of the applied activity, Table I) was loaded onto a Waters high-performance anion-exchange column for further purification.

When the enzyme obtained from IDA-Cu^{2+} was loaded onto the anion exchange column, no activity was detected in the flowthrough, indicating efficient binding to the column. All the Cu^{2+} ions which leached from the IDA-Cu^{2+} appeared in the flowthrough, as indicated by its characteristic blue color. Following application of the sodium acetate gradient, several UV peaks were observed at 280 nm. The bulk of the protein (mainly transferrin) eluted at the earlier part of the gradient, well separated from the enzyme activity peak (Fig. 1B). Only a single peak of activity (assayed with Cbz-Arg-Ser-Lys-Arg-AMC) was again observed with this purification step. A higher amount of activity was recovered from the anion exchange chromatographic column than was actually loaded (Table I), most probably due to measurement of enzyme activities in the presence of copper which leached from the IMAC column.

SDS–PAGE analysis with Coomassie staining of enzyme from the various purification steps revealed that most of the contaminating protein was eliminated during the anion exchange step (Fig. 1C). (It should be noted that the major protein detected in the IDA eluate is transferrin rather than proPC2; transferrin coincidentally possesses the same molecular weight as 75-kDa proPC2. However, preincubating the purified enzyme followed by SDS–PAGE analysis and Coomassie staining revealed that all of the 75- and 71-kDa bands converted to the 66-kDa form of PC2, thus demonstrating the absence of transferrin, Fig. 1D). Two or three Coomassie bands were observed with samples from the peak fractions of the anion exchange step. These bands, with estimated molecular weights of 66, 71, and 75 kDa, respectively, were shown by Western blot analysis to be immunoreactive to polyclonal antiserum raised against the carboxyl terminus of PC2 (Fig. 2A). Enzyme activity present in the fractions was highly correlated with the amounts of the 75- and 71-kDa bands but not with the 66-kDa band (Fig. 2B); the 66-kDa protein apparently represents an unstable form which either does not survive the collection medium or the various purification steps. However, the 75/71-kDa enzyme obtained from ion exchange spontaneously converted to the active 66-kDa protein upon incubation at pH 5.0 (results not shown), thus accounting for the high levels of enzyme activity in fractions containing these proteins. Western blot analysis for possible contamination of the purified enzyme with bovine serum albumin was negative (results not shown). As described below, the enzyme preparation contained small (nonstoichiometric) quantities of copurifying immunoreactive 21 kDa 7B2.

Time Course and Substrate Specificity of the Purified Enzyme

Time-dependent studies of purified recombinant PC1 have shown that this enzyme exhibits a lag phase before linear velocities are attained (34); immunopurified PC2 also exhibits a lag phase (15). We therefore conducted a similar study with purified recombinant PC2. The results (Fig. 3) show a similarly slow cleavage of Cbz-Arg-Ser-Lys-Arg-AMC at early time points, with an initial lag phase followed by a period in which product accumulated linearly with time.

Biochemical Characterization of Recombinant PC2

The purified recombinant enzyme was active at acidic pHs between pH 4.3 and 5.7, displaying maxi-

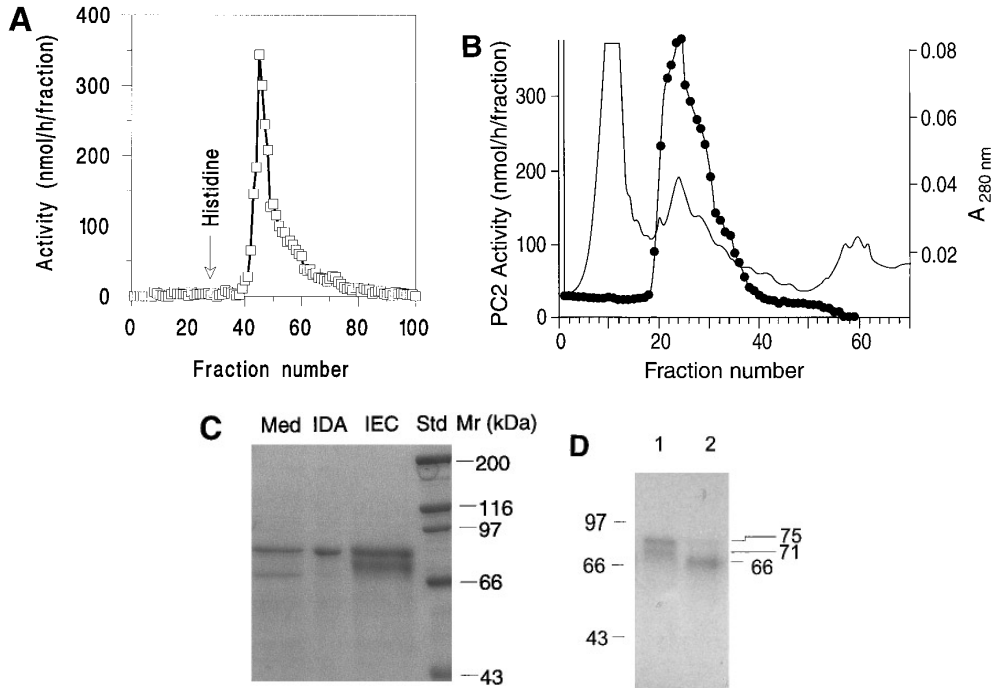

FIG. 1. Purification of recombinant PC2. (A) PC2-containing medium was pumped at 10 ml/min through a $CuSO_4$-charged IDA resin (IDA-Cu^{2+}), which was washed with 100% buffer A (20 mM Bis–Tris, pH 6.5) followed by a linear increase to buffer B (0.5 M sodium acetate in A) to 100% in 2 min; buffer B was maintained for 18 min and the column was then returned to buffer A. Concentrated proteins were eluted with a linear increase of histidine-containing buffer (shown with arrow) from 0 to 100% in 15 min. The UV absorbance of the eluate was monitored at 280 nM. Aliquots (20 μl) of each fraction were assayed for enzymatic activity as described under Experimental Procedures. (B) The enzyme peak from the IDA-Cu^{2+} column was pooled, loaded onto a Waters anion exchange column, and washed for 5 min with buffer A. This was followed by two linear increases of buffer D (1 M sodium acetate in buffer A) from 0 to 35% in 175 min and 35 to 100% in 100 min. Aliquots of each fraction (20 μl) were assayed for enzymatic activity (●), and UV absorbance was monitored at 280 nm (—). (C) SDS–PAGE of enzyme protein obtained at various stages of purification. Medium (Med), IDA-Cu^{2+} chelation resin (IDA), and ion-exchange (IEC) samples were concentrated using MicroCon-10 units before analysis by electrophoresis and Coomassie staining. (D) Spontaneous activation of purified PC2. An aliquot of fraction 25 from the ion-exchange column was either kept on ice (lane 2) or was incubated at 37°C in 0.1 M sodium acetate buffer, pH 5.0, in the presence of 5 mM $CaCl_2$ and 0.1% Brij for 2 h.

mum activity around pH 5.0 (Fig. 4). The enzymatic activity was markedly increased by Ca^{2+}, with half-maximal activity at approximately 100 μM and maximum activity close to 1 mM (not shown). A battery of proteinase inhibitors was used to obtain a profile of inhibition for PC2. The results, summarized in Table II, reveal that the purified recombinant enzyme was totally inhibited by 10 μM 7B2 CT peptide and was substantially inactivated by pCMS and EDTA. No effect on PC2 activity was observed using the metalloproteinase inhibitor 1,10-phenanthroline or the diagnostic thiol proteinase inhibitor E-64.

TABLE I

Purification of Recombinant PC2

Purification step	Total activity (units)[a]	Total protein (μg)	Specific activity (units/μg)	Yield (%)	Purification factor
Conditioned medium	9430	47700	0.20	(100)	(1)
IDA-Cu^{2+}	3160	24900	0.13	(34)	(0.64)
Ion exchange	3740	2510	1.49	40	7.5

[a] 1 unit = 1 nmol/h.

Active site titration (15) of the preactivated, 66-kDa enzyme was carried out in order to obtain an estimate for the number of active enzyme molecules in the preparation to obtain the concentration of active enzyme in the standard reaction, approximately 100 ± 5 nM. A minor population of enzyme molecules was observed to exhibit a lesser affinity for the 7B2 CT peptide; since the enzyme used for the experiment was preactivated to the 66-kDa form, this form is unlikely to represent a proteolytic variant, and its identity is currently unknown. Calculations based on total protein assay (36) and titrations with 7B2 CT peptide revealed that 27% of the enzyme protein consisted of active enzyme (assuming that only active forms of PC2 bind 7B2 CT peptide).

The titrated enzyme (26 nM active enzyme) was used in incubations with varying concentrations of Cbz-Arg-

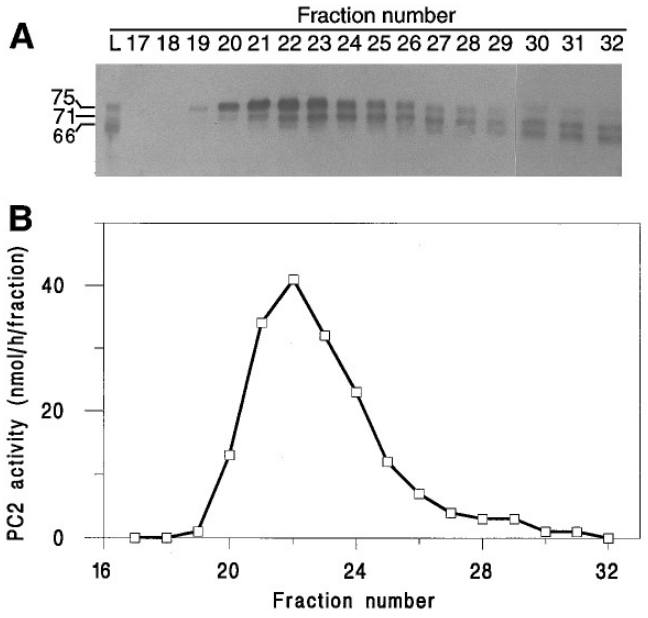

FIG. 2. Analysis of peak enzyme fractions obtained from ion exchange. (A) Western blotting of anion-exchange fractions from a purification similar to that described in Fig. 1. (B) PC2 activity in the same fractions.

Ser-Lys-Arg-AMC and Pyr-Arg-Thr-Lys-Arg-AMC and the initial rates of hydrolysis measured (Fig. 5). These experiments demonstrated K_m's of 124 and 131 μM and K_{cat}'s of 0.49 and 0.81 s^{-1} respectively for these two substrates (Table III).

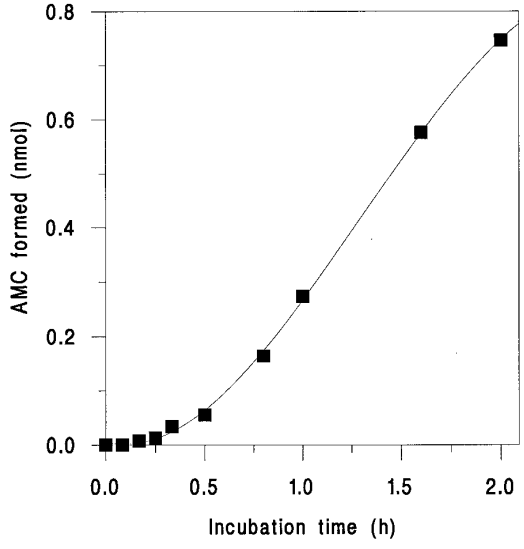

FIG. 3. Time course for the hydrolysis of Cbz-Arg-Ser-Lys-Arg-AMC by PC2. PC2 (0.22 μg) was incubated with substrate in triplicate and the rate of hydrolysis was determined by measuring the fluorescence of the released AMC at various time points.

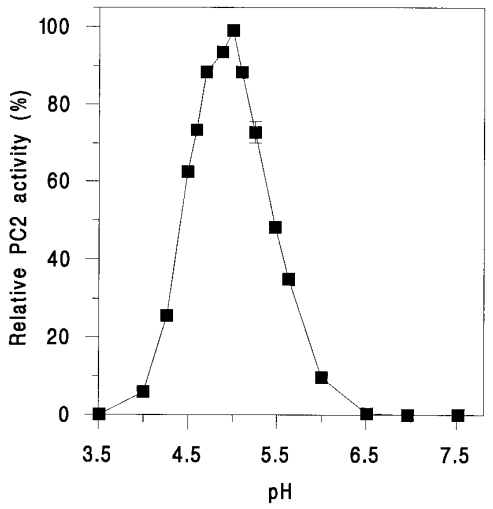

FIG. 4. Effect of pH on PC2 activity. Purified recombinant PC2 (0.22 μg) was incubated with substrate in 100 mM Bis–Tris/100 mM sodium acetate buffer adjusted to various pHs under the conditions described under Experimental Procedures.

Spontaneous Cleavage of the 75- and 71-kDa Forms of PC2 to the 66-kDa Form

Prior to incubation, the bulk of the purified enzyme was present as the 71-kDa form, with lesser amounts of the 75- and 66-kDa forms. However, upon incubation at 37°C, substantial time-dependent conversion to the 66-kDa form was observed (Fig. 6A). This experiment confirms the previous results obtained using Coomassie staining of total protein, which demonstrated that the 66-kDa protein was the final conversion product of the larger molecular weight forms (Fig. 1D). Transformation to the 66-kDa form was complete within 1 h of the start of incubation. Measurement of the initial

TABLE II

Effects of Proteinase Inhibitors on PC2 Activity

Inhibitor	Final concentration	Relative activity % of control (SE, n = 3)
Control	—	100 ± 3.3
7B2 CT peptide	10 μM	0.91 ± 0.21
pCMS	1 mM	4.89 ± 0.22
EDTA (in presence of 5 mM calcium)	10 mM	47.6 ± 1.3
Soybean trypsin inhibitor	0.1 mg/ml	112 ± 5.4
1,10-Phenanthroline	2 mM	94.5 ± 4.7
Pepstatin	10 μM	107 ± 3.01
E-64	10 μM	104 ± 4
PMSF	10 mM	120 ± 8
TPCK	2.6 mM	111 ± 1.4
TLCK	1.3 mM	93.5 ± 1.3

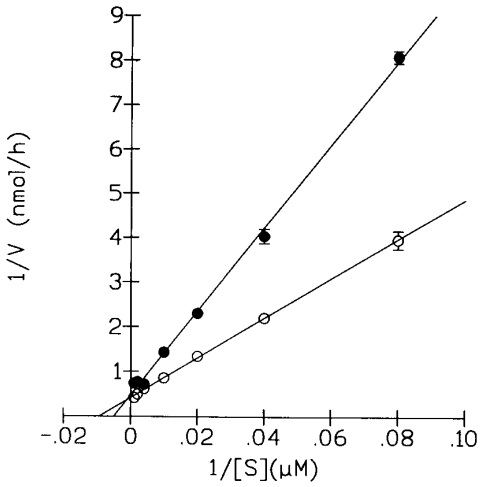

FIG. 5. Lineweaver–Burke analysis for the cleavage of Cbz-Arg-Ser-Lys-Arg-AMC (●) and Pyr-Arg-Thr-Lys-Arg-AMC (○) by PC2. Purified PC2 (0.22 μg) was preincubated in assay buffer for 1 h at 37°C and then incubated with various concentrations of substrate; initial hydrolysis rates were then measured.

activity rates of the forms produced at each of these incubation times revealed a strong correlation of enzyme activity with the production of the 66-kDa form (Fig. 6B). Over a threefold increase in initial rate was observed with a 2-h preincubation of the PC2 preparation. Since incubation resulted in the simultaneous conversion of the 75- and 71-kDa forms to the 66-kDa form of PC2, these data strongly suggest that this latter species represents the active form of PC2. The presence of substrate had no effect on this conversion, since samples containing Cbz-Arg-Ser-Lys-Arg-AMC (200 μM) but otherwise treated similarly exhibited identical kinetics for this spontaneous cleavage event (data not shown). A cocktail of inhibitors including pepstatin, TLCK, TPCK, and E-64 did not inhibit the conversion event, either in the presence or absence of 7B2 CT peptide or of recombinant rat 27 kDa 7B2 (results not shown).

Hydrolysis of Rat Proenkephalin

In order to examine the activity of the purified enzyme against a physiological substrate, rat proenkephalin was used. Recombinant rat proenkephalin was rapidly cleaved by purified recombinant PC2, resulting in five fragments (it should be noted that only peptides sufficiently large to bind to nitrocellulose will be detected with this technique). Peptides of M_rs 31.5, 26, 24.5, 21.5, and 16 kDa were detectable after 2 h of incubation followed by SDS–PAGE and Western blotting for peptide F (Fig. 7). The M_rs of the 26-, 21.5-, and 16-kDa peptides are similar those of immunoreactive proenkephalin fragments detected in chromaffin granules by Birch *et al.* (40) and by our group in transfected AtT-20 cells (41). Cleavage of proenkephalin could be completely abolished by 7B2 CT-peptide (10 μM) but not by pepstatin, TLCK, or E-64 nor by a combination of all three inhibitors (not shown). Enzyme purified by a procedure previously successfully used to obtain homogeneous PC1 (34) was unable to cleave proenkephalin (lane 9) and exhibited low specific activity against the fluorogenic substrate (not shown), possibly due to removal of bound copurifying 7B2 during phenyl–Sepharose chromatography.

21 kDa 7B2 Concentration of Purified mPC2

Radioimmunoassays for 7B2 using purified recombinant PC2 (14) revealed a molar ratio of 21 kDa 7B2 to active PC2 of about 1:100 (range, 1:60 to 1:190; PC2 values obtained by titration), with actual concentrations of 7B2 in the enzyme preparation of approximately 5 nM (it should be noted that the curve for cross-reaction of recombinant 21 kDa 7B2 in the peptide 7B2 assay is not linear, and that these numbers therefore represent only approximations). Assays of PC2 activity using enzyme purified on IDA-Cu^{2+} resin and Waters Q columns did not reveal any increase in enzymatic activity due to addition of recombinant 21 kDa 7B2, most likely due to the presence of this endogenous 7B2. However, reducing the amount of enzyme used in the enzyme assays (about 50- to 500-fold) did reveal higher activities in the presence of recombinant 7B2. The difference in activity between samples replenished with 21 kDa 7B2 and those in which no 21 kDa 7B2 was added increased with further dilution of PC2 (results not shown).

Size-Exclusion Chromatography of Purified PC2

When ion-exchange purified PC2 was preactivated and applied to a size exclusion column, a single peak of PC2 activity was observed (Fig. 8A). In contrast, the

TABLE III

Kinetic Constants for Purified PC2[a]

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Pyr-Arg-Thr-Lys-Arg-AMC	131 ± 11	733 ± 20	0.81 ± 0.02	6160 ± 153
Cbz-Arg-Ser-Lys-Arg-AMC	124 ± 9	442 ± 10	0.49 ± 0.01	3920 ± 84

[a] Triplicate determinations were conducted with 26 nM active PC2 (based on active site titration with 7B2 CT peptide). Results are given as the mean ± SE.

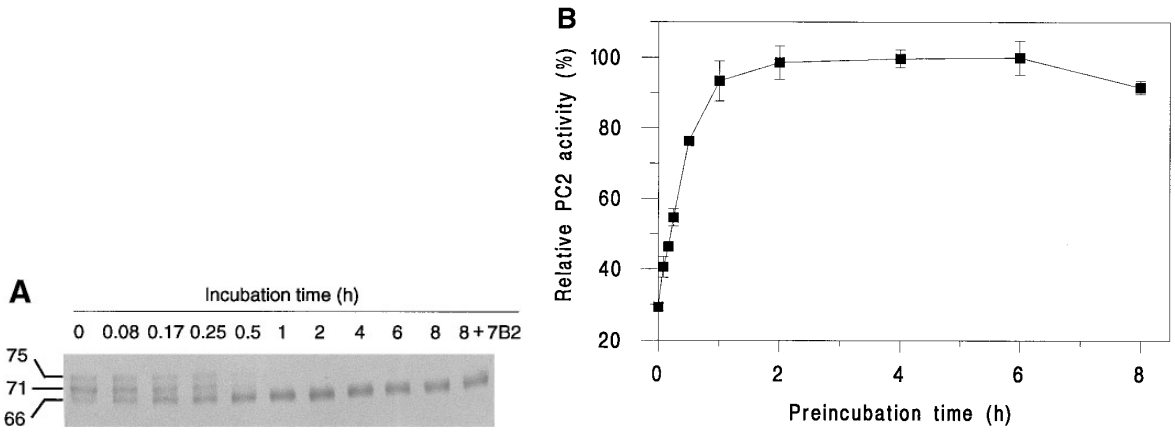

FIG. 6. Autoconversion of 75 and 71 kDa PC2 to the 66-kDa form. Purified enzyme (0.22 μg/point) was incubated in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM $CaCl_2$ and 0.1% Brij in the absence of substrate (A and B) for the times indicated and frozen until further analysis. (A) Time-dependent conversion of PC2 forms. Samples were incubated for the times indicated; Western blotting with anti-PC2 antiserum was then performed on a fifth of each sample. An incubation was also conducted with 10 μM 7B2 CT–peptide (lane 8+7B2). (B) Enzymatic activity of the molecular weight forms of PC2 shown in A. Following preincubation of samples for the times indicated in A, one-fifth of each sample was removed and reconstituted in assay buffer with substrate for the determination of initial hydrolysis rates (16 min incubation at 37°C); results are expressed as a percentage of maximum activity.

7B2 RIA revealed four peaks of apparent M_rs 3, 5, 34, and 95 kDa; one of the peaks of immunoreactive 7B2 (peak A, Fig. 8A) was always associated with enzyme peak whether or not proPC2 forms (75 and 71 kDa) were present in the samples applied to the gel filtration column. An identical elution pattern of 7B2-ir was observed when gel filtration was conducted at pHs of 6.3 and 7.5. The 95- and 34-kDa peaks (peaks A and B, respectively, Fig. 8A) may represent the 21 kDa 7B2 complex with PC2 and free 7B2, respectively, while the smaller peak (peak C) may represent a degradation product of 7B2 generated in the collection medium. Although the pattern of 7B2 immunoreactive peaks remained the same, variations in the ratios of the various peaks were observed for different enzyme preparations.

Following removal of most of the endogenous 7B2 in the purified, activated enzyme by gel filtration, addition of 100 nM 21 kDa 7B2 to the incubation mixture resulted in a significant increase in enzymatic activity (Fig. 8B). Using increasing amounts of gel filtration-derived PC2 resulted in a decreased activation effect of 21 kDa 7B2; this can possibly be attributed to the contribution of enzyme-derived residual 21 kDa 7B2. When a time course was conducted with enzyme obtained through gel filtration, the activity was linear over a longer time period in samples saturated with 21 kDa 7B2. In contrast, samples without added 21 kDa 7B2 displayed lesser activity as well as a more pronounced decrease in the rate of enzyme activity with time (Fig. 9). Bovine serum albumin was used as a control protein but itself produced no effect on the activity of PC2 (not shown).

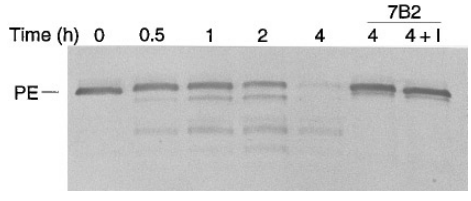

FIG. 7. Hydrolysis of rat proenkephalin. Recombinant rat proenkephalin (PE) was incubated with purified PC2. At the various time intervals shown, aliquots were frozen for analysis by Western blotting with antiserum against peptide F. 7B2 CT peptide was included in the presence (lane 4 + I) or absence (lane 4) of inhibitor cocktail.

Effect of 21 kDa 7B2 on the Thermal Stability of PC2

We found that preincubating PC2 at temperatures between 37 and 65°C resulted in severe loss of activity at temperatures above 42.5°C. As shown in Fig. 10A, adding 21 kDa 7B2 prior to heat treatment resulted in a significant increase in the stability of PC2. A time-dependent study of this protection of PC2 revealed that after 60 min of thermal denaturation, about 75% of residual enzymatic activity was still present in samples which contained 100 nM 21 kDa 7B2 prior to heating. In contrast, only around 20% of the PC2 activity remained in samples with no added 7B2 (Fig. 10B). Addition of 7B2 to samples only following the denaturation process did not exhibit a similar restoration of PC2 activity; i.e., 7B2 was apparently not able to promote spontaneous refolding of thermally denatured enzyme. The increased activity observed in samples containing 7B2 added after the heat treatment is most likely due to the stabilization of PC2 during the enzyme assay period (see Fig. 9). Millimolar concentrations of Ca^{2+}

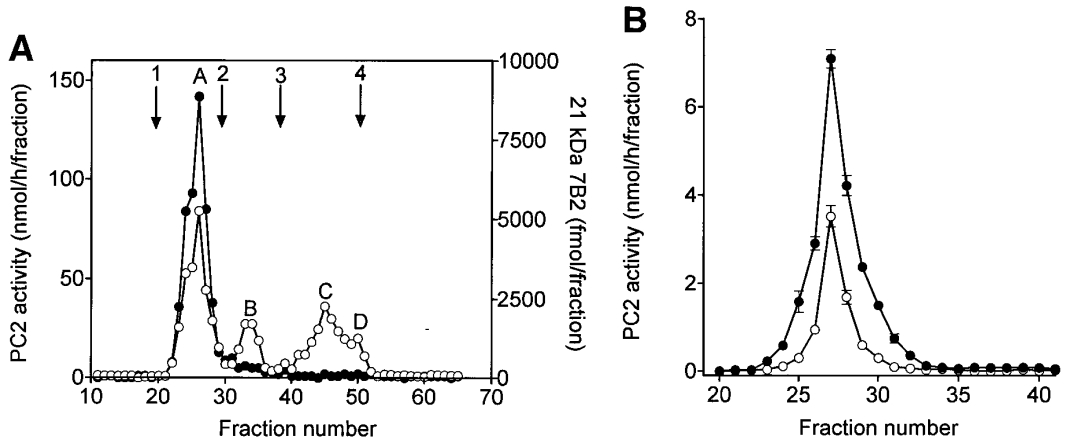

FIG. 8. Size exclusion chromatography of purified recombinant mPC2. Purified recombinant mPC2 (140 μg) was preactivated, loaded onto a Superdex 75 HR 16/60 column and eluted with 20 mM Bis–Tris, pH 5.5, containing 0.2 M sodium acetate. (A) Coelution of enzymatic activity with 7B2 immunoreactivity. Aliquots of the 2-ml fractions were assayed for total enzymatic activity (●) and immunoreactivity to 7B2 antibody (○) as described under Experimental Procedures. The elution volumes of molecular weight markers (Bio-Rad gel filtration standards) are indicated by arrows: 1, bovine γ-globulin (158 kDa); 2, chicken ovalbumin (44 kDa); 3, equine myoglobulin (17 kDa); 4, vitamin B 12 (1.4 kDa); and 7B2 immunoreactive peaks: A, 95 kDa; B, 34 kDa; C, 5 kDa; and D, 3 kDa. (B) Stimulation of enzymatic activity in individual fractions by 7B2. The effect of 21 kDa 7B2 on the enzymatic activity of the peak fractions was tested by assaying for activity in the presence of 100 nM 21 kDa 7B2 (●) or 2.1 μg BSA (○).

were required for the stabilization process (Lamango and Lindberg, unpublished data). Heat treatment at different pHs followed by measurement of residual activity demonstrated that the stabilization effect of 21 kDa 7B2 was pH-dependent, with maximal thermostabilization between pH 5 and 6 (Lamango and Lindberg, unpublished data).

DISCUSSION

The production of recombinant, active, purified PC2 has been impossible to achieve thus far despite the availability of cDNAs coding for this enzyme for the past 4 years. With the realization that the production of active PC2 requires the intracellular presence of its binding protein, 7B2 (14), we have been able to generate the large-scale CHO cell cultures similar to those previously successfully employed to produce recombinant PC1 (34). Interestingly, we have been able to generate PC2 in milligram quantities, while PC1, due to problems with aggregation, was not obtainable in nearly these quantities. The good yield of this enzyme bodes favorably for future crystallization attempts.

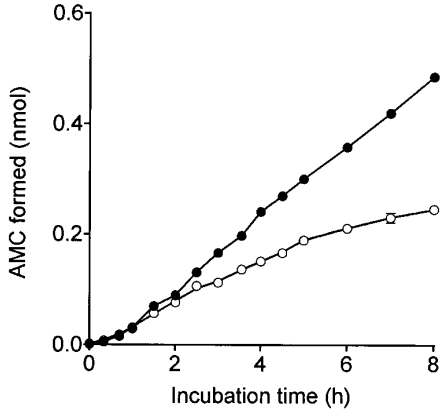

FIG. 9. 21 kDa 7B2 stabilizes PC2 obtained through size exclusion chromatography. The enzymatic activity of mPC2 (10 ng) was assayed either in the presence of 2.1 μg 21 kDa 7B2 (●) or 2.1 μg BSA (○) over the time range indicated. Individual data points represent the means ± SE, $N = 3$.

Although the enzymatic properties of naturally occurring PC2 have been previously described (23–26, 42), we thought that it was important to carry out a thorough biochemical description of the properties of recombinant PC2, since it appeared possible that recombinant and natural proteins could differ in significant ways. Indeed, recombinant PC2 expressed by oocytes has been reported to exhibit a millimolar calcium requirement (43), while all sources of naturally occurring PC2 exhibit calcium requirements in the high micromolar range (23, 42, 43). It was thus not clear *a priori* which properties our recombinant PC2 preparation might exhibit.

With regard to the purification, we employed a different, more rapid procedure to purify PC2 from conditioned medium than that previously used for the purification of PC1. Adsorption of PC2 forms from conditioned medium onto the IDA-Cu^{2+} metal chelation resin proved to be useful not only in concentrating the enzyme into a small workable volume but eliminated phenol red and other compounds that could pose prob-

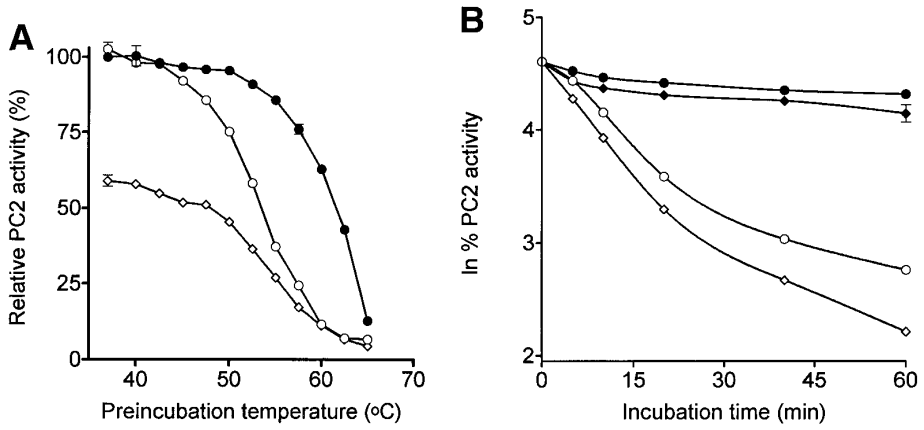

FIG. 10. 21 kDa 7B2 protects PC2 from thermal denaturation. Purified recombinant mPC2 (20 ng) obtained by gel filtration was incubated at the indicated temperatures for 5 min in 100 mM sodium acetate buffer, pH 5.0, in the presence of 6.25 mM $CaCl_2$ and 0.2% Brij. Aliquots were kept on ice and subsequently assayed for residual PC2 activity for 15 h. (A) Incubation at various temperatures. 2.1 μg 21 kDa 7B2 (●) or 2.1 μg BSA (○ and ◇). (B) Time course of denaturation. Heat treatment was conducted for various time intervals either in the presence of 2.1 μg 21 kDa 7B2 at 50°C (●) or at 52.5°C (◆); or of 2.1 μg BSA at 50°C (○) or at 52.5°C (◇). Assay for residual PC2 activity in all samples was conducted in the presence of 2.1 μg 21 kDa 7B2. Individual data points represent the means ± SE, $N = 3$.

lems during the ion exchange step. Most of the actual purification occurred during the anion exchange step, with elimination of serum-derived contaminants, including transferrin (also present in OptiMEM at 50 μg/ml) and BSA. The apparent increase in activity following the anion exchange step can possibly be linked to the elimination of the copper ions leached into the enzyme fractions during the elution phase of the IDA-Cu^{2+} step. Cu^{2+} and other heavy metal ions have previously been shown to inactivate PC2 from insulinoma granules (25, 42). The purification procedure reported in this study resulted in a 40% recovery of activity, comparable to 36% recovery for PC1 (34) and about four times that obtained for furin (29, 44). Approximately 2.5 mg of protein was obtained per liter of conditioned medium. This ratio of recovered activity to the amount of recovered protein is comparable to that obtained when furin was purified from 500 ml of conditioned medium (44). However, titration studies of purified activated PC2 with 7B2 CT-peptide indicated that only 27% of the PC2 represented active enzyme, and since it is not presently known whether this peptide can also bind to inactive enzyme forms, this number may represent an overestimate.

The K_m of purified PC2 for the substrate Cbz-Arg-Ser-Lys-Arg-AMC (124 μM) is similar to that previously obtained for PC1 (145 μM; (34)). The K_m obtained for Pyr-Arg-Thr-Lys-Arg-AMC with a soluble form of CHO-expressed furin (5.86 μM; (44)) was much better than that for PC2 obtained in this study. However, the K_{cat} for furin (0.12 s^{-1}) was substantially lower than that observed here (0.5 s^{-1}). Soluble furin prepared using the baculovirus method exhibited a K_m of 18 μM against Boc-RVRR-AMC and a K_{cat} of 0.6 s^{-1} (30). It has been speculated that the low turnover numbers of the mammalian subtilisin-like enzymes are related to their long residence times with their prohormone substrates (30).

The pH optimum of PC enzymes has been predicted to bear significance to the cellular localization of action of the various enzymes (1). Increasing evidence suggests that PC1 may act upon prohormone substrates prior to PC2, possibly in the *trans*-Golgi network (46). The optimum pH of PC2, 5.0, is lower than that of PC1 (5.5–6.5; (34)); since a decreasing pH gradient exists within the secretory pathway (reviewed in 1), the finding of a lower optimal pH for recombinant PC2 supports the notion that PC2 may act at a later stage of processing than PC1. It is curious to note that previous studies of the pH optimum of PC2 preparations have provided widely disparate results, with some studies reporting an acidic optimum similar to that found here (17, 23, 26, 42, 43, 47) and other studies reporting an optimum neutral pH (27, 48). The reason for this discrepancy is unclear at present. A recent comparison of oocyte-derived recombinant PC2 with insulinoma granule PC2 revealed a similar optimum pH for both preparations (43). However, in this study, the calcium concentration required for maximal activity was found to differ greatly between oocyte-derived recombinant PC2 and granule PC2 (43). Our data showing maximal stimulation of PC2 activity at submillimolar calcium levels are consistent with the insulinoma granule data (23, 42, 43), and potentially indicate differences in the post-translational modifications or in the folding of CHO cell PC2 as opposed to oocyte PC2.

The C-terminal fragment of 7B2 and the parent 27-kDa protein represent potent and specific natural in-

hibitors of PC2 (15, 17–19). Complete inhibition of Cbz-Arg-Ser-Lys-Arg-AMC hydrolysis by 7B2 CT–peptide thus provides evidence to indicate that all of the activity in our PC2 preparation toward this substrate can in fact be attributed to PC2. The inhibition pattern displayed by the other proteinase inhibitors, including effective inhibition by the thiol-directed reagent pCMS, is similar to results obtained for related subtilisin-like enzymes (17, 33, 34, 44).

Recombinant PC2 exhibited a distinct lag phase in the early stages of hydrolysis of fluorogenic substrates. Western blotting of the purified enzyme obtained at each time–course point revealed that this lag phase coincided with the conversion of the inactive proPC2 forms to the mature, 66-kDa form, supporting the notion that 66 kDa PC2 in fact represents the active species. The phase of maximal activity (coinciding with formation of the 66-kDa form) was followed by a phase of decreased activity. However, enzyme incubated under identical conditions but without substrate remained active when later assayed over a short period; thus either substrate or product contributes to this inactivation. The spontaneous conversion of proPC2 to 66 kDa PC2 was unaffected by a cocktail of proteinase inhibitors consisting of pepstatin, TPCK, E-64, and TLCK; although the presence of a contaminating proteinase cannot be absolutely ruled out, these data provide strong evidence against the involvement of such nonspecific contaminating proteinases and strongly suggest an autocatalytic processing event. Autocatalytic processing of proPC2 has been reported in mutagenesis studies employing translation of proPC2 mRNA in oocyte systems (12, 49). In our hands, proPC2 obtained from the parent CHO/PC2 cell line was unable to autoactivate, whereas proPC2 obtained from the CHO/PC2–7B2 cell line described here efficiently autoactivated. While it is clear that intracellular expression of 7B2 is required for this process (14), the exact mechanism by which 7B2 acts to promote activation is not understood, and potential mechanisms include assistance in folding, assistance in intracellular transport, or protection from the assumption of conformationally inactive forms. It is unclear as to why in our study neither the 7B2 CT-peptide nor 27 kDa 7B2 was able to block conversion of proPC2 to PC2, since both the 27-kDa protein (19) and the 7B2 CT-peptide (15) have previously been reported to inhibit the conversion of immunopurified proPC2 *in vitro*. The lack of effect of 7B2 in the current study may be related to the use of much greater quantities of PC2, which are soluble rather than tethered, and may indicate that the inhibitory peptide does not play a role in the prevention of zymogen activation *in vivo*. In any case, our data supporting autocatalytic maturation are similar to results indicating autocatalysis for other members of the furin/PC subfamily (6, 7, 9–11, 34) and of the oocyte results of Matthews *et al.* (12).

Our studies indicate that the higher molecular forms of PC2 can be efficiently converted *in vitro* to the 66-kDa, active form. However, this form appeared to be unstable in the culture medium and/or under our purification conditions, as fractions containing only a 66-kDa immunoreactive band (separated from the 71- and 75-kDa forms) showed no enzymatic activity. Insulinoma granule PC2 activity has been described as unstable at pHs higher than 6.6 (24) and in the presence of anions such as chloride (50).

The susceptibility of proenkephalin to hydrolysis by purified recombinant PC2 is in agreement with our earlier work involving purified prohormone convertases (17, 34, 51) and with vaccinia experiments (52) as well as with the recent work of Azaryan *et al.* (27). Further determinations of the exact cleavage sites of proenkephalin by recombinant PC2 are in progress.

In contrast to the finding of Braks and Martens (17) that PC2 only transiently associates with 7B2, our data demonstrating copurification of proPC2 forms with 21 kDa 7B2 indicate that coassociation is likely to occur *in vivo* and persists through several steps of purification. The partial coelution of 21 kDa 7B2 with both proPC2 forms as well as with activated, 66 kDa PC2 during gel filtration provides further confirmation of this coassociation.

Since 7B2-mediated activation occurred irrespective of whether mature PC2 or proPC2-containing samples were used, the possibility of a proPC2 conversion-based activation effect was deemed unlikely. Instead, the fact that, in the presence of 7B2, PC2 became more resistant to temperature denaturation implies that the association of these two proteins maintains an enzymatically active PC2 conformation, preventing enzyme denaturation. Optimal thermal stabilization of PC2 by 21 kDa 7B2 occurred between pH 5 and 6 and at millimolar calcium concentrations, conditions thought to occur within secretory granules (reviewed in 1). Since these conditions are also optimal for PC2 activity, and since 21 kDa 7B2 represents a stored secretory granule protein (21, 22), our data strongly suggest that 21 kDa 7B2 can directly modulate PC2 activity within secretory granules.

Using immunopurified enzyme and recombinant human 21 kDa 7B2, we previously were unable to detect a stimulatory effect of 21 kDa 7B2 on the hydrolysis of fluorogenic substrate (19). The reason for this discrepancy is unclear, but may involve factors such as the use of immunopurified vs recombinant enzyme, human vs rat 7B2s, or, most likely, coassociation of endogenous 7B2 with immunopurified enzyme. It should be noted, however, that while these studies were in progress Braks and Martens (53) reported that 21 kDa 7B2 can stimulate the proteolysis of proopiomelanocortin by im-

munopurified proPC2/PC2, providing further evidence that the association of this form of 7B2 with PC2 represents a physiologically important phenomenon.

In summary, we here describe a method to produce milligram quantities of purified, catalytically active recombinant PC2 from the conditioned medium of PC2 and 7B2-transfected CHO cells. The recombinant enzyme, which has kinetic properties which compare favorably with other purified recombinant enzymes in the same family, is obtained as a mixture of two zymogen forms which spontaneously convert to the mature enzyme. In addition, we have shown that 21 kDa 7B2 can associate *in vitro* with both the proforms and the mature form of PC2; since maximal stabilization of PC2 by 21 kDa 7B2 is observed under intragranular conditions, 21 kDa 7B2 may play an important role in regulating the intragranular activity of this important processing enzyme.

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