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Mapping the Pro-region of Carboxypeptidase B by Protein Engineering

CLONING, OVEREXPRESSION, AND MUTAGENESIS OF THE PORCINE PROENZYME*

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The proteolytic processing of pancreatic procarboxypeptidase B to a mature and functional enzyme is much faster than that of procarboxypeptidase A1. This different behavior has been proposed to depend on specific conformational features at the region that connects the globular domain of the pro-segment to the enzyme and at the contacting surfaces on both moieties. A cDNA coding for porcine procarboxypeptidase B was cloned, sequenced, and expressed at high yield (250 mg/liter) in the methylotrophic yeast *Pichia pastoris*. To test the previous hypothesis, different mutants of the pro-segment at the putative tryptic targets in its connecting region and at some of the residues contacting the active enzyme were obtained. Moreover, the complete connecting region was replaced by the homologous sequence in procarboxypeptidase A1. The detailed study of the tryptic processing of the mutants shows that limited proteolysis of procarboxypeptidase B is a very specific process, as Arg-95 is the only residue accessible to tryptic attack in the proenzyme. A fast destabilization of the connecting region after the first tryptic cut allows subsequent proteolytic processing and the expression of carboxypeptidase B activity. Although all pancreatic procarboxypeptidases have a preformed active site, only the A forms show intrinsic activity. Mutational substitution of Asp-41 in the globular activation domain, located at the interface with the enzyme moiety, as well as removal of the adjacent 3_{10} helix allow the appearance of residual activity in the mutated procarboxypeptidase B, indicating that the interaction of both structural elements with the enzyme moiety prevents the binding of substrates and promotes enzyme inhibition. In addition, the poor heterologous expression of such mutants indicates that the mutated region is important for the folding of the whole proenzyme.

Pancreatic carboxypeptidases are zinc metalloenzymes that hydrolyze the C-terminal amino acids from alimentary proteins and peptides (1–3). Two general types of carboxypeptidases can be distinguished: carboxypeptidase A with a preference for apolar C-terminal residues and carboxypeptidase B (CPB),[1] which displays a preference for C-terminal Lys or Arg residues. Isomorphism has also been found in human and rat tissues within the A forms, yielding CPA1 (assignable to the traditional A form) and CPA2, which is more specific for aromatic residues (4–7). Carboxypeptidases are secreted from pancreatic acinar cells as zymogens that are activated by trypsin in the small intestine. In porcine pancreatic secretions, the two major forms of pro-CPs, pro-CPB and pro-CPA1, are found in the monomeric state, making this system suitable for comparison studies on their activation processes.

The length of the N-terminal pro-segments is 94/96 residues in pro-CPA1/pro-CPA2, spanning over 1/4 of the proenzyme. This characteristic differentiates pro-CPs from the rest of digestive zymogens (*e.g.* trypsinogens or proelastases) with shorter activation pro-regions (8) and suggests that the conformational elements in their pro-segments pack in an independent globular folding unit that determines the inhibition and activation processes of these zymogens (3, 9).

The detailed three-dimensional structures of monomeric pro-CPB (porcine), pro-CPA1 (porcine), and pro-CPA2 (human) have been deduced from crystallographic studies (10–12), and the solution structure of the globular domain of porcine pro-CPB pro-segment has been derived by NMR spectroscopy (13). These studies show that conformational similarities between the 94/95 activation segments of the A1 and B forms are high despite the fact that their sequence identities reach only 32% (14). Nevertheless, important local differences in the secondary structures of the individual pro-segments are found that may account for their different inhibitory capacity and behavior upon activation.

The activation processes of porcine pancreatic pro-CPA1 and pro-CPB natural forms have been studied in detail (14–16). Both proenzymes are activated by the proteolytic cleavage of trypsin, which initially gives rise to the scissioned pro-segment and enzyme moieties. However, the generation of activity is much faster in pro-CPB than in pro-CPA1. On the other hand, intact pro-CPB is absolutely devoid of enzymatic activity, whereas pro-CPA1 is able to catalyze the hydrolysis of small

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AJ133775.

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[1] The abbreviations used are: CP, carboxypeptidase; BGA, benzoyl-glycyl-L-arginine; PCR, polymerase chain reaction; LRL, Lilly Research Laboratories; α-MF, prepro-α-mating factor; RP-HPLC, reversed phase-high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; bp, base pair(s); wt, wild type.

synthetic peptides (17, 18), a fact that indicates a different degree of coverage of the active center in both forms.

In this work, the cDNA coding for porcine pro-CPB has been cloned and overexpressed in the yeast *Pichia pastoris*, yielding large quantities of fully activable wild-type proenzyme. We describe the construction and production of site-directed mutants of porcine pro-CPB in which different residues or complete regions of the pro-segment were changed. The detailed study of the mutants allowed us to evaluate the relevance of the putative tryptic targets and to interpret the role of a number of contacts between the pro-segment and the enzyme in the maintenance of the inhibited state. From this work, the structural/functional relationships in the activation/inhibition mechanisms of pancreatic procarboxypeptidases B may be defined in detail.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T4 DNA ligase, *Taq* polymerase, deoxyribonucleotide stocks, and synthetic oligonucleotides were obtained from Roche Molecular Biochemicals. Vent DNA polymerase was purchased from New England Biolabs. Salts and mediums for *Escherichia coli* and *P. pastoris* were purchased from Difco. The *P. pastoris* strains and vectors were obtained from Phillips Petroleum and Invitrogen. Trypsin (treated with tosyl-L-phenyl chloromethyl ketone) was from Worthington. N^{α}-*p*-tosyl-L-lysine chloromethyl ketone and benzoylglycyl-L-arginine (BGA) were from Sigma.

Isolation and Cloning of Porcine Pro-CPB cDNA—Total RNA was isolated from the pancreas of young pigs by a modified acid phenol-guanidinium thiocyanate-chloroform method (19). The mRNA was isolated with the mRNA isolation kit from Roche Molecular Biochemicals.

The cDNAs for CPB and pro-CPB were synthesized from porcine pancreatic mRNA using the Uni-Zap library kit from Stratagene. The N-terminal amino acid and C-terminal amino acid sequences were determined from purified porcine pro-CPB and CPB in the LRL Protein Technology Laboratory. The cDNA was PCR-amplified using degenerate primers designed from the C-terminal amino acid sequence of CPB and the N-terminal amino acid sequences of pro-CPB, respectively. Cloning the PCR-amplified cDNAs into the vector pCR1000 (from Invitrogen) provided DNA fragments containing 975 bp for CPB and 1260 bp for pro-CPB. The 5′ end of the cDNA was cloned using 5′ rapid amplification of cDNA ends (RACE) (20). Nucleotide sequences were determined in the LRL Biotechnology Core Laboratory. Consensus nucleotide sequences were identified by comparing the sequences of multiple independent clones to eliminate sequence errors that may have been introduced during PCR. Unique restriction sites, which introduced silent base pair substitutions, were strategically placed in the consensus sequence by PCR mutagenesis near the 5′ end of the sequence, the 3′ end of the sequence, and flanking the junction between the sequence coding for the propeptide and the mature enzyme by overlap extension mutagenesis (21) to facilitate site-directed mutagenesis. Mutated DNA sequences were always confirmed by automated DNA sequencing. The plasmid, pHDM208, contained the modified cDNA sequence.

Construction of the Pro-CPB-wt Expression Vector—The pre-pro α-mating factor (α-MF) was excised from vector pPIC9 by *Bam*HI/*Xho*I restriction, and the pro-CPB gene in the pHDM208 vector was obtained after *Sfu*I/*Bam*HI restriction. The two pieces were ligated by using a *Xho*I/*Sfu*I 30-bp synthetic linker that completes the 3′ end of pre-pro α-MF and the 5′ end of pro-CPB. The resulting 1540-bp pre-pro α-MF-pro-CPB fragment was cloned into the *Bam*HI site of pBluescript SK to give the pBlue-pro-CPB vector, used for mutagenesis, and into the *Bam*HI site of pPIC3.5 to obtain pPIC-pro-CPB, used for expression of the wild-type pro-CPB. The different junctions were confirmed by sequencing on an automated DNA sequencer (Amersham Pharmacia Biotech).

PCR Mutagenesis—Using pBlue-pro-CPB as a template, site-specific mutations were introduced by a two-step PCR mutagenesis procedure using 5′-GGA GCA TTT CGA AGG GAA AAG GTG TTC CGT GTC as sense external primer (a silent Asp-700 site elimination serves for recombinant screening) and 5′-TC TCT CAC AAA CCA CTG GCA AAA TGC ATG CAT GGG AAA T as antisense external primer. Internal primers were designed to contain the single or double mutations that follow: R93Q, R95Q, R93Q/R95Q, Q89E, R83Q, D41N, and D41A. The internal primers for the Val-43–Ile-46 deletion were: sense, 5′-CCA GAT TCT AAA CCT CAC AGT ACA GTT GAC TTC; antisense, 5′-TTA ACT GAA GAC CTT TGG TCT AAG TTT GGA GTG. The different resulting DNA fragments containing the pre-pro α-MF plus the mutated pro-CPB were cloned into the *Bam*HI site of pPIC3.5 to obtain pPIC-M1 to M8. All the constructs were confirmed by sequencing on an automated DNA sequencer.

Cassette Mutagenesis—To substitute the Gln-89–Val-94 region of porcine pro-CPB for the corresponding one in porcine pro-CPA1, a 73-bp *Xho*I/*Pvu*I fragment was excised from pBlue-pro-CPB and replaced by a synthetic linker obtained after the annealing of the following 85-bp oligonucleotide 5′-TC GAG GCT **G**AG **CAG GAG CAG ATG** TTT G**C**C AGC **CAG** G**G**C CGT ACA ACT GGA CAC AGT TAT GAG AAG TAC AAC AAC TGG GAA ACG AT and the complementary strand designed to recover the restriction sites in both ends. The mutated pro-CPB was cloned into the pPIC3.5 as above to obtain pPIC-M9. The junctions were confirmed by automated DNA sequencing.

Transformation and Selection of Productive Clones—The pPIC-pro-CPB and the different expression vectors containing the mutated pro-CPB cDNAs (pPIC-M1 to M9) were linearized by *Sac*I digestion and transformed into *P. pastoris* KM71 (*arg4, his4, aox1::ARG4*) strain either by electroporation or by the spheroplast method. Histidine-independent transformants were selected and tested for production of pro-CPB. Colonies were grown in a buffered liquid medium BMGY and BMMY (1% yeast extract, 2% peptone, 90 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and either 1% glycerol or 0.5% methanol) using 10-ml test cultures at 30 °C for 5 days. The production of the clones was followed by SDS-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. Western blots were performed using 1:2000 anti-porcine pancreatic procarboxypeptidase B antibodies. The functionality of the expressed protein was analyzed with the BGA synthetic substrate after activation of the proenzyme with trypsin (at a 1:1 ratio by weight). pPIC-M8 and pPIC-M9 were also transformed into *P. pastoris* GS115 (HIS 4), and the clones that integrated by homologous recombination with the *aox*1 sequence were selected. The production of pro-CPB was tested as described above but using MM (90 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, and 0.5% methanol) as induction medium.

Expression and Purification of Wild-type Pro-CPB and Its Mutants—One-liter shake-flask cultures were grown for 3 days in buffered glycerol medium BMGY (15–20 OD units at 600 nm). Cells were collected by centrifugation at 1500 g and gently resuspended in 200 ml of a methanol-containing medium, BMMY, cultured for another 2 days to induce the production of pro-CPB. The supernatant was loaded onto a hydrophobic interaction butyl column and eluted with a decreasing gradient of ammonium sulfate. The zymogen-containing fractions were selected for their activity against BGA after tryptic activation and re-purified by fast protein liquid chromatography on an anion exchange column (TSK-DEAE) as reported previously (16). The correct processing of the different recombinant proteins was confirmed by both automated Edman degradation analysis of its N-terminal sequence and mass spectrometry. The mutants for Val-43–Ile-46 deletion and Gln-89–Val-94 pro-CPB/pro-CPA1 substitution were induced in 200 ml of methanol minimum medium. The culture medium was dialyzed against the activation buffer (50 mM Tris-HCl, 1 μM $ZnCl_2$, pH 7.5) and concentrated to 0.2 ml by ultrafiltration (Amicon).

Proteolytic Processing of the Recombinant Proenzymes—The recombinant proenzymes at 1 mg/ml in activation buffer were treated with trypsin at a 4/1 or 400/1 ratio (w/w) at 0 or 25 °C. At given times after trypsin addition, aliquots were removed for activity measurements for electrophoretic, reversed-phase HPLC and mass spectrometry analysis and for the quantitation of the released amino acids. For activity measurements, 10 μl of the activation mixture were mixed with 190 μl of aprotinin at 0.1 mg/ml in 20 mM Tris, 0.1 M NaCl, pH 7.5, and 10 μl of this new mixture were used to carry out spectrophotometric activity measurements with BGA at 254 nm. For electrophoretic analysis 20 μl of the activation mixture were mixed with 2 μl of N^{α}-*p*-tosyl-l-lysine chloromethyl ketone at 22 mM in water to reach a final trypsin inhibitor concentration of 2 mM. Each sample was immediately mixed with electrophoretic loading buffer (containing 1% SDS and 3% β-mercaptoethanol), heated at 90 °C for 1 min, and stored at −20 °C until analyzed. Electrophoresis was carried out in polyacrylamide Tricine gels (22). For HPLC and mass spectrometry analyses, 90-μl samples were removed from the activation mixture, adjusted to 0.5% trifluoroacetic acid to inhibit proteolysis, and immediately chromatographed or kept at −20 °C for subsequent analysis. For the quantitation of the amino acids released into the activation mixture, 90-μl samples were taken (2 nmols of initial pro-CPB) and mixed with trifluoroacetic acid to give a final concentration of 0.5%. Then 1.5 nmols of norleucine were added as quantitative reference before the addition of 3 volumes of ethanol to precipitate proteins and large peptides. The supernatant was lyophi-

lized and analyzed for amino acid composition. The mutants for the Val-43–Ile-46 deletion and the Gln-89–Val-94 pro-CPB/pro-CPA1 substitution, together with the wild-type zymogen as a control, were taken to 50 ng/ml in activation buffer, as measured by enzyme-linked immunosorbent assay, and treated with trypsin at a 400/1 ratio (w/w) at 0 °C. At given times after trypsin addition, aliquots were removed for electrophoresis as described above, analyzed by Western blotting, and developed with 1:2000 anti-porcine pancreatic procarboxypeptidase B antibodies.

Chromatographic Analysis and Purification of the Activation Products—Samples removed from the activation mixtures were analyzed by reversed-phase HPLC on Vydac C4 supports. A 214TP54 column, 250 × 4.6 mm, 5-μm particle size, 0.3-μm pore was used, and chromatographies were performed in 0.1% trifluoroacetic acid with a linear elution gradient between water (solvent A) and acetonitrile (solvent B), according to the following steps: 10% B from 0 to 10 min and 52% B at 130 min.

Mass Spectrometry Analysis—The activation mixture and HPLC-purified fragments were analyzed by mass spectrometry using a MALDI-TOF spectrometer (Biflex with reflectron, from Bruker) using 50% synapinic acid as a matrix for pro-CPB, CPB, and long activation fragments and 50% α-cyano 4-cinnamic acid for short fragments.

Amino Acid Analysis—Amino acid analyses were carried out by the dabsyl-derivatization method (23) using protocols and materials from Beckman. A reversed phase HPLC NovaPak C18 column was used to separate the amino acids produced during the tryptic activation.

N-terminal Sequence Analysis—Proenzymes and derived proteolytic fragments were analyzed for N-terminal sequences on a Beckman LF3000 protein sequencer using an automated Edman degradation approach after purification by fast protein liquid chromatography or RP-HPLC. Large fragments, such as the mature enzyme, were sometimes sequenced after SDS-polyacrylamide gel electrophoresis analysis and transferred onto a polyvinylidene difluoride membrane.

RESULTS

Cloning of Pre-pro-CPB cDNA—The consensus cDNA sequence of pro-CPB was cloned into pHDM147 to generate a plasmid designated pHDM208. Fig. 1 contains the consensus coding sequence for pre-pro-CPB and the amino acid sequence inferred by translating the coding sequence. The first 15 amino acids suggest a hydrophobic signal peptide, although the sequence lacks the characteristic positively charged amino acid near the N terminus of a signal peptide. The amino acid sequence of the pro-peptide agrees with the published pro-peptide amino acid sequence (14). The inferred sequence was further tested by comparing to properties of natural CPB and pro-CPB. The proteins purified from the natural source were analyzed in the LRL Protein Technology Core Laboratory by cyanogen bromide fragmentation, and determination of molecular weights was by mass spectrometry of the intact proteins and the cyanogen bromide fragments. Predictions from the inferred sequence agreed with the results obtained with the natural proteins and supported the accuracy of the cDNA sequence.[2] Comparison of the porcine amino acid sequence with the amino acid sequence of human pro-CPB shows 83.5% identity and 89% similarity between the two proteins.

Overexpression in Yeast, Purification, and Characterization of the Recombinant Pro-CPB—A DNA encoding the yeast-specific secretion signal of *Saccharomyces cerevisiae* α-MF pre-pro-signal sequence was attached in-frame to the 1260-bp DNA encoding porcine pro-CPB and incorporated into the *Bam*HI site of pPIC3.5 *P. pastoris* vector to generate pPIC3.5-pro-CPB. The recombinant plasmid was linearized by *Sac*I digestion and used to transform the KM71 (*arg4, his4, aox1::ARG4*) *P. pastoris* strain by electroporation. Transformed cells were screened for pro-CPB secretion, and the selected clones were used for large scale productions of pro-CPB. The cells were first grown in the presence of glycerol and then switched to methanol (1%) as the carbon source to induce expression of pro-CPB. The secreted proenzyme (250 mg/liter) represents more than

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                                                                  C   C
1/1 pre-pro-CPB                          31/11                  pro-CPB  C
ATG TTG GCG TTC TTG ATT CTT GTG ACT GTG ACT CTA GCA TCT GCT CAT CAT TCT GGT GAG
Met leu ala phe leu ile leu val thr val thr leu ala ser ala his his ser gly glu
61/21 C (SfuI)                           91/31
CAC TTT GAA GGT GAG AAG GTG TTC CGT GTC AAT GTT GAA GAT GAA AAT GAC ATC AGC TTA
his phe glu gly glu lys val phe arg val asn val glu asp glu asn asp ile ser leu
121/41                                   151/51
CTC CAT GAG TTG GCC AGC ACC AGG CAG ATT GAC TTC TGG AAA CCA GAT TCT GTC ACA CAA
leu his glu leu ala ser thr arg gln ile asp phe trp lys pro asp ser val thr gln
181/61                                   211/71
ATC AAA CCT CAC AGT ACA GTT GAC TTC CGC GTG AAA GCA GAA GAT ATT TTG GCT GTG GAA
ile lys pro his ser thr val asp phe arg val lys ala glu asp ile leu ala val glu
241/81                                   271/91
GAC TTT CTG GAG CAG AAT GAA CTA CAA TAT GAG GTA CTC ATA AAC AAC CTG AGA TCT GTG
asp phe leu glu gln asn glu leu gln tyr glu val leu ile asn asn leu arg ser val
  C (XhoI)                                M-CPB
CTA GAG GCT CAG TTT GAC AGC AGA GTC CGT ACA ACT GGA CAC AGT TAT GAG AAG TAC AAC
leu glu ala gln phe asp ser arg val arg thr thr gly his ser tyr glu lys tyr asn
361/121          C (PvuI)                391/131
AAC TGG GAA ACG ATA GAG GCT TGG ACT AAG CAA GTC ACC AGT GAA AAT CCA GAC CTC ATC
asn trp glu thr ile glu ala trp thr lys gln val thr ser glu asn pro asp leu ile
421/141                                  451/151
TCT CGC ACA GCC ATC GGA ACT ACA TTT TTA GGA AAC AAT ATA TAC CTC CTC AAG GTT GGC
ser arg thr ala ile gly thr thr phe leu gly asn asn ile tyr leu leu lys val gly
481/161                                  511/171
AAA CCT GGA CCA AAT AAG CCT GCC ATT TTC ATG GAC TGT GGT TTC CAT GCC AGA GAA TGG
lys pro gly pro asn lys pro ala ile phe met asp cys gly phe his ala arg glu trp
541/181                                  571/191
ATT TCC CAT GCA TTT TGC CAG TGG TTT GTG AGA GAG GCT GTT CTC ACC TAT GGA TAT GAG
ile ser his ala phe cys gln trp phe val arg glu ala val leu thr tyr gly tyr glu
601/201                                  631/211
AGT CAC ATG ACA GAA TTC CTC AAC AAG CTA GAC TTT TAT GTC TTG CCT GTG CTC AAT ATT
ser his met thr glu phe leu asn lys leu asp phe tyr val leu pro val leu asn ile
661/221                                  691/231
GAT GGC TAC ATC TAC ACC TGG ACC AAG AAC CGA ATG TGG AGA AAG ACC CGC TCT ACC AAT
asp gly tyr ile tyr thr trp thr lys asn arg met trp arg lys thr arg ser thr asn
721/241                                  751/251
GCT GGA ACT ACC TGC ATT GGC ACA GAC CCC AAC AGA AAT TTT GAT GCT GGG TGG TGC ACA
ala gly thr thr cys ile gly thr asp pro asn arg asn phe asp ala gly trp cys thr
781/261                                  811/271
ACT GGA GCC TCT ACA GAC CCC TGC GAT GAG ACT TAC TGT GGA TCT GCT GCA GAG TCT GAA
thr gly ala ser thr asp pro cys asp glu thr tyr cys gly ser ala ala glu ser glu
841/281                                  871/291
AAA GAG ACC AAG GCC CTG GCT GAT TTT ATA CGC AAC AAC CTC TCC TCC ATC AAA GCA TAC
lys glu thr lys ala leu ala asp phe ile arg asn asn leu ser ser ile lys ala tyr
901/301                                  931/311
CTG ACG ATC CAC TCA TAC TCA CAG ATG ATA CTC TAC CCT TAT TCC TAT GAT TAC AAA CTC
leu thr ile his ser tyr ser gln met ile leu tyr pro tyr ser tyr asp tyr lys leu
961/321                                  991/331
CCC GAG AAC AAT GCT GAG TTG AAT AAC CTG GCT AAG GCT GCC GTG AAA GAA CTT GCT ACA
pro glu asn asn ala glu leu asn asn leu ala lys ala ala val lys glu leu ala thr
1021/341                                 1051/351
CTG TAT GGC ACC AAG TAC ACA TAC GGC CCA GGA GCT ACA ACA ATC TAT CCT GCT GCT GGG
leu tyr gly thr lys tyr thr tyr gly pro gly ala thr thr ile tyr pro ala ala gly
1081/361                                 1111/371
GGC TCT GAT GAC TGG GCT TAT GAC CAA GGA ATC AAA TAT TCC TTC ACC TTT GAA CTC CGG
gly ser asp asp trp ala tyr asp gln gly ile lys tyr ser phe thr phe glu leu arg
1141/381                                 1171/391
GAT AAA GGC AGA TAT GGT TTT ATC CTC CCT GAA TCC CAG ATC CAG GCA ACC TGT GAG GAA
asp lys gly arg tyr gly phe ile leu pro glu ser gln ile gln ala thr cys glu glu
1201/401                                 1231/411
ACA ATG CTG GCC ATC AAA TAC GTA ACC AAC TAC GTG CTG GGC CAC CTG TAA TAG
thr met leu ala ile lys tyr val thr asn tyr val leu gly his leu ... ...
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FIG. 1. **The nucleotide coding sequence for porcine preprocarboxypeptidase B.** The coding sequence was determined as the consensus from at least three independent clones that were sequenced on both strands. The translated amino acid sequence is shown on the line below the nucleotide sequence. The *underlined sequence* labeled pre-pro-CPB is the coding sequence for the putative signal peptide. The coding sequence for the propeptide begins with the label pro-CPB and ends with CGT immediately before the coding sequence for mature carboxypeptidase B, which is labeled with *MCPB* and has the first codon *underlined*. The *dots* at the end of the sequence are beneath two tandem nonsense codons. Restriction sites labeled above the nucleotide sequence are useful for mapping the gene in recombinant plasmids and introducing site-directed mutations into the sequence. Single letters above the sequence identify nucleotide sequence changes that were introduced without changing the encoded amino acids (silent mutations) during the construction of pHDM208. Restriction sites in parenthesis after the nucleotide substitutions were created by the silent mutations. The first ATG in the sequence is half of an *Nde*I site that flanks the 5′ end of the sequence. The last G is the first nucleotide of a *Bam*HI site that flanks the 3′ end of the sequence.

90% of the protein in the culture medium, as shown by SDS-polyacrylamide gel electrophoresis. It should be mentioned here that previous expression assays of pro-CPB and mature CPB in *E. coli* rendered only insoluble and inactive enzyme.

Starting from 150 ml of culture medium, the two-step chromatographic method used to purify recombinant pancreatic pro-CPB normally yielded 30 mg of purified pro-CPB. The protein was shown to be correctly processed upon secretion by N-terminal amino acid sequence analysis. The apparent mass for the recombinant protein as analyzed by MALDI-TOF mass spectrometry was 45,817 Da (expected size for porcine pancreatic pro-CPB: 45,836 Da), confirming the integrity and the identity of the expressed product.

As expected, recombinant wild-type pro-CPB (wt-pro-CPB)

[2] Raymond Kaiser, personal communication.

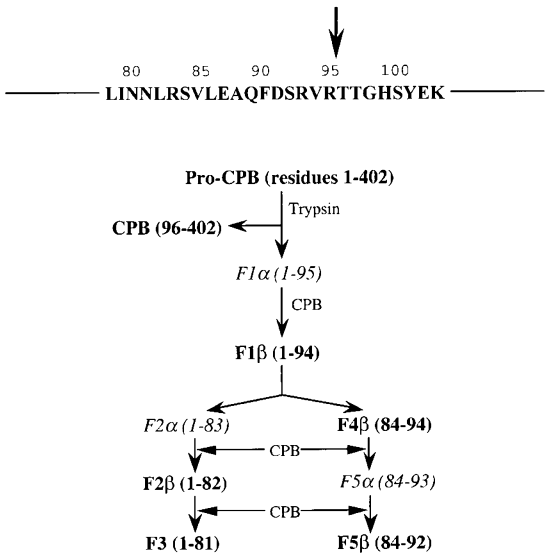


FIG. 2. **Schematic view of the sequence of cleavages observed during the proteolytic processing of pro-CPB by trypsin.** The *numbering* is that of the whole proenzyme, in such a way that residue 96 of pro-CPB would be residue 1 of CPB. At the top of the figure, the sequence around the primary and secondary processing sites is presented to serve as a guide. Modified from Villegas *et al.* (16).

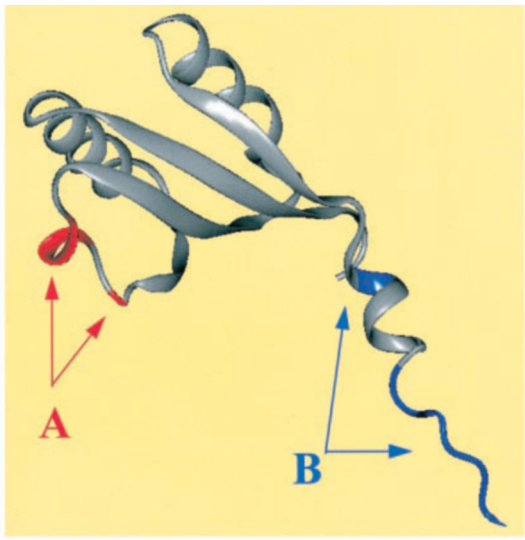
was absolutely devoid of intrinsic activity (see further in text), being the enzyme region completely inhibited by its pro-segment. The recombinant pro-CPB was fully activated when incubated at 0 °C and at 400/1 pro-CPB/trypsin (w/w) ratio. The maturation course for recombinant pro-CPB is identical to that previously reported for the natural form isolated from porcine pancreas (16). A scheme of the proteolytic maturation process is presented in Fig. 2 to serve as a reference for discussion of the results that follow.

Mapping Tryptic Targets in Pro-CPB-connecting Segment—A two-turn α-helix followed by a nonstructured flexible loop connects the globular domain of the pro-segment to the enzyme in pro-CPB (10). This connecting region contains three Arg at positions 83, 93, and 95 (see Fig. 3). Although all of them appear to be solvent-exposed, the first tryptic cleavage of the proteolytic processing of pro-CPB occurs only at Arg-95. This initial cut is followed by the trimming of the pro-segment C-terminal residue by the generated CPB. A second tryptic cleavage occurs at the Arg-83–Ser- 84 bond in the N-terminal region of the α-helix occurs (see Fig. 3). Previous studies have shown that Arg-93 may also act as a primary tryptic target when carboxypeptidase inhibitors are added to the activation mixture (16).

To individualize the effect of the different tryptic targets, different mutants were produced in which the Arg involved in the scissile bond was substituted by Gln: R83Q, R93Q, R95Q, and the double mutant R93Q/R95Q. All of these mutants were expressed at the same level and purified in the same manner that the wt-pro-CPB.

No generation of CPB activity was observed when the action of trypsin on the double mutant R93Q/R95Q was tested at 0 °C and at 400/1 pro-CPB/trypsin ratio (standard conditions) nor when the ratio of activating trypsin was increased by 100-fold and the temperature raised to 25 °C. No conversion of pro-CPB into active enzyme was detected by electrophoretic analysis of the incubation mixtures in SDS-Tricine gels, even at long times after trypsin addition (see Fig. 4*B*).

The activability of the single mutant R95Q was assayed to know whether Arg-93 can act as the initial target for proteolytic processing. No carboxypeptidase activity was detectable after trypsin addition in normal conditions, and no pro-CPB into CPB transformation was observed by electrophoresis. The appearance of peptide fragments at low concentration was detected only when the proteolysis was analyzed by RP-HPLC, indicating a slight processing of pro-CPB (less than 0.5% of the initial pro-enzyme).



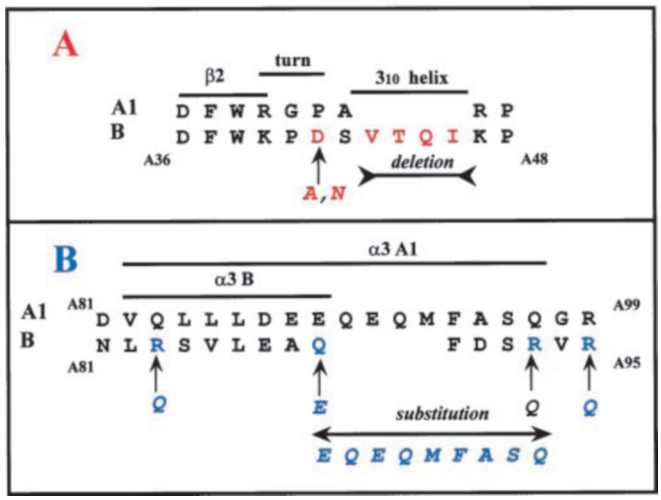


FIG. 3. **Comparison of the amino acid sequences of selected regions of procarboxypeptidases A1 and B.** In both parts of the figure, *A* refers to the "inhibitory loop," a short sequence located over the active-site cleft of carboxypeptidase, and *B* refers to the "connecting region," a sequence that links the globular activation domain to the enzyme N terminus. The secondary structure elements present in porcine pancreatic procarboxypeptidases A1 and B deduced from crystallographic studies are shown above the sequences. The point or regional mutations engineered in the present work are also indicated below the sequences. The numbering system is that of Guasch *et al.* (11), and the prefix *A* denotes that the residue belongs to the pro-segment moiety.

Under stronger proteolytic conditions, *i.e.* 25 °C and 4/1 pro-CPB/trypsin ratio, Arg-93 can act as a primary tryptic target promoting the appearance of a carboxypeptidase moiety with two additional amino acids (Val-94–Gln-95) at the N terminus, as shown by automated N-terminal amino acid sequence analysis of the CPB electrophoretic band and by mass spectrometry (MALDI-TOF) analysis. The 93-residue activation fragment released under those conditions possesses a C-terminal Arg, which is supposed to be quickly removed by the generated CPB. However, when the process was analyzed by RP-HPLC and mass spectrometry, no traces of the 1–92 fragment (F1δ) were detected, even at the shorter activation times used; instead, the products of tryptic action on Arg-83 were present (result not shown). The activation course was monotonic and correlated

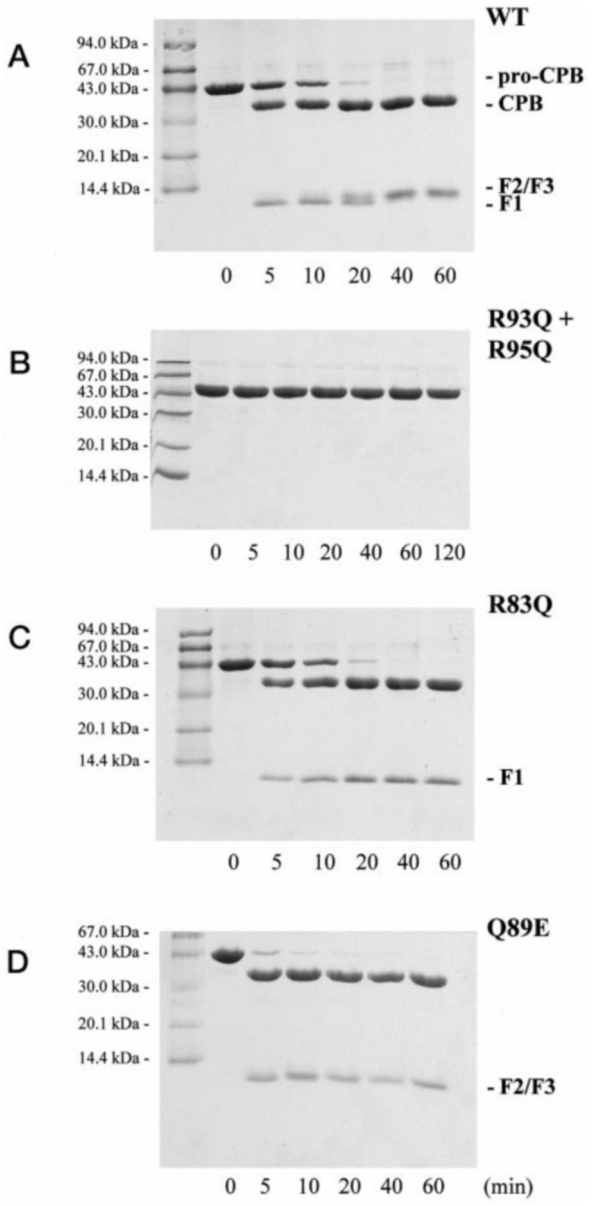


FIG. 4. **Electrophoretic analysis of the proteolytic processing of pro-CPB by trypsin.** The recombinant wild-type and mutant proenzymes at 1 mg/ml in the activation buffer were treated with trypsin at 400/1 ratio (w/w) at 0 °C. At given times after trypsin addition, aliquots were removed, made 1 mM in N^{α}-*p*-tosyl-l-lysine chloromethyl ketone, mixed with loading buffer, heated at 100 °C, and stored at −20 °C until analysis. *A*, wild type pro-CPB; *B*, R93Q/R95Q mutant; *C*, R83Q mutant; *D*, Q89E mutant. The samples were analyzed in SDS-Tricine gels. The species present in the activation mixtures are identified on the right side of the gel. On the left side, the relative masses of molecular weight markers are indicated.

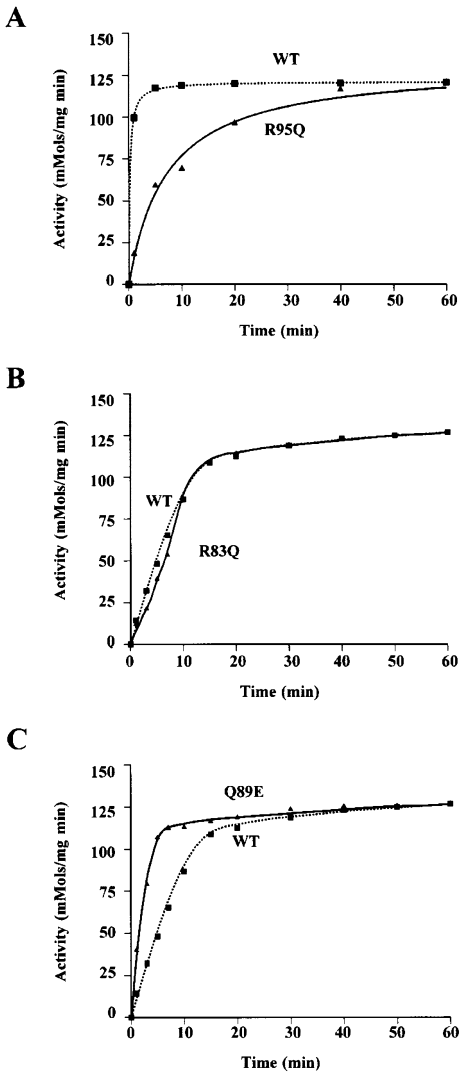


FIG. 5. **Comparison of the time course of CPB activity generation after tryptic activation in wild-type and mutant pro-CPBs.** Recombinant pro-CPBs at 1 mg/ml in 50 mM Tris-HCl, 0.01 mM $ZnCl_2$ (pH 8.0) were treated with trypsin as indicated, and at controlled times, aliquots were withdrawn and analyzed for carboxypeptidase B activity using 1 mM BGA as substrate. *A*, wild-type *versus* R95Q mutant; activation performed at 25 °C and at a proenzyme/trypsin 4:1 ratio (w/w). *B*, wild-type *versus* R83Q mutant; activation performed at 0 °C and at a proenzyme/trypsin 400:1 ratio (w/w). *C*, wild-type *versus* Q89E mutant; activation performed at 0 °C and at a proenzyme/trypsin 400:1 ratio (w/w).

well with the appearance of mature CPB observed by electrophoretic analysis. Although the generation of CPB activity was very slow when compared with wt-pro-CPB (see Fig. 5*A*), it was similar to the rate that was observed for porcine pro-CPA1 (15).

In contrast to the previous mutant, the substitution of Arg-93 in the single mutant R93Q did not induce differences in the rate of appearance of either carboxypeptidase activity or the CPB molecular species (as evaluated by electrophoresis) when analyzed under standard conditions (not shown).

Tryptic treatment of the R83Q single mutant produced a fast and complete CPB activity generation as a result of the cleavage of the bond Arg-95–Thr-1, without being affected by the loss of a secondary tryptic target (see Fig. 5*B*). However, at short times after trypsin addition, the time activation course of this mutant was slightly biphasic, as checked in different trials. This behavior is reminiscent of the clearly biphasic activation course of pro-CPA1 (15) and also correlates with the higher levels of the primary activation segment (1α, 1–95) in the activation mixture (Fig. 6). The conversion rate of the pro-CPB mutant into CPB is identical to that of the wt-pro-CPB as analyzed by electrophoresis, but as a consequence of the absence of Arg-83, only long activation fragments were produced (Fig. 4*C*). The kinetics of generation and transformation of these fragments was followed by RP-HPLC and mass spectrometry analysis and revealed a progressive C-terminal trimming of the primary activation segment (1–95) that stops at Ser-92 (see Fig. 6).

Relevance of the Connecting Segment in the Pro-CPB Tryptic Activation—An important contribution to the connecting region/active enzyme interaction forces in pro-CPA1 activation segment is attributed to a strong salt bridge between Glu-89

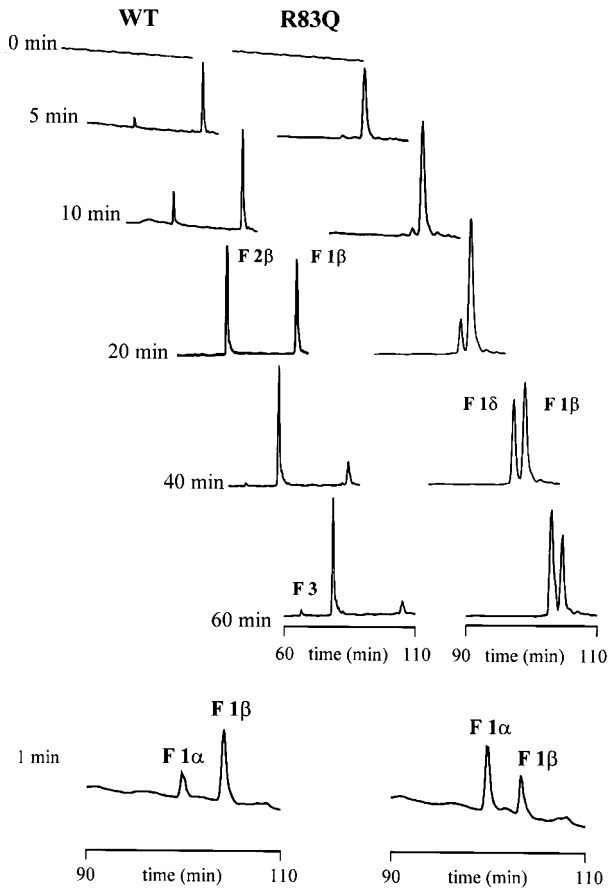


FIG. 6. **Comparative analysis of the fragments generated during tryptic processing of recombinant pro-CPB by reversed phase HPLC.** At different times after trypsin addition, aliquots were taken from the activation mixture in the same conditions as in Fig. 5*B* and analyzed by reversed phase chromatography on an HPLC system. The action of trypsin was stopped by adding trifluoroacetic acid up to 0.1%. 100 μl of each sample were loaded on a Vydac C4 reversed phase column. *Top*, the chromatographic profiles of wild-type pro-CPB and the R83Q mutant are compared at different times; labels are incorporated in some profiles for identification of the fragments. *Bottom*, the chromatographic profiles of both proenzymes 1 min after trypsin addition are shown at a different scale. Fragments 1α, 1β, and 1δ correspond, respectively, to residues 1–95, 1–94, and 1–92, and fragments 2β and 3, to residues 1–82 and 1–81.

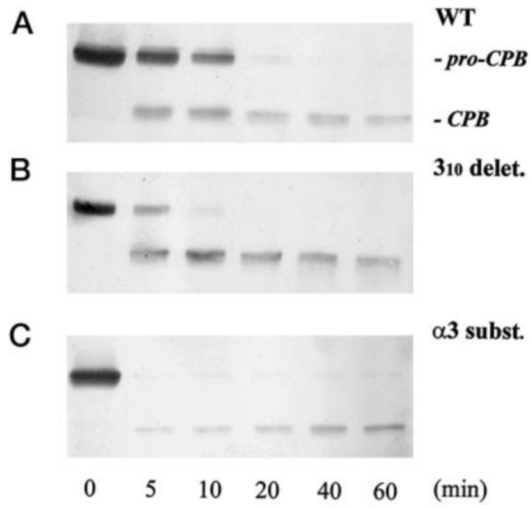


FIG. 7. **Immunoblot analysis of the transformation of pro-CPB into active enzyme in mutants with substituted or deleted secondary structures.** Proteins were expressed in minimal medium, and the supernatant was concentrated and dialyzed against 50 mM Tris-HCl, 0.01 mM $ZnCl_2$, pH 8.0. Zymogens at a 50 ng/ml were activated at 0 °C and at a proenzyme/trypsin 400:1 ratio (w/w). Samples were analyzed in 12% polyacrylamide gels, immunoblotted, and stained. *A*, wild type pro-CPB. *B*, pro-CPB mutant lacking the 3_{10} helix of the inhibitory loop (Val-43–Ile-46). *C*, pro-CPB mutant in which the C-terminal region of the connecting segment (Gln-89–Arg-94) has been substituted by the homologous sequence in porcine pro-CPA1.

and Arg-124 of CPA1 (3). Both residues are conserved in pro-CPA1 of all the studied species. The equivalent residues in porcine pro-CPB are Gln-89 and Arg-124, which interact through a hydrogen bond. Both residues are also conserved in pro-CPBs. To test the relevance of these interactions, a Q89E mutation was introduced into pro-CPB, and the recombinant proenzyme was expressed and purified as described for wt pro-CPB.

After tryptic treatment of the Q89E mutant (standard conditions), CPB activity was generated much faster than in wt-pro-CPB, reaching full activity in a significantly shorter time (see Fig. 5*C*). The monotonic activation course can be fitted to pseudo-first order kinetics. Electrophoretic analysis also showed an accelerated rate of conversion of the mutant into mature enzyme (see Fig. 4*D*). When the activation process was analyzed by RP-HPLC and mass spectrometry, it was observed not only that the first tryptic cleavage occurs faster than in the wild-type proenzyme but also that the time interval between the first cut and the hydrolysis of the Arg-83–Ser-84 bond was considerably reduced (results not shown). To test whether the Q89E substitution at the C-terminal position of the connecting segment α-helix could affect the helix stability, both wild-type and mutant sequences were analyzed by the AGADIR algorithm (24). A low propensity to adopt an α-helix conformation was predicted in both cases, the lower one corresponding to the mutated sequence and indicating the destabilizing effect of a negatively charged side chain at the C-end of the helix.

The unstructured region of pro-CPB-connecting segment (Gln-89 to Val-94) was completely substituted by the homologous sequence in the A form (see Fig. 3*B*) to obtain a procarboxypeptidase with CPB activity and with pro-CPA1-like activation behavior. The four additional residues that pro-CPA1 contains in that region contribute to a four-turn helical conformation of the connecting segment, two turns longer than the two-turn helix observed in pro-CPB. This hybrid pro-CPA1/pro-CPB was poorly secreted by KM71 *P. pastoris* strain and mostly degraded by proteases when expressed in rich medium. To increase its production and avoid proteolytic degradation, the mutant cDNA was inserted into the *AOX1* gene of GS115 *P. pastoris* strain by homologous recombination and the expression performed in minimal medium. About 200 μg/liter mutant protein were produced in this way. To avoid losses during purification, the tryptic activation of this mutant was assayed in concentrated culture medium after dialysis against the standard activation buffer. The transformation of the hybrid mutant into mature CPB was followed by electrophoresis and Western blotting using pro-CPB-specific antibodies. As shown in Fig. 7*C*, the mutant proenzyme was processed immediately after trypsin addition, indicating a complete exposure to trypsin cleavage.

Determinants of pro-CPB Inhibition—In porcine pro-CPB, the pro-region sequence located over the active site of the enzyme is formed by part of the β1 strand, a *cis*-Pro turn and one turn of 3_{10} helix, with an aspartate at position 41. This aspartate forms a salt bridge with Arg-145 in the enzyme, a residue that, in the absence of the pro-segment, binds to the C-terminal carboxyl group of substrate molecules. This interaction might partially explain the null intrinsic activity in pro-CPB against small peptides substrates (3).

To test this hypothesis, Asp-41 was replaced by Asn (D41N

mutant) or Ala (D41A mutant) by site-directed mutagenesis. A hydrogen bond contact with Arg-145 is still possible in the first mutant, whereas no polar interaction is possible in the second one. These mutants were expressed and purified following the same procedure used with wt-pro-CPB, but an important drop in the production yield was observed. Starting from 150 ml of culture broth, 1.5 mg of purified D41N and 0.75 mg of purified D41A were usually obtained.

Intrinsic activity and total CPB activity after tryptic activation were measured on small synthetic peptide substrates immediately after the second purification step. An excess of potato carboxypeptidase inhibitor was added to the solution when testing intrinsic activity to avoid detection of active enzyme traces. The results are summarized in Table I. Both mutant proenzyme forms showed clearly detectable intrinsic activity values, different from the null activity expressed by wt-pro-CPB.

The 3_{10} helix in the globular domain of the pro-segment is not present in procarboxypeptidases A1 and A2 and seemed to be a good candidate to account for the rest of the inhibitory action. Thus, the residues Val-43–Thr-44–Gln-45–Ile-46, which form the 3_{10} helix turn, were deleted by site-directed mutagenesis. This mutant was poorly expressed in KM71 *P. pastoris* strain and was expressed in the GS115 strain grown in minimal medium. About 30 μg of mutant protein at 50 ng/μl concentration were obtained after concentration and dialysis of the culture medium. Although these small quantities did not allow accurate intrinsic activity measurements, the activation behavior of this mutant could be studied by electrophoresis and Western blotting. According to these analyses, the tryptic proteolytic processing of pro-CPB was accelerated in this mutant when compared with wt-pro-CPB (see Fig. 7*B*). This last effect was also found when studying the activation process of D41N and D41A. In both cases the activation course and the appearance of active enzyme upon tryptic treatment were slightly accelerated with respect to wt-pro-CPB (data not shown).

DISCUSSION

The available information on the three-dimensional structures of porcine pancreatic pro-CPA1 and pro-CPB, together with the detailed knowledge of both tryptic activation processes, allowed the initial definition of the putative structural determinants on the activation and inhibition mechanisms of these pancreatic zymogens (3, 10, 11). In the present work a site-directed mutagenesis approach has been chosen to evaluate the previous stated hypothesis on the structure/function relationship in metallo-procarboxypeptidases.

The detailed experimental study of the activation and inhibition mechanisms in metallo-procarboxypeptidases requires considerable amounts of native and activable protein. A cloned cDNA for porcine CPB and pro-CPB expressed high levels of both proteins in *E. coli*. However, the proteins formed insoluble granules that were not successfully re-folded to active enzyme (25). Previous experience of other laboratories working on other carboxypeptidases (26, 27) indicated that expression in the yeast *S. cerevisiae* could solve the above-mentioned problems. In this work we have selected *P. pastoris* because of its higher potential expression yields (28). Expression under the control of the *P. pastoris* alcohol oxidase promoter yielded about 250 mg/liter secreted pro-CPB in shake-flask cultures, sufficient for our studies. The recombinant pro-CPB was functionally identical to the wild-type proenzyme, indicating that the protein produced in the yeast system achieves a native conformation and confirming that secretion is needed for the correct folding and disulfide bridge formation of proteins that naturally pass through the secretory pathway (29).

The high folding capability of the pro-segments in heterologous systems has been shown for different proteases (30, 31) including yeast and pancreatic procarboxypeptidases (32, 33). These pro-regions can act as intramolecular chaperones that drive the correct folding of their own protease domains. The high expression levels obtained in this work for single mutants within the pro-CPB-connecting segment indicate that changes in this region do not impair folding, probably because of the weak interactions that this region establishes with the active enzyme and the fact that it is not located in the globular core of the pro-segment. In contrast, equivalent substitutions in rat pro-CPA1-connecting segment, which exhibits strong contacts with enzyme residues, completely abolished the secretion of the recombinant protein in *S. cerevisiae* (26). On the other hand, changes in the main inhibition loop of pro-CPB, which connects β2 and β3 chains at the globular core of the pro-segment, resulted in low expression levels. This is in agreement with other studies on the folding of isolated human pro-CPA2 activation domain (34), which demonstrated that mutations in such a region affect the folding capability of the pro-segment. Returning to pro-CPB, the effects of D41N and D41A mutations and the deletion of the 3_{10} helix on secretion levels are also in line with the suggestion that interactions that stabilize binding of the prodomain to the native enzyme are important for the successful folding of procarboxypeptidases (9, 26).

The detailed study of the different tryptic targets in porcine pancreatic pro-CPB-connecting segment demonstrated that the proteolytic activation and processing of this zymogen is a highly specific and controlled process. First, it has been shown that Arg-93, preserved in human but not in rat, presents a much lower accessibility to the tryptic action than Arg-95 and may be discarded as a primary cleavage point in normal conditions. Only when Arg-95 is absent, and drastic proteolytic conditions are used, hydrolysis of the Arg-93–Val-94 bond occurs, generating a fully active carboxypeptidase with two additional residues at its N terminus. According to the crystallographic studies, Arg-95 in the pro-segment is a highly flexible and accessible residue (10). This work confirms this residue as the primary cleavage point whose hydrolysis initiates all the maturation process of pro-CPB. In the absence of this target, the zymogen cannot be attacked by trypsin and is therefore not activable. The initial cleavage at the Arg-95-Thr-1 bond is sufficient to generate full CPB activity quickly, without a need for a tryptic action on the second target. This is also in agreement with the behavior of the R93Q/R95Q mutant, which is not activated by trypsin even at very strong conditions. According to this, the secondary tryptic target, Arg-83, is not accessible in the zymogen and is only exposed to the tryptic attack after hydrolysis of the Arg-95–Thr-1 bond. The gained accessibility of the Arg-83–Ser-84 bond during the tryptic processing probably results from disturbances in the stability of the connecting region secondary structure followed by the unfolding of the α-helix to which it belongs. The sequence of this region shows low propensity to form α-helix and does not establish strong contacts with the enzyme; thus, the helical conformation can only be maintained while being part of the proenzyme.

The influence of the connecting region conformation in the kinetics of pro-CPB activation has been demonstrated by the Q89E replacement. The change of a carboxyamido by a carboxyl group in the side chain of residue 89 produced a strong acceleration of the activation process. This is probably caused by a better access of trypsin to the primary cleavage point because of destabilization of the connecting region and by an accelerated exposure of Arg-83 to trypsin after the first cut. The rapid destabilization of the connecting region in this mutant can be attributed to the loss of the original contact between Gln-89 in the pro-segment and Arg-124 in the active enzyme

moiety, as well as to the small propensity of the new sequence to adopt the expected four-turn α-helical structure at its C terminus.

Our results also suggest that the complete pro-segment (95 residues) retains a low inhibitory capability after being separated from the active enzyme. Thus, in R83Q mutant, the slightly biphasic activation course coincides with the presence of the long 1–95/1–94 (α/β) fragments in the activation mixture. On the other hand, when the first tryptic cleavage directly produces a 93-residue fragment (at high concentrations of trypsin in the case of mutant R95Q), the exposure of the secondary target and, therefore, the connecting region destabilization is so fast that primary fragments can not be detected because of a greater exposure of the secondary tryptic target and the subsequent fast release of peptide Ser-84–Ser-92. It is worth mentioning that more than half of the connecting region/enzyme contacts are established by residues in the C-terminal flexible loop of the former (10). All these results point to the fact that the complete pro-segment is capable of maintaining the original conformation of the connecting region and, therefore, the inhibitory capability for longer times than C-terminal-deleted fragments. In another system, that of porcine pro-CPA1, the primary pro-segment released by the initial trypsin action (94 residues) exhibits a 2-fold greater inhibitory power (K_I 2 nM) than the 93-residue fragment subsequently generated after CPA1 trimming of the C-terminal Arg-94 (15). The hydrolysis of the C-terminal Arg by CPA1 is a nonspecific and slow process. In contrast, the C-terminal Arg-95 in the primary tryptic fragment of porcine wild-type pro-CPB is quickly and specifically degraded by CPB, not allowing detection of its low inhibitory capacity.

We may conclude that the differences observed between pancreatic pro-CPA1 and pro-CPB proteolytic activation and processing can be partially attributed to the different conformation of the region that connects the globular domain of the pro-segment with the enzyme in these zymogens. However, because changes in the globular domain may significantly affect the tryptic processing of porcine pro-CPB, we cannot neglect the influence of the interactions between this domain and the mature enzyme, even in the B form. This is evident in the acceleration observed in the activation courses when those interactions were perturbed by deletion of the 3_{10} helix or by mutations at Asp-41. A similar effect was previously reported for the addition of carboxypeptidase inhibitors to pro-CPB during its activation process (16), probably because of a slight displacement of both moieties.

In pancreatic carboxypeptidases, enzyme inhibition results from the shielding of the large depression containing the active site by the globular part of the pro-region. This shielding is not absolute for small substrates, which have been shown to be hydrolyzed by porcine pro-CPA1 and human pro-CPA2 in the form of zymogens. The intrinsic activity of both zymogens reaches 10% of V_{max} for dipeptide substrates and decreases as the substrate volume increases (7, 35). In contrast, the pro-segment of pancreatic pro-CPB completely inhibits the active enzyme and prevents the detection of residual activity. These functional differences seem to be related to the different conformation of the pro-segments over the active site. Thus, the absence of residues 43–46 in pro-CPA1 and pro-CPA2, when compared with pro-CPB (which has a 3_{10} helix there) has important consequences. This deletion is located in the loop that connects β2 and β3 strands of the pro-segment, just over the active site. Both in pro-CPA1 and pro-CPA2, this loop adopts a straight conformation and does not physically cap the active site (11, 12), as occurs in porcine pro-CPB, where it forms a 310 helix that covers the active-site pocket (10). Pro-CPB also contains a salt bridge between Asp-41 and Arg-145 at the S1′ subsite of the active enzyme, which renders the latter residue unable to bind the C-terminal carboxylate of the substrate molecules.

When Asp-41 in the inhibitory loop of pro-CPB was changed for either Asn or Ala, the resulting zymogens showed a small intrinsic activity. This result indicates that Arg-145 is not blocked in the mutated zymogens, allowing the access of small substrates to the S1′ subsite and their anchoring to Arg-145 through their terminal carboxylate. Therefore, it seems clear that the salt bridge between Asp-41 and Arg-145 contributes to the null activity of pro-CPB. Because the intrinsic activity values are very similar for both mutants, it is also possible to infer that a hydrogen bond cannot substitute the salt bridge. However, the intrinsic activity shown by these mutant proenzymes is low when compared with those exhibited by A forms against similar substrates. This is probably because of the presence of the 3_{10} helix in pro-CPB, which physically hinders substrate diffusion. The lack of intrinsic activity in the mutated zymogens when bulkier substrates were used seems to be in accordance with this interpretation (results not shown). The low yield of production of the pro-CPB mutant with the 3_{10} helix removed did not allow the measure of intrinsic activities and the complete confirmation of the above hypothesis.

The present work used protein engineering approaches to confirm and enrich the hypotheses about the nature of the determinants of inhibition and proteolytic activation and processing of pancreatic pro-CPBs generated from enzymatic and x-ray crystallography studies. It could also facilitate future work on the overexpression and redesign of these proenzymes, their active enzymes, or other related proteins. This could be the case with human plasma pro-CPB or CPB, also called carboxypeptidase U or TAFI, which has recently generated a considerable interest because of its potential role in the stabilization of blood clots as a fibrinolysis inhibitor (36, 37). Plasma pro-CPB is significantly homologous to pancreatic pro-CPB and, therefore, amenable to similar approaches for its overproduction, engineering, and structure-based drug design.

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