Role of the Prodomain in Folding and Secretion of Rat Pancreatic Carboxypeptidase A1

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ABSTRACT: Pancreatic carboxypeptidase A1 (CPA1) is synthesized as an inactive precursor, proCPA1, which is processed to the active enzyme by the proteolytic removal of the 95-amino acid N-terminal prodomain. Purified rat proCPA1 is renatured *in vitro* after denaturation in guanidine or in guanidine plus reducing agents. In contrast, purified CPA1 is not renatured under any of the conditions tested. While proCPA1 is secreted in yeast when fused to the α-factor signal sequence in place of its endogenous signal sequence, mature CPA1 is not secreted and is trapped and degraded intracellularly. Thus, in addition to maintaining CPA1 in the inactive state, the prodomain promotes folding and secretion of the proenzyme. Neither of these functions can be restored by supplying the prodomain to CPA1 in trans. The three-dimensional structure of porcine proCPA reveals a number of extensive contacts made between the prodomain and the enzyme active site which account for its inhibitory properties [Guasch et al. (1992) *J. Mol. Biol. 224*, 141−157]. Among these contacts are salt bridges formed between Arg-71 and Asp-*A36* and between Arg-124 and Asp-*A89*. Mutation of any of these four residues inhibits secretion of proCPA1 from yeast and results in its intracellular degradation. The effect of the mutations on secretion suggests that interactions which stabilize the binding of prodomain to the native enzyme active site may also be important for the successful folding of proCPA1.

Proteases are most commonly secreted as inactive precursors or proenzymes. The propeptide maintains the protease in the inactive state until delivery to the site of action where the propeptide is cleaved off to produce active enzyme (Neurath, 1984). This mechanism of enzyme regulation is found for proteases from both eukaryotic and prokaryotic sources and for all mechanistic classes of proteases. Two types of propeptides have been identified, and their functions and properties have been recently reviewed (Baker et al., 1993). The first type of propeptide is illustrated by trypsin; the propeptide is small (16 amino acids), it functions by maintaining the enzyme in an inactive conformation, and it is not required for *in vitro* refolding or for secretion of trypsin from yeast or bacteria (Higaki et al., 1989). The propeptides of the second type are large enough to form an independent domain (e.g., 77 amino acids for subtilisin and 166 for α-lytic protease), function by binding to the enzyme active site as an inhibitor, and are required for folding *in vitro* and for secretion *in vivo* [e.g., subtilisin (Ikemura et al., 1987; Zhu et al., 1989), α-lytic protease (Baker et al., 1992; Silen & Agard, 1989), and yeast carboxypeptidase Y (Ramos et al., 1994; Winther & Sorensen, 1991)]. The secretion deficit is believed to be an indication of failure of these proteins to properly fold along the secretory pathway. For α-lytic protease and subtilisin, these functions can be restored if the prodomain is supplied in trans. In the absence of the prodomain, α-lytic protease folds into an intermediate state which is prevented from folding into the native structure by a large kinetic barrier; upon addition of the prodomain, the intermediate is rapidly converted to the active enzyme (Baker et al., 1993). The requirement of the prodomain for secretion has also been demonstrated for papain in insect cells (Vernett et al., 1990) and for *Yarrowia lipoytica* alkaline extracellular protease from native cells (Fabre et al., 1991); in the latter case, enzyme secretion is restored when the prodomain is supplied in trans (Fabre et al., 1992).

The role of the propeptide in folding and secretion of the pancreatic carboxypeptidases (CPs) has not been experimentally studied. It appears likely however that both classes of propeptides are found within the family of Zn^{2+}-dependent CPs, which includes the pancreatic and mast cell CPs as well as the more distantly related regulatory CPs. The propeptides of the pancreatic and mast cell CPs share similarities to the "subtilisin like" class of proenzymes, suggesting that these prodomains will be required for folding. In contrast, the regulatory CPs have short propeptides which are not required for expression of the active enzyme (e.g., CPE has a 16-amino acid propeptide; Manser et al., 1990).

The prodomain of pancreatic CPA1[1] is 95 amino acids, and it inactivates the enzyme by binding as an inhibitor to the fully formed and functional active site (San segundo et al., 1982). The structure of porcine proCPA reveals that extensive contacts are made between CPA active site residues and the prodomain, providing the structural basis of pro-

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[1] Abbreviations: proCPA1, rat procarboxypeptidase A1, amino acid residues *A1*−*95* plus residues 1−307; CPA1, activated rat carboxypeptidase A1, amino acid residues 1−307. Mutant proCPA1s are abbreviated by their single letter codes; e.g., R71D is proCPA1 with Arg-71 replaced with Asp. Prodomain residues (*A1*−*95*) are proceeded by an A and displayed in italics, and amino acid residues from the active enzyme domain (1−307) are displayed in normal type.

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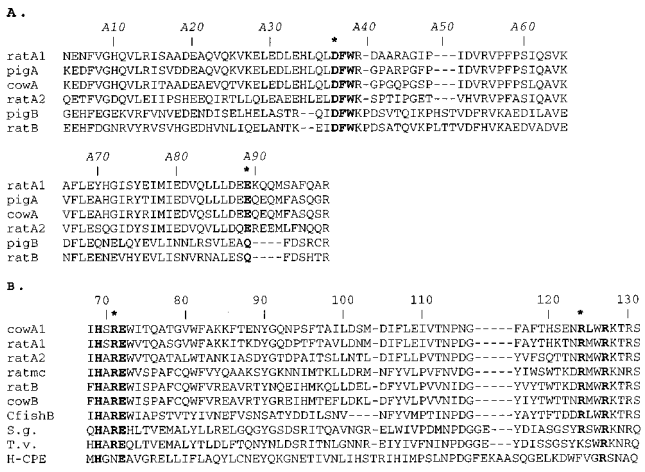

FIGURE 1: Alignment of selected carboxypeptidases: (A) prodomains and (B) partial alignment of the enzyme domain. Standard bovine pancreatic CPA numbering is used; the prodomain is numbered *A1*−*95* and the CPA domain 1−307. Sequences were obtained from the Prosite entry PS00132: A1, carboxypeptidase A1; A2, carboxypeptidase A2; A, carboxypeptidase A; B, carboxypeptidase B; ratmc, rat mast cell carboxypeptidase A; CfishB, crayfish carboxypeptidase B; S.g., *Streptomyces griseus* carboxypeptidase; T.v., *Thermoacitnomyces vulgris* carboxypeptidase; and H-CPE, human carboxypepitidase E (enkephalin convertase or CPH). Residues which have been mutated are indicated by an asterisk, essential active site residues His-69 and Asp-72 (Zn^{2+} binding residues) and Arg-127 (stabilizes the oxyanion intermediate; Phillips et al., 1990) are displayed in bold.

domain inhibition (Guasch et al., 1992). The triad Asp-*A36*, Phe-*A37*, Trp-*A38*, invariant among all known pancreatic and mast cell CP prodomains (Figure 1A), is the site of several interactions between the prodomain and the S1 subsite of the enzyme, including a salt bridge between Arg-71 and Asp-*A36*. A salt bridge is also observed between Glu-*A89*, which is conserved in the proenzymes with A-type specificity, and Arg-124. Arg-71 and Arg-124 are invariant among the pancreatic CPs but are not conserved in the related regulatory CPs (Figure 1B). As the prodomain structure is not conserved between the pancreatic and regulatory CPs, this observation suggests that one possible function of Arg-71 and Arg-124 in the pancreatic enzymes is to stabilize the interaction with the prodomain.

In this paper, we demonstrate that the prodomain of rat pancreatic CPA1 is required to facilitate folding *in vitro* and for secretion in yeast. Neither of these functions is restored by supplying the prodomain in trans. Mutation of residues which form ionic interactions between the active site (Arg-71 and Arg-124) and the prodomain (Asp-*A36* and Gln-*A89*) causes a loss of secretion in yeast. In the cells where the enzyme is not secreted, proteolytic fragments of CPA1 are found intracellularly by immunoblotting, suggesting that the enzyme is improperly folded and is targeted for degradation.

MATERIALS AND METHODS

Expression of proCPA1, CPA1, and the Prodomain in Yeast

proCPA1. Rat proCPA1 was expressed and secreted in yeast DLM101α cells from the ADH/GAPDH promoter as a fusion protein with the α-factor leader sequence as described (Phillips et al., 1990).

CPA1. A yeast vector for the expression of CPA1 in the absence of its prodomain was constructed as follows. A 250 bp *Kpn*I/*Bst*EII fragment was generated by PCR and contains the protease site for α-factor leader cleavage (Lys-Arg at −2, −1 below) followed by the coding sequence for CPA1 starting at Ala-1 and ending at amino acid 74, the site of a natural *Bst*EII site in the gene. The 5′-sequence of this fragment which corresponds to the 5′-PCR primer is

```
      Kpn site                1
        Val Pro Leu Asp Lys Arg Ala Leu Ser Thr Asp Ser Phe Asn Tyr Ala
5'-GGG GTA CCT TTG GAT AAA AGG GCC TTG TTC ACT GAC TCT TTC AAT TAT GCC-3'
```

The 3′-PCR primer was 5′-AGCCTGGGTGACCCACTC-CCT-3′, with the *Bst*EII site underlined. The *Kpn*I/*Bst*EII fragment was verified by sequence analysis and cloned into the *Kpn*I/*Bst*EII-digested shuttle vector (pMP36; Phillips et al., 1990) in frame to the α-factor leader sequence and downstream from the ADH/GAPDH promoter element to create pMP70. The *Bam*HI fragment from pMP70 was cloned into the yeast 2 μm circle YEp420 (Rose & Broach, 1991) which contains the URA selectable marker for maintenance of the plasmid in yeast. This construct (pMP72) was transformed into yeast DLM101α cells, and the protein was expressed as described for proCPA1 except that the selective media was SD-ura.

Prodomain. The 300 bp *Kpn*I/*Bgl*I fragment from pMP36 which contains the α-factor proteolysis site fused to the prodomain ending at amino acid 92 of the prodomain (Phe-*A92*) was cloned into the *Kpn*I/*Sal*I fragment of pMP36 in a three-piece ligation with a *Bgl*I/*Sal*I adapter (below), and the stop codon is underlined.

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5'-AGGCCAGGTGAG-3'
3'-AGGTCCGGTCCACTCAGCT-5'
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The *Bam*H1 fragment from this construct (pMP71) was cloned into the yeast 2 μm circle YEp13 (Rose & Broach, 1991) which contains the Leu selectable marker. The 2 μm circle containing the prodomain (pMP73) was transformed into yeast DLM101α cells, and the protein was expressed as described for proCPA1.

Coexpression of the Prodomain and CPA in Yeast

pMP72 and pMP73 were simultaneously transformed into yeast DLM101α cells, and the two plasmids were selected and maintained by plating transformants on SD-ura, -leu.

Purification of Wild-Type CPA1 from Yeast after Expression as proCPA1

CPA1 was purified from the media by phenyl-Sepharose column chromatography followed by trypsin activation to remove the prodomain, affinity column chromatography on benzylsuccinic acid-Sepharose, and Mono Q column chromatography as described (Phillips et al., 1990).

Purification of proCPA1 from Yeast

The first step in the purification of proCPA1, phenyl-Sepharose column chromatography, was performed in a manner identical to what is described above for active CPA1. proCPA1-containing fractions, as monitored by SDS−PAGE analysis were pooled and dialyzed against buffer A [20 mM Tris-HCl (pH 7.5) and 20 mM NaCl]. The pooled fractions were applied to a DEAE-Sepharose column (20 mL). The

column was washed in buffer A, and the protein was eluted using a salt gradient from 20 to 250 mM NaCl. The proCPA1-containing fractions were again dialyzed against buffer A and applied to a Mono Q column, where the protein was again eluted with a 20 to 250 mM NaCl gradient. Approximately 4 mg of pure proCPA1 was obtained from 2 L of culture.

Expression and Purification of the Prodomain from Escherichia coli

The *Hin*dIII/*Sal*I fragment from MP71 which contains the prodomain was cloned into the pFlag vector (IBI), cut with the same restriction enzymes, where both *Hin*dIII sites were blunted with T4 polymerase. The vector, which directs the expression of the prodomain as a fusion protein to the Flag epitope into the periplasm of *E. coli*, was transformed into DH5α cells. An overnight culture grown at 30 °C was diluted 10-fold, and protein expression was induced by the addition of 1 mM IPTG. After induction, cells were grown for 4 h at 30 °C.

Cells were lysed by resuspending the cell pellet in water and lysozyme (1 mg/mL) for 15 min at room temperature. The cell pellet was removed by centrifugation, and the supernatant which contains the periplasmic fraction was mixed with an equal portion of 10 mM Tris-HCl (pH 7.5) and 300 mM NaCl. The periplasmic fraction was heated to 65 °C for 15 min, and the precipitated protein was removed by centrifugation. The soluble fraction which contains the prodomain was applied to a Mono Q HR 5/5 column (Pharmacia), and the protein was eluted with a 0.15 to 1 M NaCl gradient. The fractions which contained CPA inhibitory activity were pooled and applied to a phenyl-Superose HR 5/5 column (Pharmacia) after mixing with an equal volume of 20 mM Tris-HCl (pH 7.5) and 3 M ammonium sulfate. The protein was eluted with a 1.5 to 0 M ammonium sulfate gradient. The prodomain was approximately 98% pure by SDS−PAGE analysis. Propeptide purification from the periplasmic fraction was monitored by following the inhibition of CPA1 activity and by Western analysis with the M1 and M2 antibodies to the Flag epitope as recommended by the manufacturer (IBI).

SDS−PAGE and Western Blot Analysis

Samples were prepared for analysis as follows. Yeast cultures (2 mL) harboring the indicated proCPA1 construct were grown and induced for CPA1 expression as described above. The yeast pellet was resuspended in SDS−PAGE sample buffer (200 μL), and the pellet was disrupted by vortexing in the presence of glass beads. The cell media was precipitated with 10% trichloroacetic acid (TCA) and resuspended in $^1/_{10}$ of volume of SDS−PAGE sample buffer. The cell pellet (3 μL) and media (2 μL for wild-type proCPA1 and 10 μL for mutant constructs) were analyzed by SDS−PAGE (Laemmli, 1970) followed by Western blot analysis using the ECL kit (Amersham) as recommended by the manufacturer. Antibodies to CPA1 were generated to enzyme purified from rat pancreatic acetone powder as described (Gardell et al., 1988) by Berkeley Antibodies, Inc., and were used at a 1:1000 dilution.

Refolding Conditions

proCPA1 or CPA1 was denatured for 2−4 h in 4−8 M guanidine hydrochloride at a final protein concentration of 0.35−1.3 mg/mL (10−40 μM) both in the absence and in the presence of the reducing agent DTT (8 mM) or β-mercaptoethanol (1 mM). Several different methods were employed to refold the proteins. (1) The first was rapid dilution of the denatured protein sample by renaturing buffer. An excess of renaturation buffer (50−200-fold) was added to 5 μL of denatured protein sitting in a 1.5 mL Eppendorf tube. The ionic strength and pH of the renaturation buffer were varied as described in Figure 2. Unless otherwise indicated, the standard renaturation buffer was 50 mM Tris-HCl (pH 7.5) and 500 mM NaCl. (2) For samples which were reduced with β-mercaptoethanol, the renaturation buffer included 200 mM β-mercaptoethanol and 50 mM sodium thiosulfate. (3) For samples which had been reduced with DTT, the sample was first brought to 2.5 mM cystine by the addition of $^1/_{10}$ of volume of 250 mM cystine dissolved in 0.5 N NaOH. An equal volume of 50 mM Tris-HCL (pH 7.5) and 5 mM cysteine was added and the reaction incubated overnight. The sample was than dialyzed against 50 mM Tris-HCl (pH 8.0) and 250 mM NaCl. (4) The last method was dialysis of the denatured protein (0.6 mg/mL) against buffer containing successive decreases of guanidine hydrochloride in 50 mM Tris (pH 8.0). The guanidine hydrochloride steps were 8, 4, 1, and 0.25 M and no guanidine hydrochloride plus 250 mM NaCl. Dialysis was for 4−12 h. After refolding, the samples of proCPA1 were incubated for 30 min with trypsin (2 μg/mL) before assay.

For attempts to renature CPA in the presence of the prodomain supplied in trans, 0.35 mg/mL (10 μM) CPA denatured in 6 M guanidine was rapidly diluted with a 100-fold excess of standard renaturation buffer containing propeptide (0.1−10 μM). Trypsin was added at the end of the reactions to degrade the propeptide. Control samples were fully active after this procedure.

Mutagenesis

Oligo-directed mutagenesis was performed as described (Kunkel, 1985) in the wild-type proCPA construct pMP36 (Phillips et al., 1990). The *Bam*H1/*Sal*I fragment from this construct, containing proCPA1 mutants in frame to the α-factor leader sequence and downstream from the ADH/GAPDH promoter element, was cloned into the yeast 2 μm circle pBS24.1 [a derivative of pC1/1 (Rose & Broach, 1991) which contains both URA3 and leu2-d selectable markers]. The expression constructs were transformed into yeast DLM101α cells, and protein expression was induced as described for wild-type proCPA1.

Enzyme Assays

CPA1 activity was measured by following the hydrolysis of hippuryl-L-phenylatic acid (250 μM) spectrophotometrically at 254 nm in 50 mM Tris-HCl (pH 7.5) and 500 mM NaCl at 25 °C.

Molecular Modeling

Structures were displayed with the INSIGHT program (Biosym Technologies). Coordinates for the structures of cow CPA were obtained from the Protein Data Base file

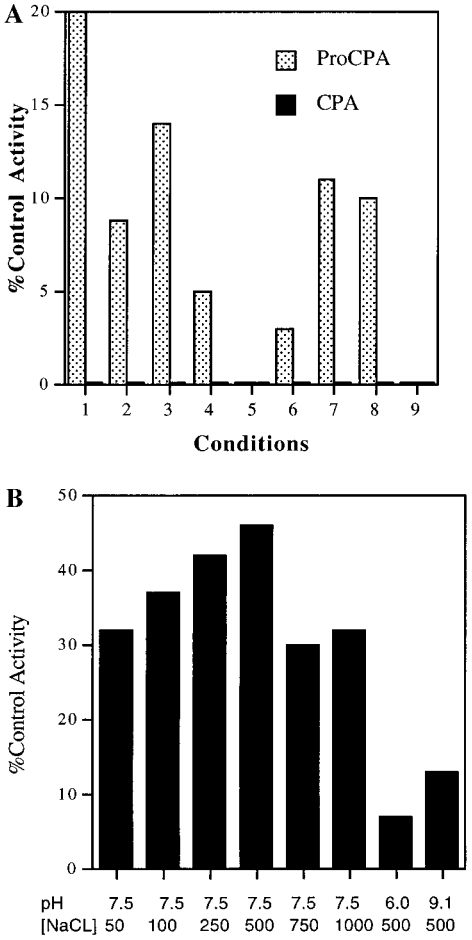

FIGURE 2: Testing of *in vitro* renaturation conditions. (A) Comparison of conditions for the refolding of proCPA (dotted boxes) with those of CPA (solid boxes). The results are presented in pairs with proCPA first and CPA second. No CPA activity was observed under any conditions for the refolding of CPA. Protein was denatured in 8 M guanidine for 2 h at a protein concentration of 1.2 mg/mL; reducing agents were included when indicated. Renaturation for columns 1−6 was by rapid dilution of the denatured protein sample with a 100-fold excess of renaturation buffer. The renaturation temperature was 25 °C, and renaturation was carried out for 2 h prior to assay unless indicated. The renaturation buffer was varied as follows: column 1, 50 mM Tris-HCl (pH 7.5) + 500 mM NaCl; column 2, as in column 1 except renaturation was performed at 4 °C; column 3, as in column 1 plus 10^{-6} M $ZnCl_2$; column 4, as in column 1 plus 10^{-5} M $ZnCl_2$; column 5, as in column 1 plus 10^{-3} M $ZnCl_2$; column 6, sample denatured in guanidine plus 2 mM β-mercaptoethanol and renatured as described in Materials and Methods; column 7, denatured in guanidine plus 8 mM DTT, renatured with cysteine/cystine as described in Materials and Methods; column 8, renature by slowly adding denatured protein to a 100-fold excess of 50 mM Tris-HCl (pH 7.5) + 500 mM NaCl; and column 9, renatured by stepwise dialysis as described in Materials and Methods. (B) Further analysis of the conditions for proCPA renaturation. Protein was denatured in 6 M guanidine and renatured by rapid dilution with a 100-fold excess of renaturation buffer at 25 °C. Renaturation buffer included either 50 mM Tris (pH 7.5, 9.1) or 50 mM Mes (pH 6.0) at the indicated NaCl concentration. Samples were treated with trypsin and assayed for CPA activity as described in the Materials and Methods.

6CPA, and coordinates for porcine proCPB were kindly provided by Dr. R. Huber.

RESULTS

The Prodomain Facilitates Renaturation of proCPA in Vitro. Purified proCPA1 and CPA1 were denatured in guanidine (6−8 M) in the absence or presence of reducing agents (8 mM DTT or 1 mM β-mercaptoethanol). The standard renaturation condition was rapid dilution of the denatured protein sample (5 μL) with a 100-fold excess of renaturing buffer. The composition of the renaturing buffer was varied as described in Figure 2 and in Materials and Methods. Stepwise dialysis was also tested (Figure 2A). The extent of renaturation was monitored by following the restoration of CPA1 activity; samples containing proCPA1 were activated with trypsin prior to assay. Control samples which had not been denatured were incubated under identical renaturation conditions.

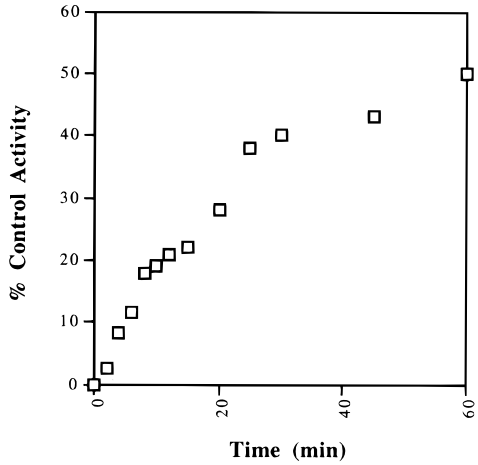

FIGURE 3: Time course of proCPA1 renaturation. proCPA (0.35 mg/mL) denatured in 6 M guanidine was renatured by rapid dilution with a 100-fold excess of 50 mM Tris-HCl (pH 7.5) and 500 mM NaCl. Aliquots were removed at the specified time point, treated with trypsin, and assayed for CPA activity.

proCPA1 is renatured under a variety of conditions after denaturation in guanidine hydrochloride (6−8 M) or in guanidine hydrochloride plus reducing agents (Figure 2). In contrast, CPA1 is not renatured under any of the tested conditions (Figure 2), supporting the hypothesis that the prodomain is required for folding. The optimal conditions for proCPA1 refolding are rapid dilution of the sample by a 100-fold or greater excess (a range from 50- to 400-fold was tested) of renaturation buffer at 25 °C and pH 7.5 (Figure 2). Folding occurred over a range of NaCl concentrations, but the highest yield of refolded proCPA1 was obtained at 500 mM NaCl (Figure 2B). The addition of $ZnCl_2$ did not improve the yield of refolded protein and at high concentrations had an inhibitory effect. Higher recovery of activity was also observed after the sample had been denatured in 6 M guanidine as compared to that in 8 M guanidine. The single disulfide bond could be reduced during denaturation and reformed during renaturation, though this procedure lowered the yields of refolded material by about 50% (Figure 2A). A time course for the renaturation of proCPA1 under optimal conditions is displayed in Figure 3.

To test if the prodomain could supply its folding function to CPA1 in trans, the prodomain was expressed and purified from the periplasm of *E. coli* DH5α cells as described in Materials and Methods. The isolated prodomain inhibited rat CPA1 activity (in the low nanomolar range), in agreement with previous reports for porcine and bovine CPA (San segundo et al., 1982). The addition of prodomain (0.1−10 μM) to the renaturation buffer did not aid in the refolding of CPA1, and again no enzyme activity was restored. To test if small molecules which bind the active site could

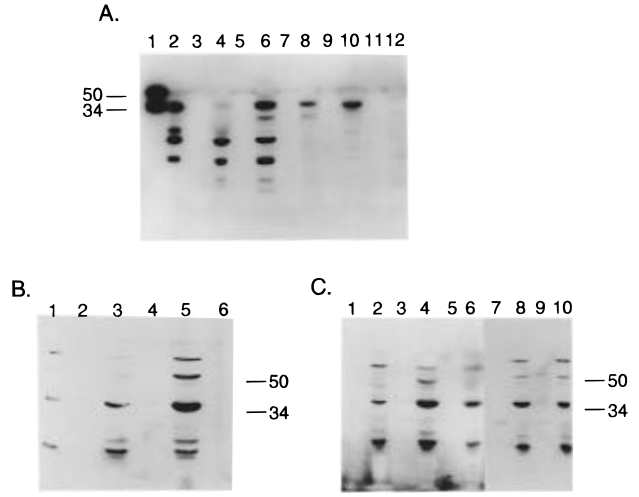

FIGURE 4: Secretion of proCPA1, CPA1, and proCPA1 mutants from yeast. Samples of media and intracellular protein extracts were prepared as described in Materials and Methods. Samples were analyzed by SDS−PAGE and Western blot analysis with rabbit anti-rat CPA1 antibody. Detection was by the ECL kit (Amersham): (A) lanes 1 and 2, wild-type proCPA1, media and pellet; lanes 3 and 4, R71M, media and pellet; lanes 5 and 6, R124M, media and pellet; lanes 7 and 8, wild-type CPA1, media and pellet; lanes 9 and 10, coexpressed wild-type CPA1 and prodomain in trans, media and pellet; lanes 11 and 12, prodomain alone, media and pellet; (B) lanes 1 and 2, E*A89*R, pellet and media; lanes 3 and 4, R124E/E*A89*R, pellet and media; lanes 5 and 6, R124E, pellet and media; (C) lanes 1 and 2, D*A36*R, media and pellet; lanes 3 and 4, R71D, media and pellet; lanes 5 and 6, R71D/D*A36*R, media and pellet; lanes 7 and 8, D*A36*F, media and pellet; lanes 9 and 10, D*A36*A, media and pellet.

promote the refolding of CPA1, substrate, Cbz-Gly-Gly-Phe (25 and 250 μM), and a CPA inhibitor, D,L-benzylsuccinic acid (10 μM), were added to the renaturation buffer and again no CPA activity was recovered. SDS−PAGE analysis of samples at the end of the folding reactions was done to demonstrate that CPA1 had not been degraded during the course of the reaction; CPA was recovered intact and in the same quantities as for the control samples.

The Prodomain Is Required for Secretion in Yeast. Additional yeast expression vectors were constructed independently for the expression and secretion of CPA1 and the prodomain (see Materials and Methods for details). The constructs were transformed into DLM101α cells. To test if the prodomain could supply its function in trans, these plasmids were also cotransformed into DLM101α cells. Cells containing these constructs were induced for protein expression (see Materials and Methods), and the cell pellet and media were examined by Western blot analysis for evidence of CPA1 expression. While proCPA1 (50 kDa band) is abundantly secreted into the yeast media (Figure 4A, lanes 1 and 2; lane 1, the 34 kDA band represents partial processing of proCPA1 to CPA1 by the yeast), CPA1 was not secreted in the absence of the prodomain (Figure 4A, lanes 7 and 8) or when the prodomain was supplied in trans (Figure 4A, lanes 9 and 10). CPA1 expressed from this construct is found intracellularly; the presence of multiple bands which react with CPA1 antibody suggests that the intracellular CPA1 has been targeted for degradation (Figure 4A, lanes 8 and 10). Enzyme activity is not detectable in the intracellular extracts, suggesting that the intracellular CPA1 is not properly folded.

Mutagenesis of Residues Which Form Ionic Interactions between the Prodomain and the Active Site Inhibits Secretion of proCPA1. The structure of proCPA1 elucidated specific interactions which occur between the prodomain and the enzyme active site (Guasch et al., 1992). The importance of two salt bridges, Arg-71 with Asp-*A36* and Arg-124 with Glu-*A89*, to prodomain function was examined by site-directed mutagenesis. Mutant proCPAs (R71M, D; R124M, E; D*A36F*, *A*, *R*; E*A89R*) were cloned into the yeast expression vector, and DLM101α cells harboring the plasmid were induced for protein expression. The distribution of proCPA or CPA between the secreted fraction (media) and the intracellular fraction (pellet) was analyzed by SDS−PAGE and Western blotting for the wild-type and mutant enzymes (Figure 4). The substitution of any one of these residues with neutral (Figure 4A) or amino acids of the opposite charge (Figure 4B,C) inhibited the secretion of the proenzyme in yeast, resulting in the intracellular degradation of the expressed product. Attempts to restore secretion by reversing the charge pairs (Figure 4B, lanes 3 and 4, and Figure 4C, lanes 5 and 6) or by replacing both members of the charge pair with hydrophobic amino acids (data not shown) were unsuccessful.

DISCUSSION

In this paper, we demonstrate that, in addition to regulating enzyme activity, the prodomain of pancreatic CPA1 promotes *in vitro* folding and secretion of the enzyme from yeast. These observations extend previous reports which demonstrated the importance of prodomains for the folding of some bacterial and yeast proteases to proteases from mammalian cells. In addition, the lack of CPA1 secretion from yeast cells in the absence of its prodomain is likely to be caused by the inability of CPA1 to fold *in vivo*. In a number of examples, mutation or deletion of prodomains leads to the accumulation and degradation of the precursor proteins *in vivo*, suggesting that the proteins were not properly folded. Deletion mutants of the prodomains of carboxypeptidase Y (Ramos et al., 1994) and of *Yarrowia lipolytica* alkaline extracellular protease (Fabre et al., 1991) were not secreted *in vivo*. The deletion mutants of *Y. lipolytica* alkaline extracellular protease accumulated in the ER (Fabre et al., 1991), while the carboxypeptidase Y mutants were not transported to the Golgi apparatus and were instead degraded (Ramos et al., 1994). Analogously, the lack of secretion of CPA1 and of the salt bridge proCPA1 mutants suggests that these enzymes are retained in the ER and targeted for degradation because they are improperly folded.

These data suggest that all members of the metallo CP family (Figure 1) which have prodomain structures similar to pancreatic CPA1 (e.g., approximately 95 amino acids in length) will also require their prodomains for folding. In support of this hypothesis, mast cell CPA1 could not be expressed in COS cells as an active enzyme in the absence of its prodomain (Dikov et al., 1994). Recently, mRNA transcripts of CPA2 which lack the prodomain and the first 18 amino acids of the mature enzyme were found in the brain (Normant et al., 1995), and our results would suggest that these transcripts are unlikely to encode functional CP. Interestingly, while the regulatory CPs (Figure 1) are members of the metallo CP family, they do not have similar prodomain structures and do not require the prodomain for secretion (e.g., CPE; Manser et al., 1990). This observation

suggests that an additional structural element in the regulatory CPs is able to substitute for the prodomain in directing the folding of the mature enzyme. The regulatory enzymes contain several large insertions in the primary sequence which map to the rim of the catalytic site at positions 241 and 271 (Osterman et al., 1992) and may be functioning in this capacity.

Analysis of the structure of subtilisin complexed to its prodomain suggests that the prodomain promotes the folding reaction by binding to and stabilizing the central $\alpha\beta\alpha$ substructure of the enzyme domain, the formation of which is the rate-limiting step in the folding process (Bryan et al., 1995). Random PCR mutagenesis of the subtilisin prodomain localized several regions (hot spots) where amino acid substitution interfered with prosubtilisin secretion. For one mutation, a second site mutation in the subtilisin domain restored secretion (Kobayashi & Inouye, 1992). Additionally, mutant prodomains which bind poorly to subtilisin are unable to promote the folding reactions *in vitro*; a direct correlation was observed between the binding affinity of mutant prodomains for subtilisin and their ability to catalyze the folding reaction (Li et al., 1995; Wang et al., 1995). These results suggest that interactions which are important for stabilizing the native interaction between the prodomain and the enzyme active site are also essential for stabilizing important folding intermediates and further that an essential folding intermediate has native-like structure.

The observation that mutation of proCPA1 residues which form interactions between the prodomain and CPA1 in the native structure inhibits secretion suggests that similar mechanisms are operating in the folding of proCPA1. Arg-71 in CPA1 is positioned on a loop structure between strand 3 and helix 2, both of which run through the central core of the molecule. This loop also positions two of the essential Zn^{2+} ligand residues, His-69 and Asp-72. Arg-124, which is positioned on a loop structure adjacent to helix 4, interacts with the Arg-71 loop structure via stacking of its aliphatic carbons against Trp-73. Thus, similar to the effect of the prodomain of subtilisin, the interactions of Arg-71 with Asp-*A36* and Arg-124 with Glu-*A89* may promote the folding reaction by stabilizing the core $\alpha\beta$ structure of CPA1. Further, Trp-73 is a surface residue in active CPA1, and it also forms a contact with the prodomain residue Glu-*A89* (Guasch et al., 1992). Thus, a role of the prodomain in folding could be to protect this hydrophobic patch from aggregation during the folding reaction. The interactions between the two active site Arg residues and the prodomain may be essential to position the prodomain to achieve this goal. Unlike subtilisin and α-lytic protease, the prodomain of CPA1 was unable to complement the folding deficiency when supplied in trans. In CPA1, the folding process may be in competition with aggregation caused by the presence of the surface-exposed Trp-73; thus, when the prodomain is supplied in trans, it may be unable to catalyze the folding process fast enough to compete with the potential aggregation.

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