Dipeptidyl aminopeptidase processing and biosynthesis of alkaline extracellular protease from Yarrowia lipolytica

Sam Matoba, Kevin A. Morano, Daniel J. Klionsky, Keunsung Kim and David M. Ogrydziak

Institute of Marine Resources and Section of Microbiology, University of California, Davis, CA 95616, USA

Alkaline extracellular protease (AEP) from Yarrowia lipolytica is synthesized as a precursor with a 157 aa prepro-region. Signal peptide cleavage was shown to occur after Ala₁₅ by N-terminal amino acid radiosequencing of the largest intracellular AEP precursor. AEP proteolytic activity was not required for AEP processing. After a change of the putative active site Ser to Ala, inactive AEP with the same mobility on SDS-PAGE as wild-type mature AEP was secreted. The role of dipeptidyl aminopeptidase (DPAPase) activity in AEP processing was also investigated. Mutations early in the -X-Ala- and -X-Pro- dipeptide stretch (Pro₁₇ to Met which should prevent DPAPase processing and Ala₁₅ to Val which should allow removal of only the first dipeptide) did not prevent synthesis of active mature AEP nor did use of the DPAPase inhibitor Pro-boroPro. Deletion of the entire dipeptide stretch (Ala₁₅ to Pro₁₇) resulted in intracellular accumulation of an AEP precursor, which surprisingly was not glycosylated, and little or no secretion of AEP-related polypeptides. Expression of AEP in wild-type and dpp₁ dpp₂ Saccharomyces cerevisiae strains (lacking both the Golgi and vacuolar DPAPases) resulted in secretion of only mature AEP and no AEP precursors. Transit times and levels of AEP secretion were similar for both strains. These results indicate that the KEX₂-like cleavage after Lys₁₅₀-Arg₁₅₇, which yields mature active AEP can occur in the absence of DPAPase processing and that DPAPase processing is not necessary for secretion of mature active AEP.

Keywords: Yarrowia lipolytica, protease, dipeptidyl aminopeptidase

INTRODUCTION

The dimorphic yeast Yarrowia lipolytica secretes high levels of a 32 kDa subtilisin-like alkaline extracellular protease (AEP) (Tobe et al., 1976; Ogrydziak & Scharf, 1982; Matoba et al., 1988). AEP has been used as a reporter molecule for in vivo studies of functions of the signal recognition particle of Y. lipolytica (He et al., 1992; Yaver et al., 1992), and the XPR2 gene (encoding AEP) promoter and prepro-region have been used for heterologous protein secretion. AEP is synthesized with a 157 aa prepro-region that begins with a signal sequence followed by a stretch of nine X-Ala, X-Pro dipeptides (Fig. 1). The prepro-region contains a consensus sequence for N-linked glycosylation and ends with a Lys-Arg pair of basic amino acids (Davidow et al., 1987; Matoba et al., 1988; Nicaud et al., 1989). Removal of the signal peptide and addition of 2 kDa of N-linked carbohydrate in the pro-region are thought to occur as prepro-AEP is translocated across the ER membrane yielding a 55 kDa AEP precursor (Matoba et al., 1988; Fabre et al., 1991). Removal of the X-Ala, X-Pro dipeptides by dipeptidyl aminopeptidase (DPAPase) activity, which yields a 52 kDa precursor, and cleavage after Lys-Arg at the end of the pro-region by the KEX₂-like XPR6 processing endoprotease, which yields 32 kDa mature AEP, presumably occur in the late Golgi (Matoba et al., 1988; Matoba & Ogrydziak, 1989; Fabre et al., 1991; Enderlin & Ogrydziak, 1993; Lopez et al., 1994).
The role of the nine X-Ala, X-Pro dipeptides (dipeptide stretch) in AEP processing and secretion is not clear. The location of the dipeptide stretch in AEP (over 100 aa upstream of the N terminus of mature AEP) is unusual in that in other cases, such as honey bee prepromelittin and prepro-α-factor, the dipeptides are located directly upstream of the mature polypeptide and their removal results in activation of the polypeptide (Kreil, 1990). The lack of activity of the 52 kDa AEP precursor secreted by xpr6 mutants indicates that removal of the dipeptide stretch does not result in AEP activation (Matoba & Ogrydziak, 1989). We speculated that XPR6 processing, which does result in AEP activation, was inhibited until the dipeptide stretch was removed and that this provided a mechanism for preventing premature activation of AEP in the secretory pathway (Matoba & Ogrydziak, 1989).

The pro-region seems to serve several functions. Its removal is necessary for enzyme activation. The pro-domain may also function as an internal chaperone to promote AEP folding to secretion-compatible structures and as a template for the final folding involved in AEP activation. Three different deletions of the AEP pro-region (removal from the end of the dipeptide stretch to Asp128, removal of all the pro-region after the dipeptide stretch and removal of the region between Lys-Arg1 and the end of the pro-region) resulted in intracellular accumulation of AEP precursors of the predicted size but no AEP activity or AEP-related proteins were secreted (Fabre et al., 1991). These AEP precursors were translocated into the ER but did not undergo DPAPase or Xpr6p processing, suggesting they had not reached the late Golgi (Fabre et al., 1991). Supplying the AEP prepro-region in trans resulted in significant but slower secretion of partially active AEP from the deletion constructs (Fabre et al., 1992).

N-Linked glycosylation in the pro-region is needed for AEP secretion at higher temperatures (Fabre et al., 1991). After elimination of the N-linked glycosylation site at Asn299, AEP was secreted at 18 °C but not at 28 °C. A 53 kDa intracellular AEP precursor was detected at 28 °C but not at 18 °C, suggesting that misfolding was occurring at the higher temperature and that the misfolded protein was retained in the secretory pathway somewhere before the late Golgi.

Because of the high levels of secretion of AEP, the XPR2 promoter and parts of the prepro-region have been used to secrete foreign proteins in Y. lipolytica, including prochymosin (Franke et al., 1988), Saccharomyces cerevisiae invertase (Nicaud et al., 1989), porcine α1-interferon (Heslot et al., 1989), hepatitis B virus middle surface antigen (Hamsa & Chattoo, 1994) and bovine β-lactoglobulin (M. Huang & D. M. Ogrydziak, unpublished results). Interestingly, of six XPR2-human blood coagulation factor XIIIa fusion constructs containing increasing portions of the AEP prepro-region, only one containing the signal peptide and dipeptide stretch secreted human blood coagulation factor XIIIa (Tharaud et al., 1992). Improved understanding of the role of the dipeptide stretch will facilitate production of commercially desirable heterologous proteins by Y. lipolytica.

In this study we have examined several aspects of AEP processing and secretion. We determined the site of signal peptide cleavage and that AEP does not undergo autocatalytic processing during biosynthesis. We were particularly interested in determining the role of the dipeptide stretch and DPAPase processing in AEP maturation. These were investigated by altering the dipeptide stretch, using Pro-boroPro to inhibit DPAPase processing, by deleting the dipeptide stretch and by expressing AEP in S. cerevisiae strains with and without DPAPase activity. The two major processing events carried out by DPAPase and XPR6 seem to occur independently.

**METHODS**

**Materials.** The Random Primed DNA Labelling Kit and proteinase K were purchased from Boehringer Mannheim. L-[4,5-3H]Leu (4.44 x 10^11-7.03 x 10^13 Bq mmol^-1) and L-[35S]Met (2.96 x 10^12 Bq mmol^-1) were purchased from Amersham. T4 DNA polymerase and T4 polynucleotide kinase were from New England Biolabs. Restriction enzymes and T4 DNA ligase were purchased from all the above suppliers and from BRL and Stratagene. Oligonucleotides were purchased from Operon Technologies Inc. Galactose and Triton X-100 were from Sigma and bovine serum albumin, fraction V from USB. PMSF, casein (Hammersten), polypropylene glycol, DMSO, Triton X-100, EDTA, Protein A-Sepharose 4 Fast Flow, endoglucosidase H and 5-fluoroorotic acid (FOA) were obtained from the same sources as described previously (Yaver et al., 1992). AEP antiserum was prepared as described previously (Matoba et al., 1988).

**Media.** LB, 2XYT, H medium and glucose-minimal medium were used for growing Escherichia coli and in vitro mutagenesis (Sambrook et al., 1989; Bio-Rad Muta-Gene M13 In vitro
Table 1. Yeast strains used in this study

<table>
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<tr>
<th>Strain</th>
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<td>a his3Δ1 leu2 trp1-289 ura3-52</td>
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<td>614</td>
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<td>1308</td>
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<td>T. Stevens, University of Oregon</td>
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<tr>
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<td>DO613</td>
<td>A ade1 xpr6-13</td>
<td>Enderlin et al. (1993)</td>
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Mutagenesis Kit manual). Y. lipolytica was maintained on YM or YM Δ (Lee & Ogrydziak, 1977) and S. cerevisiae on YPD (Sherman et al., 1986). Y. lipolytica competent cells were prepared in YPDC medium. The selective medium for transformants was YLT [SM (Sherman et al., 1986) plus 0-087% drop our mixtures] lacking a specific amino acid or base. YPGA is YPD with 2% (w/v) galactose substituted for 2% (w/v) glucose. GXY, GPP & GC media were used for growing and labelling Y. lipolytica cells (Matoba et al., 1988, Yaver et al., 1992).

Strains. E. coli MV1190 and CJ236 were used for in vitro mutagenesis (Bio-Rad Muta-Gene M13 In vitro Mutagenesis Kit manual) and JM105 was used for DNA manipulations (Sambrook et al., 1989). Yeast strains used or constructed for this study are described in Table 1. SMY1 and SMY2 contain deletions of URA3 and of both URA3 and XPR2, respectively, in a wild-type (CX161-1B A ade1) background. A 0.4 kb EcoRV fragment from URA3 in pIMR41 was deleted to construct pIMR105 (ura3-ΔE flanked by SalI sites in pUC13). The NcoI site in ura3-ΔE in pIMR105 was destroyed by addition of BamHI linkers to form pIMR116. Filling in the BamHI site of pIMR107 containing the BamHI/SalI 4.3 kb ADE1 fragment in pBR322 yielded pIMR115. The 1.3 kb SalI fragment from pIMR116 containing the modified ura3-ΔE was integrated into the SalI site at the end of the ADE1 gene in pIMR115 to yield pIMR112. Plasmids containing the mutations were cut with MluI to target upstream of xpr2-ΔA in SMY2. Ura' transformants were plated on 5-FOA medium to select for Ura' loop-outs. Replacement of xpr2-ΔA by the mutagenized version of XPR2 was confirmed by Southern blotting.

Protease assays. Skim milk (SKM) plates for detection of production of AEP by Y. lipolytica were prepared as described previously (Ogrydziak & Mortimer, 1977; Enderlin & Ogrydziak, 1993). SKM plates for detection of production of AEP by S. cerevisiae were prepared in a similar manner except for the addition of 1% (w/v) glucose. Extra-cellular protease activity secreted by S. cerevisiae strains was measured using the A260 casein hydrolysis method (Ogrydziak & Scharf, 1982).

Pulse-chase immunoprecipitation. Pulse-chase labelling with [35S]Leu, disruption of cells, immunoprecipitation of cell extracts with AEP antibody, SDS-PAGE of immunoprecipitates on 10-15% (w/v) gradient gels and fluorography was based on previously described procedures (Matoba et al., 1988).

S. cerevisiae strains 614 and 1308 were grown to mid-exponential phase in synthetic minimal medium (0.67% yeast nitrogen base, 30 mg adenine l-1, 20 mg His l-1, 5 mg Trp l-1 and 20 mg Lys l-1) with 4% galactose. Cells were harvested by centrifugation (5000 g for 5 min) and resuspended in 0.25 ml of the same medium. A 2-5 min pulse with 1:02 x 107 ExpreS38 kDa label (NEN) was followed by the addition of unlabelled Met and Cys to final concentrations of 50 mM and 25 mM, respectively, and addition of 0.2 ml fresh medium with yeast extract to 0.2%. At various times after initiation of chase, 0.1 ml samples were removed and cells quickly separated from the medium by a pulse spin in a...
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Endoglycosidase H digestion. Immunoprecipitated samples were digested with endoglycosidase H as described previously by Yaver et al. (1992).

Radiosequencing. Radiolabelled cell extracts were prepared as described for pulse–chase immunoprecipitation with the following modifications: 40 ml cells were labelled with 9.25 x 10^6 Bq [3H]Leu and 3.7 x 10^7 Bq [35S]Met. Six millilitre samples were taken at 2 and 4 min and cell extracts immunoprecipitated. The immunoprecipitates were combined and run on a 10–15% (w/v) SDS-PAGE gel. Quantification was done with phosphorimager analysis using the Fuji FUJIX BAS1000 Bioimaging Analyzer (Fuji Medical Systems).

Results and Discussion

Signal peptide cleavage site

AEP is cleaved at several sites within the N-terminal prepropeptide, but the location and function of some of these processing events has not been determined. Most likely the largest AEP precursor (55 kDa) detected in cell extracts in pulse–chase immunoprecipitation experiments lacks the signal peptide (Matoba et al., 1988; Fabre et al., 1991). The predicted site of cleavage is after Ala15 (S value is 7.0) (von Heijne, 1986) and this has been shown to be the cleavage site in the P17M version of XPR2 (Yaver et al., 1992). To determine if this was also the cleavage site for wild-type AEP, N-terminal amino acid radiosequencing of immunoprecipitated [3H]Leu- and [35S]Met-labelled 55 kDa AEP precursor was done. Leu is predicted at positions 3 and 10 and Met at position 1 for an AEP precursor still containing the signal peptide, and only Leu at position 3 and no Met for a precursor cleaved after Ala15. The highest [3H] counts were in cycle 3 with only background [3H] counts in cycle 10 (Fig. 2) and [35S] counts in all cycles including cycle 1 were at background (not shown), suggesting that the wild-type 55 kDa AEP precursor lacks the signal peptide.

Role of AEP activity in AEP processing

Based on sequence homology, AEP is a subtilisin-like protease with Ser99 as part of the active site triad (Davidow et al., 1987). Two putative intracellular precursors of 44 and 36 kDa have been seen in many pulse–chase immunoprecipitation experiments (Matoba et al., 1988). The 55 kDa AEP precursor lacks the signal peptide and no Met for a precursor cleaved after Ala15. The highest [3H] counts were in cycle 3 with only background [3H] counts in cycle 10 (Fig. 2) and [35S] counts in all cycles including cycle 1 were at background (not shown), suggesting that the wild-type 55 kDa AEP precursor lacks the signal peptide.
et al., 1988). The 44 kDa polypeptide contains about 2 kDa of N-linked carbohydrate and is of the appropriate size to result from cleavage after LyS188-Arg190 (Matoba et al., 1988). The 36 kDa polypeptide contains no N-linked carbohydrate and there is no potential dibasic cleavage site appropriately located downstream of the glycosylation site at Asn123 (Matoba et al., 1988). The possibility that AEP activity was needed for AEP processing and was involved in producing one or more of these precursors was investigated by changing Ser397 to Ala (S397A). As for SMY2, a strain containing a deletion of the XPR2 gene which encodes AEP, SMS397A, produced no zone of clearing on a SKM plate (Fig. 3). Processing of AEP in pulse–chase immunoprecipitation experiments was similar to wild-type except that less mature AEP was secreted and little or none of the 44 and 36 kDa precursors was present (Fig. 4a). The production of mature AEP implies that AEP activity was not necessary for processing of AEP precursors. The absence of the 44 and 36 kDa precursors suggests that they may be degradation products produced by AEP activity during sample preparation and not in vivo AEP processing intermediates.

The fact that no labelled AEP precursor accumulated in the extracellular medium when PMSE, which inhibits AEP activity, was included during labelling of wild-type cells is also consistent with AEP activity not being needed for AEP processing (Matoba et al., 1988). It also suggests that AEP activity is not necessary for extracellular processing of AEP, i.e. no AEP precursor is secreted which is quickly proteolytically processed by AEP activity.

Role of -X-Ala-, -X-Pro- dipeptide stretch in AEP processing

For DPAPase to remove a dipeptide, it requires a free N-terminus and either Ala or Pro as the second amino acid (Kreil, 1990). The P17M and A19V mutations were constructed either to completely prevent DPAPase processing (P17M) or to allow removal of only the Ala16-Pro17 dipeptide (A19V) (Fig. 1b). If removal of the dipeptide stretch is needed for KEX2-like processing to occur, then it would be predicted that an AEP precursor(s) of molecular mass identical (P17M) or very similar (A19V) to the wild-type 55 kDa AEP precursor would be secreted. For P17M, the unexpected result of post-translational translocation was observed (Yaver et al., 1992). Also production of AEP activity was reduced compared to wild-type (Fig. 3). For strain S Mint19V, production of AEP activity (SKM zones) (Fig. 3) and AEP processing and secretion kinetics (Fig. 4b) were very much like wild-type (Matoba et al., 1988; see Figs 1 and 5). The A19V 55 kDa AEP precursor had mobility on SDS-PAGE and N-linked carbohydrate content indistinguishable from the wild-type 55 kDa AEP precursor (data not shown). As expected, the 52 kDa precursor, a product of DPAPase processing, was not detected.

The role of the dipeptide stretch was also investigated by deleting the region encoding the dipeptides. In SMADP the nine dipeptides starting with Ala16 were deleted and Glu-Val inserted (Fig. 1b). Based on S values (von Heijne, 1986), signal peptide cleavage is much more likely to occur after Ala16 (as in wild-type) if Ala16 is followed by Glu-Val than if it was followed by Glu-Gly as would occur without the insertion. If the dipeptide stretch functions to delay AEP activation by the XPR6 processing endoprotease, then AEP might be activated prematurely in SMADP and the cells might be sick or unviable. In pulse–chase immunoprecipitation experiments with SMADP, only one AEP precursor (ca 51 kDa) was detected intracellularly (Fig. 4c). No mature AEP or polypeptides in the 50–55 kDa range were detected extracellularly. The material at 65 kDa is most likely also secreted by other strains but not detected because the fluorographs were only exposed for 3 d as opposed to 15 d for SMADP. There was very weak clearing on the skimmed milk plate (Fig. 3), indicating that a very low level of AEP activity was secreted. These results suggest that most of the ADP precursor had never reached the late Golgi where XPR6 processing presumably occurs. We suspected that it was misfolded and retained in the ER. Surprisingly, the mobility of the ADP AEP precursor did not change after treatment with endoglucosidase H, indicating that, unlike other translocated AEP precursors, it did not contain N-linked carbohydrate (Fig. 5a). Folding and glycosylation reactions can compete in vivo and, in some cases, slowing folding can increase the level of glycosylation (Holst et al., 1996). In this case, we suspect altered folding makes the normal N-linked glycosylation site (Asn123 in the wild-type pro-region) unavailable.
Fig. 4. (a) Mature AEP is secreted by SMS397A. (b) SMA19V behaves much like wild-type, except that no 52 kDa AEP precursor was detected. (c) For SMΔDP only a 51 kDa AEP precursor is detected which is not processed further, and little or no mature AEP is secreted. Pulse-chase immunoprecipitation was done by labelling each of the strains with [3H]Leu for 45 s. Cell extracts were immunoprecipitated and supernatant samples TCA-precipitated. The precipitates were analysed by SDS-PAGE and fluorography. The position of mature AEP (mAEP) and estimated molecular masses of AEP precursors and pro-region, and of some secreted proteins are indicated. The time after chase (min) is shown above each lane.

The deletion of 16 aa could account for an approximately 2 kDa decrease in size. Because both a 55 kDa translocated AEP precursor lacking both the 16 aa and the 2 kDa of N-linked carbohydrate and a 53 kDa untranslocated (and therefore unglycosylated) AEP precursor lacking the 16 aa would be expected to have mobilities similar to that of the ΔDP AEP precursor, a protease protection experiment was done to determine if the SMΔDP AEP precursor was translocated. The SMΔDP AEP precursor was not degraded by proteinase K in the absence of Triton X-100 (Fig. 5b, lane 2) but it was in its presence (Fig. 5b, lane 3), suggesting that the precursor had been translocated into a membrane-enclosed compartment. These results suggest that the dipeptide stretch is necessary for folding into a form competent for translocation out of the ER.
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Fig. 5. (a) The SMΔDP 51 kDa AEP precursor does not contain N-linked carbohydrate. 3H-Labelled immunoprecipitated intracellular samples from wild-type (lanes 1–4) and SMΔDP (lanes 5–8) were taken either 3 min (lanes 1, 2, 5 and 6) or 5 min (lanes 3, 4, 7 and 8) after addition of the chase. Samples in lanes 2, 4, 6 and 8 were incubated with endoglycosidase H. (b) The SMΔDP 51 kDa AEP precursor is enclosed in a membrane-bound compartment. Spheroplasts were prepared, lysed and either subjected to a mock protease treatment (lane 1) or incubated with proteinase K in the absence (lane 2) or presence (lane 3) of Triton X-100. Lane 4, Triton X-100 with no proteinase K. After Western blotting, the SMΔDP 51 kDa AEP precursor was visualized using antiserum to AEP and chemiluminescence.

Fig. 6. AEP is processed and mature AEP secreted in the presence of Pro-boroPro. Cells were labelled with [3H]Leu either in the presence (lanes 1, 2, 5, 6, 9 and 10) or absence (lanes 3, 4, 7, 8, 11 and 12) of Pro-boroPro. Supernatant samples were collected 10 min (lanes 1, 3, 5, 7, 9 and 11) and 20 min (lanes 2, 4, 6, 8, 10 and 12) after the start of labelling and TCA-precipitated. Twenty microlitres of resuspended sample (equivalent to 60 µl extracellular medium) were applied per lane. Lanes: 1–4, wild-type (CX161-1B) samples; 5–8, DO613; 9–12, P17M. The positions of the 55 and 52 kDa AEP precursors, mature AEP (mAEP) and the 20 kDa AEP pro-region are indicated. The filled arrowhead and circle indicate putative AEP propeptide fragments which possibly differ in the number of dipeptides retained.

Role of DPAPase activity in AEP processing

Results with strain A19V suggest that removal of the dipeptide stretch is not needed for AEP processing by Xpr6p. To eliminate the possibility that the A19V mutation caused an altered conformation of AEP, we examined the susceptibility to Xpr6p processing of wild-type protein using the DPAPase inhibitor Pro-boroPro (Flentke et al., 1991). If removal of the dipeptide stretch is required for XPR6 processing, then inhibition of

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before labelling to stabilize the secreted AEP propeptide (Matoba & Ogrydziak, 1989).

Although it is not known which intracellular compartments of the secretory pathway are accessible to this inhibitor, results with strain DO613 indicate that Pro-boroPro did inhibit DPAPase activity in vivo (Fig. 6, lanes 5–8). xpr6-13 is a leaky mutation in the gene encoding XPR6, the KEX2-like processing endoprotease responsible for the cleavage after Lys-Arg at the end of the AEP propeptide (Enderlin & Ogrydziak, 1993). Strain DO613 secretes a 52 kDa AEP precursor which has undergone DPAPase processing (Matoba & Ogrydziak, 1989). As expected if the dipeptide stretch was not removed in the presence of Pro-boroPro, the larger 55 kDa AEP precursor was secreted (Fig. 5, lanes 5–6).

Even when DPAPase activity was inhibited by Pro-boroPro, there was no evidence for secretion of a 55 kDa AEP precursor by the wild-type, providing additional evidence that removal of the dipeptide stretch was not necessary for KEX2-like processing of AEP (Fig. 6, lanes 1–2).

**Secrected AEP propeptide**

Several results are consistent with the 19–20 kDa doublet secreted by wild-type being the AEP propeptide: (i) its secretion kinetics were similar to AEP; (ii) it is near the expected size; (iii) like the 55 and 52 kDa AEP precursors, it contains about 2 kDa of N-linked carbohydrate; and (iv) it lacks Trp, Met and Cys as predicted for the AEP propeptide (Matoba et al., 1988). Originally, we proposed that the 19–20 kDa doublet contained the intact propeptide with the 20 kDa form retaining some dipeptides and the 19 kDa form devoid of dipeptides (Matoba et al., 1988). Results obtained for SMA19V and P17M and for all strains tested with Pro-boroPro are most consistent with both the 19 and 20 kDa bands being derived from the AEP propeptide. One interpretation is that since the mobility of the major secreted form of the AEP propeptide is unaffected in P17M and by treatment with Pro-boroPro, in all cases it contains the dipeptide stretch. For DO613, Pro-boroPro inhibition resulted in accumulation of a precursor larger than the 52 kDa precursor which lacks most of the dipeptides (Matoba & Ogrydziak, 1989). If this mobility difference reflects the extent of removal of the dipeptide stretch, then the major secreted propeptide synthesized with the 20 kDa form retaining some dipeptides and the 19 kDa form devoid of dipeptides (Matoba et al., 1988). Results obtained for SMA19V and P17M and for all strains tested with Pro-boroPro are most consistent with both the 19 and 20 kDa bands being derived from the AEP propeptide. One interpretation is that since the mobility of the major secreted form of the AEP propeptide is unaffected in P17M and by treatment with Pro-boroPro, in all cases it contains the dipeptide stretch. For DO613, Pro-boroPro inhibition resulted in accumulation of a precursor larger than the 52 kDa precursor which lacks most of the dipeptides (Matoba & Ogrydziak, 1989). If this mobility difference reflects the extent of removal of the dipeptide stretch, then the major secreted propeptide synthesized in the presence of Pro-boroPro (Fig. 6, lanes 5–6) should be larger than in its absence (Fig. 6, lanes 7–8). In fact, they have similar mobilities and this is consistent with the possibility that the 20 kDa bands contain little or none of the dipeptide stretch. In the 13–17 kDa region a larger band is found in the Pro-boroPro-treated sample (Fig. 6, lanes 5–6) compared to the untreated sample (Fig. 6, lanes 7–8), suggesting that these bands differ in the number of -X-Ala-, -X-Pro- dipeptides they contain. Since these bands were precipitated by TCA and not by antibodies to the AEP pro-region, however, it is not certain that they are AEP-related. The first model in which the 20 kDa propeptides contain the dipeptide

DPAPase activity should result in secretion of a 55 kDa AEP precursor. For three strains Pro-boroPro was added before labelling in a pulse-chase immunoprecipitation experiment and extracellular samples TCA-precipitated 10 and 20 min after the chase. PMSF was also added...
The immunoprecipitation experiments suggested that the levels of AEP secreted by the S. cerevisiae strains were comparable. This was confirmed by assaying AEP activity of the supernatant medium from 614(pIMR88) and 1308(pIMR88) grown in synthetic minimal medium containing 0.1% bovine serum albumin and either 2% glucose or 4% galactose. As expected no AEP activity was detected during growth on glucose. With galactose the growth rates were similar and AEP activity was almost identical $-5.1 \pm 0.4$ U (mg dry wt cells)$^{-1}$ for 614(pIMR88) and $5.3 \pm 0.2$ U (mg dry wt cells)$^{-1}$ for 1308(pIMR88) (means $\pm$ SEM, $n = 4$). In GPP medium Y. lipolytica produces 135 U (mg dry wt cells)$^{-1}$ (Ogrydziak & Scharf, 1982). Thus, much less AEP was secreted by S. cerevisiae strains with multiple copies of XPR2 than by a Y. lipolytica strain with a single copy. These results, however, confirm that DPAPase processing is not required for maturation and secretion of the enzyme.

To examine the role of DPAPase activity in AEP processing, the multicopy $2\mu$ plasmids pIMR88 (wild-type XPR2) and pIMR89 (xpr2 with the S397A mutation) were transformed into S. cerevisiae strain 1308 containing disrupted copies of both DPP1 (STE13, encoding the Golgi DPAP A) and DAP2 (encoding vacuolar DPAP B), and the related strain 614, wild-type for both DPAPase activities. No zones of clearing were detected for any of the transformants grown on G1a-SKM plates. On Gal-SKM plates, clearing was detected for both 614 and 1308 with pIMR88 but not with pIMR89, suggesting that, at least in S. cerevisiae, DPAPase processing is not required for production of AEP activity (data not shown). Clearing was only detected around areas of heavy growth and not around single colonies, and zones of clearing were significantly larger for cultures incubated at 23 °C than at 30 °C.

To determine if a kinetic defect in AEP secretion was occurring in the DPAP mutant strain, radiolabelled pulse–chase immunoprecipitations were performed. Cells were labelled for 2.5 min, the label chased and samples of the extracellular medium taken at various times and immunoprecipitated with AEP antibody. The major band detected migrates with the same mobility on SDS-PAGE as mature AEP produced by Y. lipolytica (Fig. 7a). There was no delay in appearance of mature AEP in 1308(pIMR88) versus 614(pIMR88) (Fig. 7b) and no evidence for secretion of AEP precursors in either strain (Fig. 7a). Therefore, it seems that a lack of DPAPase activity does not affect transit time or delay KEX2 processing of AEP. Preliminary results of immunoprecipitation of labelled cell extracts reveal that, unlike the case for Y. lipolytica, in S. cerevisiae AEP precursor(s) of about 35 kDa are still cell-associated 15–30 min after the start of the chase (data not shown). If AEP precursors were trapped in the cell wall, some leakage into the extracellular medium might be expected. This was not found, suggesting that the AEP precursor(s) are intracellular and that their movement through the secretory pathway is slowed in S. cerevisiae whether or not DPAPase activity is present.

The immunoprecipitation experiments suggested that

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