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Biogenesis of the yeast vacuole (lysosome)

Active site mutation in the vacuolar aspartate proteinase yscA blocks maturation of vacuolar proteinases

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The activation process of vacuolar (lysosomal) proteinases in the yeast *Saccharomyces cerevisiae* is initiated by the *PRA1* (*PEP4*) gene product, proteinase yscA. To elucidate the activation process of proteinase yscA the catalytically active amino acid Asp^{294} of the enzyme was exchanged with Ala^{294} using site directed mutagenesis. The resulting proteinase yscA-Ala^{294} showed no proteolytic activity against the substrate hemoglobin. The mutant protein did not undergo processing in vivo. This phenomenon is in good agreement with the hypothesis that maturation of proteinase yscA is due to self-processing of pro-proteinase yscA. Furthermore, proteinase yscA-Ala^{294} is not able to convert the zymogens pro-proteinase yscB and pro-carboxypeptidase yscY to the mature enzymes.

Proteolysis; Aspartate proteinase; Active site mutant; Yeast; *Saccharomyces cerevisiae*

1. INTRODUCTION

The yeast vacuole, the lysosome-like organelle of this organism, contains a multitude of proteolytic enzymes. A functional vacuolar proteolytic system in *Saccharomyces cerevisiae* is essential for cell survival under nutrient deprivation and the differentation process of sporulation [1]. The vacuolar proteinases yscA (PrA) and yscB (PrB) not only have an important function in protein degradation but also play a crucial role in specific proteolytic processing and enzyme precursor activation events. The soluble PrA, PrB and carboxypeptidase yscY (CpY) are synthesized as precursors of higher molecular weight. Maturation occurs in the vacuole or a post-Golgi pre-vacuolar compartment by proteolytic cleavage of the respective pro-peptide segments (for reviews see [2–5]).

PrA occupies a key position in these activation events. A mutation (*pep4-3*) in the structural gene of PrA has been described which leads to the accumulation of pro-PrB and pro-CpY in the vacuole [6]. PrA is homologous to other aspartyl proteinases. Mature PrA shows 40% amino acid identity with human pepsin and 46% identity with the lysosomal human cathepsin D. PrA is synthesized as a pre-pro-enzyme of 44 kDa [7,8]. The signal sequence is cleaved off after transfer into the ER by signal peptidase between amino acids 22 and 23 [9] as predicted by the rules of von Heinje [10]. During transit through the ER two *N*-linked core oligosaccharides are added to pro-PrA, which are trimmed and extended in the Golgi complex resulting in a molecular weight of 52 kDa for the pro-enzyme [11]. During or upon delivery to the vacuole the pro-peptide is cleaved off. Pro-PrA is processed in two steps: in a first step the 52 kDa precursor is transferred into a 43 kDa form which is further processed to the mature 42 kDa protein. The first processing step is thought to be autocatalytic in nature, whereas the second step is dependent on PrB activity (H.H. Hirsch, H. Schiffer and H. Müller, in preparation). Amino acid 77 of pre-pro-PrA constitutes the amino terminus of the mature enzyme [12]. The pro-peptide fragment of PrA is able to direct the enzyme invertase into the vacuole and thus contains vacuolar targeting information [9].

In an analogy to pepsin an autocatalytic activation caused by an acidic environment had been suggested for PrA [7,8]. Recent experiments, however, using yeast strains completely lacking a subunit of the vacuolar ATPase (*VAT2* or *VMA3* gene product), resulted in a neutral vacuolar environment and showed the mature active forms of PrA and CpY [13,14]. To elucidate the activation process of pro-PrA we introduced a mutation

Abbreviations: ER, endoplasmatic reticulum; CpY, carboxypeptidase yscY; PrA, proteinase yscA; PrB, proteinase yscB.

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into the enzyme at amino acid 294 (*pra1*-Ala^{294}) which is predicted to be one of the catalytically active amino acid residues. The outcome of this mutation on PrA maturation and on the maturation events of other vacuolar enzymes was studied.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All enzymes used in DNA manipulation were obtained from Boehringer-Mannheim (Germany), Bethesda Research Laboratories (Basel, Switzerland) or Pharmacia (Freiburg, Germany). Dideoxysequencing [15] was carried out according to the instructions of the T7 polymerase sequencing kit (Pharmacia, Freiburg, Germany). Purified PrA was purchased from Sigma (Deisenhofen, Germany). Pepstatin, chymostatin and zymolyase 100 000 were obtained from the Peptide Institute (Osaka, Japan). [^{35}S]methionine and [α-^{35}S]-labeled dATP were obtained from Amersham (Braunschweig, Germany). All other chemicals were obtained from Roth (Karlsruhe, Germany), Pharmacia (Freiburg, Germany) or Merck (Darmstadt, Germany). The *PRA1* gene was provided by Dr A. Hinnen (Basel, Switzerland).

2.2. *Media*

E. coli strains were grown in LB Medium [16] with or without ampicillin (50 μg/ml). The following yeast growth media were used: YPD medium (2% glucose, 1% yeast extract and 2% peptone) and MV medium (2% glucose, 0.67% yeast nitrogen base without amino acids and supplements required by auxotrophic strains). [^{35}S]methionine labeling medium was used as described in [11].

2.3. E. coli *and yeast strains*

E. coli strains: JM 109 {*reA1, endoA1, gyrA96, thi$^-$, hsdR17, supE44, relA1* (*lac, pro*) *F'traD36, proAB, lacI^q, lacZ, ΔM15*} [17], RZ 1032 (*dut$^-$, ung$^-$*) and BMH 71-18 {d(lac-proAB) thisup-E[F'proAB(lacI^{q}ZdM15)]} were used [18]. Yeast strains: YS18 (*MATα, his3-11,15, leu2-3,112, ura3Δ5*, can^R) [19], YHH65 (*MATα, pra1ΔEN::HIS3, his3-11,15, leu2-3,112, ura3Δ5*, can^R) (H.H. Hirsch, unpublished), SR1 (*MATα, pra1ΔEN::HIS3, his3-11,15, leu2-3,112, ura3Δ5, can^R, pDP83pra1-Ala^{294}*) *and SR2 (MATα, pra1ΔEN::HIS3, his3-11,15, leu2-3,112, ura3Δ5*, can^R *pDP83PRA1*).

2.4. *Molecular biological techniques and plasmids*

Purification, restriction, ligation, analysis of DNA and transformation of *E. coli* and *S. cerevisiae* were carried out according to standard procedures [16,20]. Introduction of the 1.9 kbp *SacI/XhoI PRA1* and *pra1-Ala^{294}* alleles into yeast was performed in the vector pDP83 (*CEN14, ARS1, URA3*, amp^R, lacZ) (D. Pridmore, unpublished). Bluescript KS$^+$ M13 (Stratagene) was used for mutagenesis and dideoxysequencing. For in vitro transcription the *PRA1* and *pra1-Ala^{294}* genes were subcloned as 1.5 kbp *EcoRI/XhoI* fragments into the vector pGEM2 (Promega Biotec).

2.5. *Site directed mutagenesis and DNA-sequencing*

Site directed mutagenesis replacing amino acid Asp at position 294 of the PrA sequence with Ala was carried out as described by Kunkel [18] using the mutagenic 18-mer 5'-GCCATCGCGACTGGTACT-3'. The plasmid Bluescript M13 KS$^+$, containing a 642 bp *EcoRV/XhoI* fragment of the *PRA1* gene in the respective polylinker restriction sites, was used for site directed mutagenesis. Single stranded DNA was prepared in *E. coli* RZ 1032 using the helper virus R408 (Stratagene, San Diego, USA) [21]. The mutated *pra1-Ala^{294}* gene fragment was subcloned into the *PRA1* wild-type gene and the nucleotide sequence of both strands over the entire open reading frame of the resulting *pra1-Ala^{294}* gene was confirmed by the dideoxy-chain terminating method [15] using T7 polymerase and [α-^{35}S]-labeled dATP.

2.6. *In vitro transcription-translation*

For in vitro transcription of the *PRA1* and *pra1-Ala^{294}* genes *pGEM2PRA1* and *pGEM2pra1-Ala^{294}* were linearized downstream of the open reading frame by digestion with *PstI*. SP6 polymerase directed transcriptions, wheat germ extract mediated translation and SDS-PAGE of the radiolabeled proteins was performed as described in [22].

2.7. *Pulse-chase experiments*

Labeling of cells, preparation of cell extracts, immunoprecipitation of proteins, SDS-PAGE and fluorography were done as outlined by Mechler et al. [11,23]. Previously characterized antisera against PrA, PrB and CpY have been used [11,23].

2.8. *Enzyme assays*

PrA was tested against acid hemoglobin as substrate as described by Saheki and Holzer [24]. α-Mannosidase was tested with *p*-nitrophenyl-α-D-mannopyranoside as described by Van der Wilden et al. [25]. NADPH cytochrome *c* oxidase was assayed as described by Kubota [26] and glucose-6-phosphate dehydrogenase was measured as described by Deutsch [27]. The amount of protein was determined by the method of Lowry et al. [28].

2.9. *Isolation of vacuoles*

Cells were grown for 18 h at 30°C in YPD medium. Spheroplast formation, lysis of spheroplasts with DEAE-dextran and purification were performed as described by Wiemken et al. [29] with the modifications outlined by Mechler et al. [30]. SDS-PAGE and electro-immunoblotting were done as described in [30]. The enrichment of a marker enzyme of the vacuolar membrane, α-mannosidase could be determined to be 7-fold for strain SR1 and 15-fold for strain YHH65 while the activity of NADPH cytochrome *c* oxidase, an ER-marker enzyme, was reduced 4-fold for both vacuolar preparations. Glucose-6-phosphate dehydrogenase activity, a cytosolic marker enzyme, was not detectable in either of the vacuolar fractions.

3. RESULTS

3.1. *Introduction of the mutation* pra1-Ala^{294} *into the* PRA1 *gene*

Among aspartate proteinases the amino acid sequences containing the catalytic aspartate residues are conserved. The two putative catalytic aspartates of proteinase yscA are located at position 109 and 294 of the peptide sequence. Using site directed mutagenesis Asp^{294} was changed to Ala^{294} [31]. Sequencing data of both strands of the entire open reading frame confirmed the exchange of Asp^{294} to Ala^{294} as described in section 2.5. (not shown).

3.2. *The mutation* pra1-Ala^{294} *prevents maturation of pro-PrA-Ala^{294}*

Analysis of cellular and vacuolar extracts of strain SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala^{294}*) showed no proteolytic activity against hemoglobin, a routinely used PrA substrate.

To follow processing of the PrA-Ala^{294} mutant protein, cells of strain SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala^{294}*), and for control, cells of strains YS18 (*PRA1*) and YHH65 (*pra1ΔEN::HIS3*) were labeled with [^{35}S]methionine for 30 min and chased for 2 h with non-radioactive methionine [11,23]. In contrast to wild-type YS18 (Fig. 1, lanes 1 and 2) no processing of the mutant pro-PrA-Ala^{294} to the mature enzyme was observed (Fig. 1, lanes 3 and 4). In addition, unprocessed pro-PrA-Ala^{294} migrated as a somewhat smaller molec-

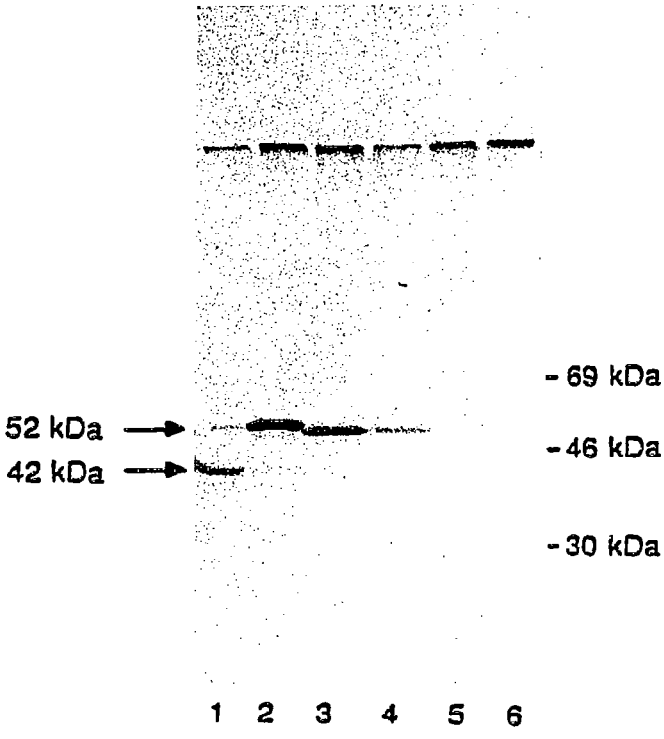

Fig. 1. Immunoprecipitation of labeled PrA-antigen in cell extracts of wild-type strain YS18 (*PRA1*), mutant strain YHH65 (*pra1ΔEN::HIS3*) and SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala*^{294}). Cells were labeled at 30°C for 30 min with [^{35}S]methionine. Extract mixtures containing 90 μCi of radioactivity were applied on each lane. Chase time was 120 min. The chase solution contained 60 μg cycloheximide/incubation mix. Immunoprecipitation was done using anti-PrA-serum; SDS-PAGE (10% gel): (Lane 1) YS18, 120 min chase; (lane 2) YS18, pulse; (lane 3) SR1, pulse; (lane 4) SR1, 120 min chase; (lane 5) YHH65, pulse; (lane 6) YHH65, 120 min chase.

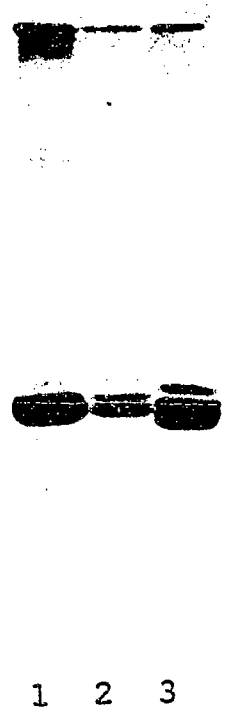

Fig. 2. Glycosylation pattern of PrA-Ala^{294}. The yeast strains YS18 (*PRA1*), SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala*^{294}) and SR2 (*pra1ΔEN::HIS3, pDP83PRA1*) were preincubated for 20 min at 30°C with 60 μg/ml tunicamycin prior to labeling with [^{35}S]methionine for 30 min. Extract mixtures containing 100 μCi of radioactivity were applied on each lane. Immunoprecipitation was done using anti-PrA-serum; SDS-PAGE (12% gel): (Lane 1) SR2; (lane 2) YS18; (lane 3) SR1.

ular weight species (51 kDa) than did the wild-type PrA precursor (52 kDa). Reasons for this shift in molecular weight might be: (i) altered trimming of the polysaccharide chains of the mutated protein; (ii) an altered primary sequence due to limited proteolysis of pro-PrA-Ala^{294}; or (iii) an altered mobility of the mutated pro-PrA-Ala^{294} protein in the SDS-PAGE due to the amino acid exchange.

To evaluate the nature of this shift in molecular weight in more detail, we did the following experiments:

(i) The molecular weight of the unglycosylated mutant PrA and its glycosylation pattern was determined. Radioactive labeling of cellular protein was done after incubation of mutant strain SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala*^{294}) with tunicamycin, an inhibitor of *N*-acetylglucosaminyl-pyrophosphoryldolichol formation. As control wild-type YS18 (*PRA1*) and plasmid encoded wild-type SR2 (*pra1ΔEN::HIS3, pDP83PRA1*) cells were treated in the same way. Since PrA contains two *N*-glycosylation sites, the occurrence of three different molecular species of PrA is expected if tunicamycin-repressed *N*-glycosylation is not complete [11] as can be seen in Fig. 2. PrA-Ala^{294} of tunicamycin-treated cells (Fig. 2, lane 3) showed the same glycosylation pattern as wild-type pro-PrA (Fig. 2, lane 2) but for each PrA-Ala^{294} species a smaller apparent molecular weight of about 1 kDa could be observed. Evidently a difference in molecular weight even between the completely unglycosylated wild-type and mutant species does exist. These observations show that altered trimming of polysaccharide chains cannot account for the difference in molecular weight.

To exclude (ii) we compared in vitro synthesized wild-type PrA and mutant PrA-Ala^{294} proteins. Synthesis of both proteins was achieved by transcription-translation of the wild-type and mutant genes, *PRA1* and *pra1-Ala*^{294}, cloned into the vector pGEM2. The mRNA of both genes was prepared by in vitro transcription with SP6 polymerase. Translation of the mRNA was done in the presence of [^{35}S]methionine in a wheat germ system (kindly provided by H.O. Krebs, [22]). Addition of the capped mRNAs to the translation reaction mixture resulted in a 44 kDa protein for the *PRA1* wild-type gene (Fig. 3, lane 1), as expected for the unglycosylated full-length polypeptide chain [7,8], and a 43 kDa protein for the *pra1-Ala*^{294} mutant gene (Fig. 3, lane 2). The molecular weights of wild-type PrA and mutant PrA-Ala^{294} show an apparent difference of about 1 kDa. (Several

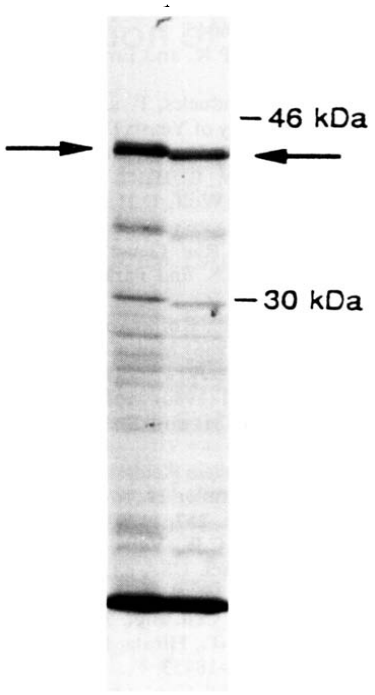

Fig. 3. In vitro translation of wild-type PrA and PrA-Ala^{294}. For analysis of the [^{35}S]methionine in vitro translation products of *PRA1* and *pra1-Ala^{294}* mRNA, 2 μl of mRNA were added to each translation mixture; SDS-PAGE (10% gel): (Lane 1) *PRA1*; (lane 2) *pra1-Ala^{294}*.

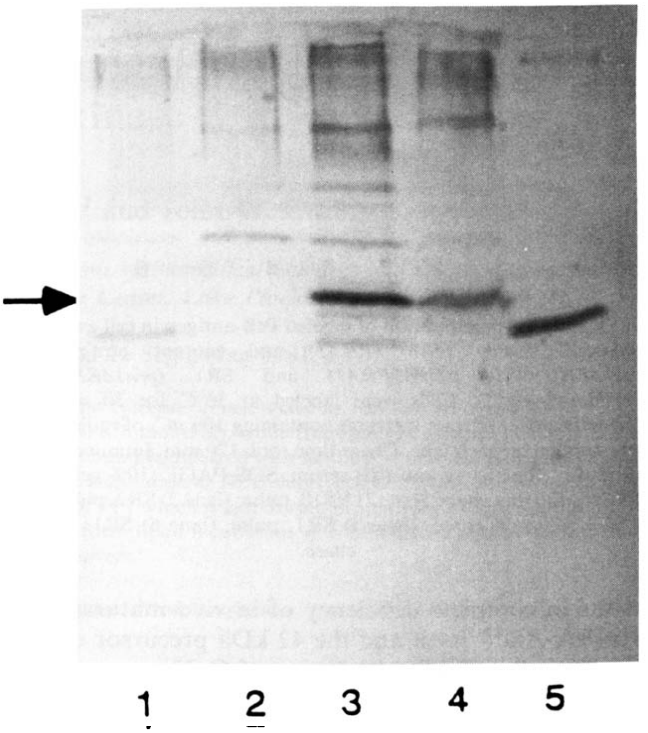

Fig. 4. Immunoblotting of PrA antigenic material in spheroplasts and vacuoles of strain YHH65 (*pra1ΔEN::HIS3*) and strain SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala^{294}*). Each lane contained 50 μg total protein. Arrow marks PrA-Ala^{294} mutant protein; SDS-PAGE (10% gel): (Lane 1) YHH65, spheroplasts; (lane 2) YHH65, vacuoles; (lane 3) SR1, vacuoles; (lane 4) SR1, spheroplasts; (lane 5) YS18, crude extract.

proteins of smaller molecular mass were also synthesized in a *pra1-Ala^{294}* or *PRA1* mRNA dependent manner. They could be the result of non-standard usage of internal AUG codons at start of protein synthesis and/or premature termination [32]. Thus the apparent shift in molecular weight of the mutant pro-PrA-Ala^{294} cannot be due to an altered length in the peptide chain of the protein.

These experiments leave us only with possibility (iii), the altered mobility of mutant PrA-Ala^{294} protein due to the exchange of the acidic amino acid aspartate against the neutral/hydrophobic amino acid alanine. Similar effects have been reported by Rosinski-Chupin et al. [33].

3.3. *The mutant protein PrA-Ala^{294} is delivered to the vacuole*

The fact that PrA-Ala^{294} is sensitive to the *N*-glycosylation inhibitor tunicamycin (Fig. 2) indicates that the mutant protein travels through the secretory pathway [2]. Since neither the ER-directing pre-sequence nor the vacuole directing pro-sequence [9] of pre-pro-PrA have been changed by mutagenesis we expected that the PrA-Ala^{294} mutant protein is delivered to the vacuole. This was indeed the case: the isolation of the vacuolar fraction from mutant strain SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala^{294}*) showed enrichment of PrA antigenic material (Fig. 4, lane 3).

3.4. *The* pra1-Ala^{294} *mutation blocks maturation of pro-PrB and pro-CpY*

Mature PrA has been shown to be the trigger for maturation of other vacuolar hydrolases [6–8]. PrB and CpY are among the best examples studied [30,34–36].

As can be seen in Fig. 5 presenting in vivo labeling experiments using [^{35}S]methionine, PrA-Ala^{294} is unable to initiate maturation of the 42 kDa pro-PrB to the authentic mature 33 kDa PrB species (Fig. 5, lanes 5 and 6). As control, wild-type strain YS18 (Fig. 5, lanes 1 and 2) and strain SR2, chromosomally deleted in PrA but carrying a plasmid-encoded PrA wild-type gene (Fig. 5, lanes 3 and 4) both show maturation of PrB. Also immunoblotting of vacuolar extracts of strain SR1 carrying the mutated *pra1-Ala^{294}* allele showed only the presence of the unprocessed 42 kDa PrB precursor (not shown). As expected the loss of the PrA activity which prevents PrB maturation also abolishes processing of the 69 kDa CpY precursor to the authentic mature 61 kDa CpY in the mutant strain SR1 (*pra1ΔEN::HIS3, pDP83pra1-Ala^{294}*) (not shown).

This mutation of the amino acid Asp^{294} seems to confirm its predicted role as part of the catalytic site and

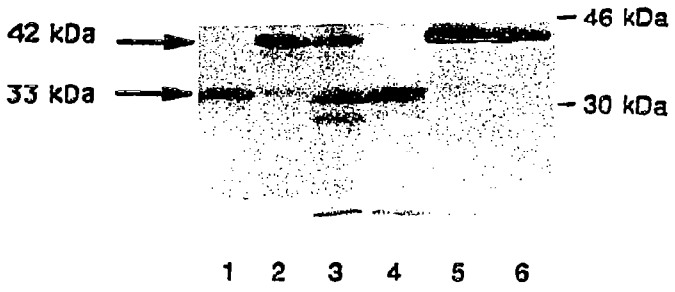

Fig. 5. Immunoprecipitation of labeled PrB-antigen in cell extracts of wild-type strain YS18 (*PRA1*) and mutant strains SR2 (*pra1ΔEN::HIS3*, *pDP83PRA1*) and SR1 (*pra1ΔEN::HIS3*, *pDP83pra1-Ala*^{294}). Cells were labeled at 30°C for 30 min with [^{35}S]methionine. Extract mixtures containing 100 μCi of radioactivity were applied on each lane. Chase time took 120 min. Immunoprecipitation was done using anti-PrB-serum; SDS-PAGE (10% gel): (Lane 1) YS18, 120 min chase; (lane 2) YS18, pulse; (lane 3) SR2, pulse; (lane 4) SR2, 120 min chase; (lane 5) SR1, pulse; (lane 6) SR1, 120 min chase.

results in complete deficiency of in vivo maturation of pro-PrA-Ala^{294} itself and the 42 kDa precursor of PrB, as well as the 69 kDa precursor of CpY.

4. DISCUSSION

We have shown that exchange of Asp^{294} of yeast PrA against Ala^{294} (*pra1-Ala*^{294}) by site directed mutagenesis destroys the in vitro activity of the enzyme and results in deficiency of processing of pro-PrA-Ala^{294} itself and two other vacuolar hydrolase precursors, the 42 kDa pro-PrB and the 69 kDa pro-CpY. Like wild-type PrA, the mutated PrA species (PrA-Ala^{294}) travels through the secretory pathway to the vacuole (Figs. 2,4). Since the mutated protein (PrA-Ala^{294}) is located in the vacuole the processing deficiency of itself and of other vacuolar hydrolases due to mis-sorting effects can be excluded. These results are in agreement with the prediction that Asp^{294} is one of the two catalytically active aspartate residues found in aspartate proteinases. An analogy to pepsinogen self-processing of pro-PrA has been proposed [7,8]. We could not observe any processing of pro-PrA-Ala^{294} to the 43 kDa or the 42 kDa form of the mature enzyme. Our results strongly indicate that processing of pro-PrA is indeed autocatalytic in nature. However, we cannot completely exclude that the mutation *pra1-Ala*^{294} leads to an altered structure of the PrA-Ala^{294} mutant protein which cannot be processed by an as yet unknown proteinase. However, we consider this possibility rather unlikely since the mutant protein, like the wild-type protein, is correctly glycosylated and correctly delivered to the vacuole.

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