

Mutant defective in processing of an enzyme located in the lysosome-like vacuole of *Saccharomyces cerevisiae*

(proenzyme/posttranslational modification/proteinases/multiple hydrolase deficiency/intracellular localization)

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ABSTRACT Carboxypeptidase Y, a vacuolar enzyme in *Saccharomyces cerevisiae*, is synthesized as a larger precursor whose apparent molecular mass is approximately 67,000 daltons. We have characterized a recessive mutation, *pep4-3*, that prevents maturation of this precursor. The accumulated precursor does not possess enzymatic activity. We have shown that the precursor accumulating in the *pep4-3* mutant is not produced in a doubly mutant strain that also bears a mutation in the carboxypeptidase Y structural gene that eliminates production of carboxypeptidase Y. We have also shown that a nonsense fragment of carboxypeptidase Y is processed. Although there is evidence that proteinase B can catalyze the conversion of the precursor to a mature form *in vitro*, nonsense mutations in the structural gene for proteinase B, *PRB1*, do not affect the levels of carboxypeptidase Y activity, and strains bearing these mutations produce a carboxypeptidase Y of apparently normal size. Hence, proteinase B is not essential for the maturation of carboxypeptidase Y precursor *in vivo*. The *pep4-3* mutation affects at least five vacuolar enzymes. This suggests that there is a processing event common to all of these enzymes.

The posttranslational cleavage of peptide fragments from precursor proteins has been shown to play a crucial role in the assembly of viral capsids (1) and collagen molecules (2), in the activation of various proenzymes (3, 4) and prohormones (5–15), and in the cascade of events that leads to coagulation (16, 17) and complement fixation (18) in blood. In addition, the scission of a hydrophobic amino-terminal segment from secreted proteins has been hypothesized to be an integral component of the process that results in the cellular localization of proteins (19, 20). The observation that many secreted proteins contain an oligosaccharide moiety has invited speculation concerning the importance of this carbohydrate to the secretion process (21, 22).

The yeast *Saccharomyces cerevisiae* synthesizes a spectrum of proteins that become localized either within the vacuole or outside of the plasma membrane. In yeast cells the vacuole is a prominent organelle and it contains a variety of hydrolytic enzymes, amongst them proteinase A (EC 3.4.23.6), proteinase B (EC 3.4.22.9), and carboxypeptidase Y (23–27).

Carboxypeptidase Y is a glycoprotein with a molecular mass of about 61,000 daltons, of which approximately 10,000 daltons is contributed by the oligosaccharide (28–34). Hasilik and Tanner have shown that carboxypeptidase Y is synthesized as a 67,000-dalton precursor, and that this precursor contains mannose. The maturation of the precursor occurs proteolytically and the same or a very similar cleavage can be catalyzed by proteinase B or trypsin, *in vitro* (35, 36).

Sixteen genes have been described whose function is essential for the production of carboxypeptidase Y in yeast (37). Of

these, *PRC1* is the structural gene for carboxypeptidase Y (38). Mutations at the *PEP4* locus are recessive and pleiotropic (37). The *pep4-3* mutation reduces proteinase A, B, and carboxypeptidase Y levels to 10%, 7%, and 3% of normal (39). Because *PEP4* is not the structural gene for carboxypeptidase Y, and because mutations at this locus are recessive and pleiotropic, we imagined that this locus might encode a product essential for the posttranslational modification of carboxypeptidase Y and have tested this hypothesis. Also, since we have defined the structural gene for proteinase B, *PRB1*, and have isolated strains bearing nonsense mutations at this locus (40), we have tested whether proteinase B is essential for the conversion of the 67,000-dalton carboxypeptidase Y precursor to mature enzyme, *in vivo*, as had been hypothesized (36).

MATERIALS AND METHODS

Materials. L-[4,5-³H(N)]Leucine (60 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) and the ¹⁴C-labeled molecular weight standards phosphorylase B (93,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome *c* (12,000) were obtained from New England Nuclear. Triton X-100 and electrophoresis materials were purchased from Bio-Rad. Yeast nitrogen base was purchased from Difco. Carboxypeptidase Y was obtained from Pierce and Boehringer Mannheim.

Yeast Strains. All mutant strains were derived from a *trp1* derivative of X2180-1B (*α gal2*) and are isogenic to it. The *trp1* mutation produces a requirement for tryptophan. The *prb1-1122* mutation is an ochre mutation in the structural gene for proteinase B and results in an absolute deficiency for proteinase B (40). The *prc1-36*, *prc1-229*, and *prc1-407* mutations lie in the structural gene for carboxypeptidase Y (38); *prc1-229* and *prc1-407* are ochre and amber mutations, respectively (unpublished results). The *pep4-3* mutation is a pleiotropic mutation that lowers the levels of proteinases A, B, and C to 10%, 7%, and 3% respectively, of the corresponding wild-type activities. It is allelic to *pep4-1* (37). Mutant strains carrying more than one of these mutations were produced from crosses made with an isogenic series of strains.

Cultivation and Labeling. Growth medium contained 2% glucose and 0.7% yeast nitrogen base. For experiments involving the immunoprecipitation of carboxypeptidase Y antigen, cells were labeled with [³H]leucine. Because the level of carboxypeptidase Y rises in the stationary phase of growth, 1–2 μCi of [³H]leucine was added per ml of culture at the end of the logarithmic phase of growth. The cells were incubated for a fur-

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then 6–8 hr with label prior to harvesting. Cells were harvested by centrifugation and stored at -70°C .

Preparation of Crude Extracts. Cell suspensions were made by the addition of 1 ml of 0.1 M Tris·HCl pH 7.5 buffer per gram of wet cells. The suspensions were passed three times through a French pressure cell at 20,000 pounds/inch² (138 MPa) and the debris was removed by centrifugation at $27,000 \times g$ for 1 hr at 4°C . The resulting supernatants were adjusted to pH 7.5 with 0.5 M NaOH and are referred to as crude extracts.

Enzyme Purification and Preparation of Antiserum. Carboxypeptidase Y was purified by the method of Hata *et al.* (41) as reported by Hasilik and Tanner (33). The enzyme was pure as judged by electrophoresis in the presence of sodium dodecyl sulfate. The immunization procedure for obtaining rabbit antiserum against carboxypeptidase Y has been described (33). The IgG fraction of the antiserum was obtained by fractionation of the antiserum with ammonium sulfate followed by gel filtration of the dissolved precipitate (33).

Immunoprecipitation. One milliliter of crude extract (4–15 mg of protein), to which 1–2 μg of the commercially obtained carboxypeptidase Y had been added, was mixed with KCl, Tris·HCl, pH 7.5, and Triton X-100 to give final concentrations of 0.4 M, 0.1 M, and 1% (vol/vol), respectively. After the addition of antiserum, the mixture was incubated at room temperature for 30 min and then at 4°C for 16 hr. The precipitate was pelleted by centrifugation at $8000 \times g$ for 10 min and washed as described (42).

Electrophoresis and Fluorography. The washed immunoprecipitates were solubilized in the presence of sodium dodecyl sulfate and dithiothreitol (35). Electrophoresis was performed according to the method of Laemmli (43) on 10% or 12.5% polyacrylamide gels. After electrophoresis, the slab gels were treated as described by Laskey and Mills (44).

Enzyme Assays and Protein Determination. Carboxypeptidase Y was assayed according to Jones (37) except that the sodium dodecyl sulfate activation was omitted and the assays were carried out at 30°C . The determination of protein concentration was made according to Lowry *et al.* (45) or Bradford (46). One unit of carboxypeptidase Y activity corresponds to the cleavage of 1 μmol of substrate per min at 30°C . Carboxypeptidase Y specific activities are reported in milliunits/mg of extract protein.

RESULTS

To test the possibility that the *pep4-3* mutation interrupted the maturation of carboxypeptidase Y, we immunoprecipitated the carboxypeptidase Y antigen present in wild-type and mutant cells that had been labeled with [³H]leucine. The immunoprecipitates were solubilized and sized on sodium dodecyl sulfate/polyacrylamide gels and the resulting bands were visualized by fluorography (Fig. 1). The carboxypeptidase Y immunoprecipitated from the wild-type strain is shown in lane 5. The antigen immunoprecipitated from a strain carrying *pep4-3* is shown in lane 2. The molecular masses of the antigens immunoprecipitated from the wild-type and *pep4-3*-bearing strains are approximately 62,000 daltons and 69,000 daltons, respectively. A mixture of these two immunoprecipitates was run in lane 7 to demonstrate this molecular mass difference directly. These results indicate that a protein that crossreacts with an antiserum raised against purified carboxypeptidase Y and is approximately the size of the glycosylated precursor described by Hasilik and Tanner (36) accumulates in the mutant. The accumulated precursor apparently possesses little or no enzyme activity, because mutant cells show 3% or less of the wild-type carboxypeptidase Y specific activity, whereas incubation of crude extracts of the

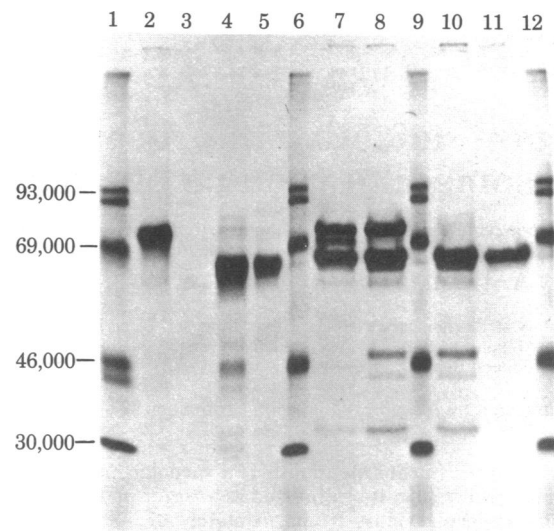


FIG. 1. Electrophoresis (10% gel) of antigens precipitated by carboxypeptidase Y antiserum from extracts of strains of various genotypes. Molecular mass standards are in lanes 1, 6, 9, and 12. The molecular masses are presented in the left margin. Lane 2, *pep4-3*; lane 3, *prb1-1122 prc1-36*; lane 4, *prb1-1122*; lane 5, wild type; lane 7, wild type plus *pep4-3*; lane 8, *prb1-1122* plus *pep4-3*; lane 10, *prb1-1122* plus *prb1-1122 prc1-36*; lane 11, wild type plus *prb1-1122 prc1-36*.

pep4-3 mutant with trypsin (1 mg/ml at 4°C for 18 hr) results in production of active carboxypeptidase Y at a specific activity at least 35% of that for wild-type extracts treated similarly. Trypsin treatment of extracts of the *pep4-3 prc1-407* double mutant does not result in the appearance of active carboxypeptidase Y as expected.

On the basis of the observation that proteinase B could convert the 67,000-dalton carboxypeptidase Y precursor, *in vitro*, to a form indistinguishable in size from that of the mature enzyme, Hasilik and Tanner (36) postulated that this function was provided by proteinase B *in vivo*. Zubenko *et al.* (40) found, however, that strains carrying mutations in the structural gene for proteinase B did not have altered levels of carboxypeptidase Y activity. We resolved this apparent discrepancy by immunoprecipitating the carboxypeptidase Y present in a strain carrying an ochre mutation, *prb1-1122*, in the structural gene for proteinase B (40). The result of this is shown in lane 4. Because the carboxypeptidase Y present in this strain is of the mature form, we conclude that proteinase B is not essential for the maturation of the carboxypeptidase Y precursor *in vivo*. A mixture of the immunoprecipitates obtained from extracts made from the mutant bearing the *pep4-3* mutation and that bearing the *prb1-1122* mutation was run in lane 8 to demonstrate the difference in the molecular weights of the carboxypeptidase Y antigens present in these strains. We believe that the band of intermediate molecular weight is artifactual. No labeled antigen could be immunoprecipitated from a strain carrying both the *prb1-1122* and *prc1-36* mutations (lane 3). This was not inconsistent with our expectations, because *prc1* is the structural gene for carboxypeptidase Y.

To test whether the aberration in posttranslational modification of carboxypeptidase Y was the result of the *pep4-3* mutation, we determined the segregation patterns for the proteinase A, proteinase B, carboxypeptidase Y, and processing defects in three meiotic tetrads produced by the sporulation of a diploid that was heterozygous for *pep4-3*. The proteinase deficiencies

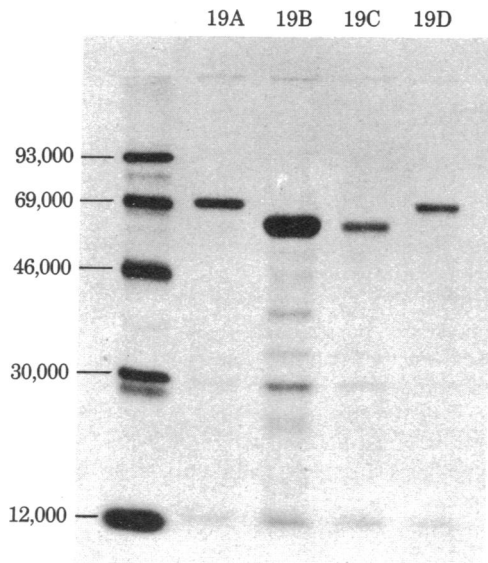


FIG. 2. Electrophoresis (12.5% gel) of antigens precipitated by carboxypeptidase Y antiserum from extracts made from the four meiotic products of one tetrad derived from sporulation of a diploid heterozygous for *pep4-3*. Labeled molecular mass standards are in lane 1. The specific activities of carboxypeptidase Y found in the extracts of these segregants were, in milliunits/mg: 19A, 0.08; 19B, 2.77; 19C, 1.61; 19D, 0.16.

cosegregate 2:2 (47). In addition, high molecular weight carboxypeptidase Y precursor accumulated in each spore clone that exhibited a deficiency for the three proteinases in these three tetrads. Immunoprecipitates for spore cultures for one such tetrad are shown in Fig. 2. Spore cultures 19A and 19D accumulate the precursor and have low carboxypeptidase Y specific activities of 0.08 and 0.16 milliunit/mg, respectively. Spore cultures 19B and 19C show processed enzyme only and show high carboxypeptidase Y levels of 2.77 and 1.61 milliunits/mg. We concluded that the defect in posttranslational modification of carboxypeptidase Y that we observed was the result of the *pep4-3* mutation. In some of the *pep4-3*-bearing segregants, however, a variable amount of immunoprecipitated antigen of a molecular weight slightly less than that of mature carboxypeptidase was observed; it was not accompanied by a coincident increase in carboxypeptidase Y activity. We assigned no significance to this and attributed the finding to artifactual cleavage of the precursor.

We attempted to visualize the nonsense fragments that result from *prc1-229*, an ochre mutation, and *prc1-407*, an amber mutation, both of which lie in the structural gene for carboxypeptidase Y. The results of this experiment are shown in Fig. 3. The immunoprecipitates obtained from extracts made from strains carrying *prc1-229* or *prc1-407* were run in lanes 5 and 8, respectively. The antigen precipitated from the mutant carrying *prc1-229* was not significantly smaller than the wild-type mature carboxypeptidase Y. To distinguish whether the antigen present in this mutant was processed, we constructed a strain carrying both the *prc1-229* and the *pep4-3* mutations and assessed the size of the carboxypeptidase Y antigen present in this strain. The result is shown in lanes 4, 6, and 7. Because the antigen produced in this doubly mutant strain was larger than that produced by the strain carrying the *prc1-229* mutation, we conclude that the nonsense fragment was not processed in the double mutant but was processed in the single mutant bearing *prc1-229*. No antigen was immunoprecipitated from the extract made from the mutant bearing the *prc1-407* mutation (lane 8) or that made from the mutant bearing both the *prc1-407* and

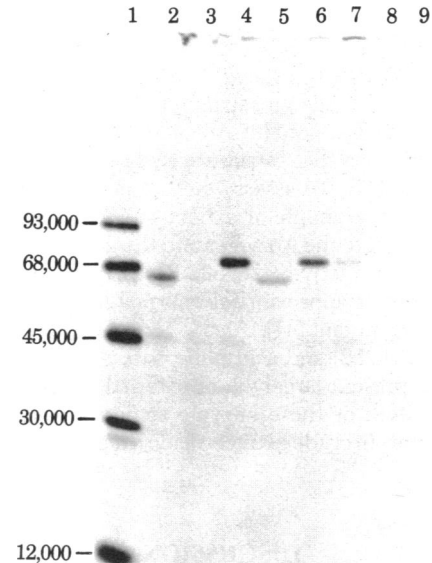


FIG. 3. Electrophoresis (12.5% gel) of antigens precipitated by carboxypeptidase Y antiserum from extracts made from strains of various genotypes. Labeled molecular mass standards are in lane 1. Lane 2, *prb1-1122*; lanes 3 and 5, *prc1-229*; lanes 4, 6, and 7, *prc1-229 pep4-3*; lane 8, *prc1-407*; lane 9, *prc1-407 pep4-3*.

the *pep4-3* mutations (lane 9) and, hence, we conclude that *prc1-407* is epistatic to *pep4-3*. This epistasis argues that the high molecular weight carboxypeptidase Y antigen is a precursor of the mature carboxypeptidase molecule—i.e., that they are products of the same gene.

Because the *pep4-3* mutation affected three different enzymes, all of which were localized to the vacuole (23–27), we decided to determine whether the *pep4-3* mutation resulted in a deficiency for other enzymes localized to particular compartments within the yeast cell. These experiments are summarized in Table 1. RNase and alkaline phosphatase activities have been shown to be localized within the yeast vacuole (23, 27, 48), although some alkaline phosphatase activity is probably not in the vacuole (27). In extracts of the *pep4-3* mutant, the specific activity of RNase is 8–10% of that found in extracts made from the isogenic parent, a level comparable to the proteinase levels in the mutant. The alkaline phosphatase level is about 30% of the wild-type level (47). The deficiencies of these two activities cosegregated with the *pep4-3* mutation in meiotic tetrads and, therefore, we assign these effects to the *pep4-3* mutation as well (47). Preliminary results from our laboratory and others' laboratories (23, 27) suggest that there may be multiple RNase and alkaline phosphatase species in yeast. If this is the case, it is possible that one or more of these species is more drastically

Table 1. Effect of the *pep4-3* mutation on a number of vacuolar and extracellular enzymes

Enzyme	Cellular location*	% of normal activity levels†
Proteinase A	Vacuole	10
Proteinase B	Vacuole	7
Carboxypeptidase Y	Vacuole	3
RNase	Vacuole	8–10
Alkaline phosphatase	Vacuole and cytoplasm	25–30
α -D-Mannosidase	Vacuolar membrane	100
Invertase	95% in extracellular space	100
Acid phosphatase	Extracellular space	100

* Evidence for localization is cited in the text.

† Summary of data from ref. 47.

affected by the mutation than are others. The *pep4-3* mutation had no effect on the specific activity of α -D-mannosidase (47), an enzyme located in the vacuolar membrane (49).

It has been reported that catalase A is located in the vacuole, whereas catalase T is not (26). Wiemken *et al.*, however, have presented evidence that catalase activity is not present in purified vacuoles (27). To assess whether *pep4-3* affected either of these species, a sample of extract was subjected to electrophoresis to separate the isozymes and the gel was subsequently stained for catalase activity (26, 50). Both catalase A and catalase T were present at approximately normal levels in the extract made from the mutant (47).

Invertase (51, 52) and acid phosphatase (53) have been reported to be present largely in or external to the yeast plasma membrane. Both of these enzyme activities were present in normal amounts in crude extracts made from the *pep4-3*-bearing mutant (47).

DISCUSSION

These results demonstrate that the *pep4-3* mutation blocks the conversion of a larger carboxypeptidase Y precursor to a smaller active form. It is clear that the precursor has little if any enzymatic activity and that processing of the precursor does not require proteinase B *in vivo*. The simplest explanation for the conversion event is that a terminal peptide is cleaved from the precursor. Because mature carboxypeptidase Y is a single polypeptide (28, 31, 32), it is unlikely that the cleavage removes an internal peptide. The results presented for the *prc1-229*- and *prc1-229 pep4-3*-bearing strains favor the possibility that the putative terminal peptide is NH₂-terminal. If the terminal peptide were COOH-terminal, a larger precursor form of carboxypeptidase Y antigen should not occur in the *prc1-229 pep4-3* bearing strain because *prc1-229* is an ochre mutation (presumably subterminal) and would prevent synthesis of the putative COOH-terminal peptide. By this argument, the peptide removed must be NH₂-terminal. It is possible, however, that an ochre mutation near the terminus of a putative COOH-terminal extension peptide might result in an improper scission of the additional segment, leading to nearly normal-sized but inactive carboxypeptidase Y molecules. Because this latter hypothesis would require a novel cleavage to occur, we consider it less likely, but we cannot exclude it.

PEP4 and *PRC1* are two separate genes, the latter being the structural gene for carboxypeptidase Y (38). Mutations in the two loci complement and segregate independently in meiosis. Therefore, the phenotype of the *pep4-3*-bearing mutant must result from a defect in the processing machinery rather than in the primary structure of the precursor.

It is likely that the precursor that accumulates in the *pep4-3* mutant is glycosylated, as is the precursor described by Hasilik and Tanner (35, 36), because the two precursors are similar in molecular weight. We have not tested directly whether the precursor accumulated by the *pep4-3* mutant cells is glycosylated. In animal cells glycosylation of the lysosomal enzyme cathepsin D apparently occurs during cotranslational segregation into the lumen of the endoplasmic reticulum (54). If the accumulated precursor of the *pep4-3* mutant is glycosylated, it is likely that the precursor has entered the lumen of the endoplasmic reticulum, because the enzymes for dolichol-mediated glycosylation in yeast are located in the membrane fractions in which microsomal enzymes are located (55).

For all of the above reasons it seems likely that the precursor accumulated by the *pep4-3* mutant is a proenzyme form of carboxypeptidase Y that must be activated, possibly within the

vacuole. Precursors of lysosomal enzymes are common in animal cells (56, 57).

Synthesis of active forms of carboxypeptidase Y and of acid phosphatase and invertase, all of which are glycoproteins of the asparagine-*N*-acetylglucosamine type (28–34, 58, 59), are perturbed when core glycosylation is blocked (33, 60). The fact that acid phosphatase and invertase are not affected by the *pep4-3* mutation implies either that the glycosylation pathways for secretory and vacuolar proteins are different or that a common glycosylation pathway for both classes of proteins is not seriously perturbed in the mutant strain. We cannot yet distinguish between these two possibilities.

Because the activities of five enzymes, all located in the vacuole, are affected by the *pep4-3* mutation, it is tempting to speculate that all of the affected enzymes are synthesized as larger precursors that must be processed to be activated. According to this hypothesis, the *pep4-3* mutant is defective in this activation process.

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