In Vivo Biosynthesis of the Vacuolar Proteinases A and B in the Yeast Saccharomyces cerevisiae

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Proteinase A and proteinase B, two vacuolar enzymes in Saccharomyces cerevisiae, are synthesized as larger precursors with apparent molecular weights of approximately 52,000 and 42,000, respectively. These precursor molecules are processed to their mature forms of 42,000 molecular weight for proteinase A and 33,000 molecular weight for proteinase B. In the presence of tunicamycin, an inhibitor of the synthesis of protein-asparagine linked carbohydrate moieties, two smaller molecular forms each of precursor and mature proteinase A were synthesized, indicating that proteinase A contains N-linked carbohydrate which is apparently not required for processing. Tunicamycin interferes also with the glycosylation of the proteinase B precursor, whereas no unglycosylated mature proteinase B could be detected.

Very little is known about the biogenesis and functioning at the molecular level of mammalian lysosomes and of lysosome-like organelles of other species. These organelles were found to represent the locus of hydrolitic enzymes (1, 2). In the yeast Saccharomyces cerevisiae, four of the known proteinases (the two endoproteinases A and B, carboxypeptidase Y, and aminopeptidase I) have been shown to reside in the vacuole (3, 4), the organelle functionally equivalent to the mammalian lysosome (5). Carboxypeptidase Y, a glycoprotein (6–8), has been shown in vivo to have two forms of molecular weight, 67,000 and 61,000 (9). It was shown that a precursor-product relationship exists between these two forms, the high molecular weight protein being converted into the low molecular weight product (9). The maturation process involves proteolytic cleavage of a peptide (9) at the NH₂ terminus of the enzyme (10). In vitro studies suggested proteinase B as the converting enzyme (9), however, studies on proteinase B mutants ruled out this possibility (10–12). A variety of questions must be answered to understand this aspect of vacuole biogenesis, for instance: (i) Is the occurrence of a pro-enzyme form only restricted to carboxypeptidase Y or is this a general phenomenon of vacuolar enzymes? (ii) What is the function of the pro-enzyme forms? This study shows that two other vacuolar hydrolases of yeast, proteinase A, a glycoprotein of 42,000 molecular weight (13), and proteinase B, a 33,000 molecular weight glycoprotein (14), are synthesized as precursor proteins in vivo.

Experimental Procedures

Materials—[⁴⁺S]Methionine (1000 Ci/mmole) and the [¹⁴C]-labeled molecular weight standards phosphorylase b (M₅₀ = 92,500), bovine serum albumin (M₅₀ = 68,000), ovalbumin (M₅₀ = 46,000), carbonic anhydrase (M₅₀ = 29,000), and lysozyme (M₅₀ = 14,300) were obtained from Amersham-Buchler (Braunschweig, F.R.G.). Protein A-Sepharose CL-4B was from Pharmacia (Freiburg, F.R.G.). Carboxypeptidase Y, proteinase A, and tunicamycin were purchased from Sigma (Taufkirchen, F.R.G.). Endoglycosidase H was obtained from Seikagaku Biochemicals (Tokyo, Japan). Endopeptidase 2.4G10, from Miles Laboratories (Kankakee, F.R.G.). Purified proteinase B was a gift of H. Hoffschulte (Biochemisches Institut der Universität Friburg). Suc d’Ouchterlony was from Pharmindustrie (Villejuve-Le-Gauroc, France). All media were from Difco (Roth, Karlsruhe, F.R.G.).

Yeast Strains—The haploid wild type strain S288C h(a, mal-) was obtained from Dr. G. R. Fink (Cornell University).

Growth, Labeling, and Preparation of Extracts—Cells were grown in yeast extract (1%), peptone (2%), glucose (2%) medium into late logarithmic phase. Spheroplasts were prepared according to Ref. 15, pulse-chase labeled in a medium containing 0.3% glucose and 1 M sorbitol with 130 μCi of L-[³⁵S]methionine/ml of cell suspension and lysed essentially as described (16). Samples were always mixed with phenylmethylsulfonyl fluoride, pepstatin, p-hydroxymercuribenzoate (final concentration, 1 mM each) and chymostatin (final concentration, 1 μg/ml) to prevent proteolytic artifacts and boiled in SDS' according to Ref. 16. In experiments, where inhibition of dolichol-phosphate-dependent glycosylation of proteinases A and B and carboxypeptidase Y was tested, tunicamycin was added to the cells 30 min prior to the pulse period and the addition was renewed during the chase period.

Antibodies—Rabbit antibodies against sodium dodecyl sulfate-denatured carboxypeptidase Y were prepared as described earlier (15). Antibodies against proteinase A were raised in rabbits using the purified enzyme (13). The antisera was prepared was shown to be specific for proteinase A. Using the double diffusion technique of Ouchterlony (17) a single precipitation line was observed when antiserum was allowed to react with purified proteinase A or wild type cell crude extracts (18). Rabbit antibodies against proteinase B were a generous gift of Dr. E. Kornmami (Biochemisches Institut der Universität Friburg). Protein A-Sepharose mediated precipitation of an antiserum-protein complex from crude extracts followed by dissociation and SDS electrophoresis resulted in a protein with the identical mobility as purified proteinase B under the same conditions. Precipitation of the labeled 33,000-dalton protein could be reduced by adding increasing amounts of highly purified proteinase B before immunoprecipitation.

Immunoprecipitation and Electrophoresis—SDS lysates from spheroplasts (60 mg) were diluted, immunoprecipitated (20 μl of antiserum), and SDS electrophoresis of the immunoreactive products was done as outlined in Ref. 15. Radioactivity on gels was visualized by fluorography (19).

In Vitro Deglycosylation—In vitro deglycosylation of proteinases A and B and, for comparison, of invertase and carboxypeptidase Y by endoglycosidase H was done by incubating the purified enzymes (20 μg in 40 μl of 0.04 M Na citrate buffer, pH 5.5) with 4 millimolar
RESULTS AND DISCUSSION

To analyze whether different molecular forms appear in the biosynthetic pathway of the vacuolar proteinases A and B, antibodies raised against the two enzymes were incubated with SDS-lysatexof yeast spheroplasts which had been labeled with L-[35S]methionine. The immunocomplexes formed were bound to protein A-Sepharose, washed, eluted, sized on SDS-polyacrylamide gels, and visualized by fluorography. Fig. 1A shows the appearance of immunoreactive forms of proteinase A which can be isolated from intact yeast spheroplasts during a pulse-chase labeling. Following a 20 min pulse with L-[35S]methionine, an antibody cross-reacting protein appeared (lane 1). During the following chase period (10 min, lane 2; 30 min, lane 3; 180 min, lane 4) the higher molecular weight protein disappeared in favor of the $M_r = 42,000$ mature enzyme suggesting a precursor-product relationship between the two proteins. The antigen and antibody were separated in the presence of diethiothreitol (16), which led to the appearance of the light and heavy chain of the immunoglobulin molecule. The presence of a large excess of heavy chain immunoglobulin in the sample, a molecule with a similar molecular weight to pro-proteinase A, could interact during SDS electrophoresis with the precipitated protein (20). This might lead to a false estimation of the molecular weight of the protein in question. Such a situation proved to be the case with the precursor protein of proteinase A under the above conditions. Therefore, molecular weight estimation of the precursor protein was done on SDS-gels omitting diethiothreitol to dissociate the immunoglobulins (Fig. 1B, lane 1). Under these conditions, a molecular weight of 52,000 was found for the proteinase A precursor.

A similar pulse-chase experiment was performed in vivo to trace the immunologically reactive forms of the vacuolar proteinase B from yeast spheroplasts (Fig. 2). After 20 min of pulse labeling with L-[35S]methionine a 42,000 dalton cross-reacting protein appeared with the specific antibodies against proteinase B (lane 1). The subsequent chase period (10 min, lane 2; 30 min, lane 3; 120 min, lane 4; 180 min, lane 5) led to the appearance of a protein identical in molecular weight to proteinase B ($M_r = 33,000$) while the higher molecular weight component completely disappeared, indicating also here a precursor-product relationship between these two proteins. These experiments strongly suggest the existence of a pro-proteinase A and a pro-proteinase B in the biosynthetic pathway of these two enzymes, which are processed into the mature enzyme forms found in the vacuole.

The molecular weight difference between the pro-protein and the mature enzyme is $10,000$ for proteinase A and $9,000$ for proteinase B. Enzymes pro-forms in vivo which are processed into the respective mature forms have also been found for lysosomal enzymes (21-24) and the vacuolar yeast carboxypeptidase Y (9). Where analyzed in more detail (9, 23) the maturation processes were found to be due to a cleavage of a peptide chain. We propose that the maturation process observed for yeast proteinase A and proteinase B is at least in part due to proteolytic removal of a peptide. Lysosomal hydrolases (25-27) as well as the vacuolar yeast carboxypeptidase Y (7) are glycoproteins with asparagine-linked carbohydrate. Proteinase A and proteinase B were found to be glycoproteins containing 8.5 and 10% carbohydrate, respectively (13, 14). Tunicamycin, an inhibitor of N-acetylgalcosaminylpyrophosphoryl dolichol formation (28) should block asparagine-carbohydrate linkage in these two proteinases if present. As can be seen from Fig. 3, the treatment of yeast cells in vivo with tunicamycin under conditions which lead only to unglycosylated carboxypeptidase Y (not shown), resulted in two additional proteins of smaller molecular weight for each of precursor and mature proteinase A (Fig. 3A, lanes 2, 3, 5, and 6). This indicates that proteinase A contains asparagine-linked carbohydrate, which, however, may not be involved in proenzyme maturation. The amount of radioactive material found in the presence of tunicamycin under chase conditions (lanes 5 and 6) is much less than that found without tunicamycin treatment of spheroplasts (lane 4). This might be due to enhanced proteolytic degradation of the proteins formed in the presence of tunicamycin under chase conditions. Tunicamycin interfered also with the glycosylation of the proteinase B precursor (Fig. 3B, lanes 2 and 3), whereas the authentic
In vivo Biosynthesis of Yeast Vacular Proteinases

proteinase B molecule remained unchanged (Fig. 3B, lanes 5 and 6).

One possible explanation for the appearance of a smaller molecular weight form in the presence of tunicamycin of the proteinase B precursor but not of authentic proteinase B is an asparagine-linked carbohydrate being part of the pro-sequence which is cleaved off during maturation. As mature proteinase B is proposed to be a glycoprotein (14), our result might indicate that linkage of this carbohydrate to the mature protein is tunicamycin insensitive. The sensitivity of purified mature proteinase A and proteinase B under non-denaturating conditions to endoglycosidase H, an enzyme cleaving high mannose oligosaccharide chains (29), gave essentially the same results (not shown). Proteinase A was nearly completely deglycosylated in two consecutive steps leading to 37,500 and 36,000 dalton forms, similar to those found after tunicamycin treatment of cells and indicating a different structural environment of the carbohydrate residues within the molecule. Proteinase B was not attacked by endoglycosidase H under these conditions.

Taken together, these results might indicate that the carbohydrate linkage on mature proteinase B is of a completely different nature. However, failure to detect asparagine-linked oligosaccharide on mature proteinase B due to high resistance to tunicamycin treatment in vivo or endoglycosidase H attack in vitro cannot completely be excluded. Different carbohydrate linkage on proteinase B might also be reflected by the observation of Hasilik and Tanner (30), who found loss of activity of proteinase A and carboxypeptidase Y after tunicamycin treatment of cells, whereas proteinase B activity remained nearly unchanged under those conditions.

We consider the precursor forms of proteinase A and proteinase B found here functionally identical to that of the proform of carboxypeptidase Y. The finding of two more vacuolar enzymes existing as precursor proteins in vivo leads us to assume a general function of the pro-sequence(s) in vacuolar biogenesis. We do not know yet whether this function resides in the necessity to keep the hydrolytic enzymes inactive during the transport from the locus of biosynthesis to the locus of action or whether this pro-sequence has a leader or recognition function to guide the protein into the right cell compartment or whether it serves both functions. Recently, it was found that carbohydrate-free carboxypeptidase Y could be segregated into the vacuoles, indicating that some possible recognition marker for segregation might be part of the protein moiety (31).

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In vivo Biosynthesis of Yeast Vacuolar Proteinases