Several lines of evidence suggest that soluble peptide: N-linked glycanase (PNGase) is involved in the quality control system for newly synthesized glycoproteins in mammalian cells. Here we report the occurrence of a soluble PNGase activity in Saccharomyces cerevisiae. The enzyme, which was recovered in the cytosolic fraction, has a neutral pH optimum, and dithiothreitol is required for activity. All of these properties were similar to those of earlier described for mammalian PNGases. Interestingly, the yeast enzyme activity was found to be present almost exclusively in cells in stationary phase; little activity was detected in logarithmic growth phase cells. Upon incubation of a glycosylatable peptide R-Asp-X-Thr-R' with permeabilized yeast spheroplasts, we detected formation of both glycosylated peptide and the peptide product expected from PNGase-mediated deglycosylation of this glycopeptide, namely, R-Asp-X-Thr-R'. Recent findings that yeast have an active system for the retrograde transport of unfolded (glyco)proteins and glycopeptides out of the endoplasmic reticulum (ER) into the cytosol raise the possibility that this PNGase may participate in an early step in degradation of these molecules following their export from the ER.

### MATERIALS AND METHODS

**Yeast Cell Culture Conditions**—The yeast strain used in this study was W303-1a (MATa ade2-1 ura3-1 his 3-11 trp1-1 leu2-3, 112 can1-100). Cells were grown at 25 °C in 1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextan, and 0.003% adenine sulfate (YPD). Unless noted, 10 ml of cells were grown at 25 °C with shaking in a 50-ml centrifuge tube.

**Glycopeptide Substrates and PNGase Activity Assay**—The following 14C-labeled glycopeptides were used in this study: fetuin glycopeptide I, (14C(CH3)2Leu-Asn(GlcNAc5Man3Gal3NeuAc3)-Asp-Ser-Arg; ovalbumin high mannose glycopeptide, (14C(CH3)2Glu-Glu-Lys-Tyr-Asn(GlcNAc5Man3)3-Leu-Thr-Ser-Val-Leu-Hse, where Hse represents homoserine; and glycoasparaginase GP-IVD, (14C(CH3)2AsnGlc NAcoMan)). The methods for preparation of these glycopeptides were reported previously (11, 21). Desialylation of fetuin glycopeptide I was carried out as described (21). PNGase activity was identified by paper electrophoresis and paper chromatography as reported previously (11, 21). Unless otherwise noted, 6 μl of reaction mixture in a polyethylene microtube contained 2 μl (20 μg of total protein) of the enzyme fraction from yeast prepared as below, 5 mM DTT, 70 mM Hepes-NaOH buffer (pH 7.2), and 25 μg asialofetuin glycopeptide I. Incubation was at 25 °C for 16 h. Quantitation of radioactivity was carried out by using a PhosphorImager (Molecular Dynamics). One unit was defined as the amount of enzyme that cleaved 1 nmol of asialofetuin glycopeptide I/h. Endo-β-N-acetylglucosaminidase activity and amidease activity, which act upon GlcNAc-peptide, were also assayed as described previously (21).

**Preparation of Crude Extract from Yeast Cells**—Yeast cells in a 10-ml culture grown to a total A600 of 50–60 (A600/ml of 5–6) as described above were harvested by centrifugation at 1,500 × g for 5 min at 4 °C. The cell pellet (200 μl) was resuspended in a 1.5-ml polyethylene tube with 300 μl of chilled B88 buffer (20 mM Hepes-KOH (pH 6.8), 150 mM NaCl, 10% glycerol, 0.05% NaN3, 0.25% BSA). The cell suspension was disrupted by sonication and centrifugation as described previously (21). The cell pellet (200 μl) was resuspended in a 1.5-ml polyethylene tube with 300 μl of chilled B88 buffer (20 mM Hepes-KOH (pH 6.8), 150 mM NaCl, 10% glycerol, 0.05% NaN3, 0.25% BSA). The cell suspension was disrupted by sonication and centrifugation as described previously (21).
potassium acetate, 5 mM magnesium acetate, and 250 mM sorbitol) with various protease inhibitors (aprotinin (Sigma), 2 μg/ml; leupeptin, (Sigma) 2 μg/ml; antipain hydrochloride (Sigma), 4 μg/ml; benzamidine hydrochloride (Sigma), 20 μg/ml) and 4 mM DTT. Glass beads (0.5-mm diameter; 0.3 g) were added, and the cells were lysed by 4 × 15 s periods of agitation in a Fisher Vortex Genie 2 vortex mixer at full speed. Then the samples were quickly frozen in liquid nitrogen and stored at –80 °C until use. The protein concentration was measured by the BCA protein assay (Fierce) on an aliquot of the cell extract taken prior to addition of DTT. Bovine serum albumin was used as a standard.

Optimization of Yeast PNGase Activity—To study the effect of pH on activity the cell extract (2 μl, 20 μg of protein) and 2 μl of asialofetuin glycopeptide (75 μg) were added to 2 μl of 400 mM Mes-NaOH buffer (pH 5.0–7.0) or Hepes-NaOH buffer (pH 6.6–8.4) containing 10 mM DTT and incubated at 25 °C for 16 h. The molarity of the reaction mixtures was adjusted to 0.17 M by addition of NaCl. For substrate specificity studies fetuin glycopeptide I, asialofetuin glycopeptide I, or ovalbumin high mannose-type glycopeptide (25 μg each) was added to the reaction mixture and assayed for PNGase activity as described above. The Km value for PNGase was determined using asialofetuin glycopeptide I as the substrate.

Subcellular Localization of PNGase Activity—Stationary phase yeast cells (total A600 of 140) were used. Preparation of spheroplasts was carried out by zymolyase digestion (Zymolyase-100T; ICN) as described above. The extract thus obtained was clarified by centrifugation at 15,000 g for 5 min at 4 °C, and the supernatant was centrifuged at 55,000 rpm in a TLA 100.3 rotor (Beckman) for 1 h, and the reaction product was analyzed using the same analytical methods.

Preparation of Permeabilized Cells and N-Glycosylation/De-N-glycosylation Assay—Preparation of permeabilized spheroplasts in which the plasma membrane was disrupted but both cytosol and intracellular membrane vesicles remain intact was carried out as described earlier (25) with the following modifications. A 300-ml culture in a 1-liter Erlenmeyer flask was grown until the A600nm was 4.7. The cells were then harvested and digested with zymolyase as described (23) and then incubated in 0.7 mM sorbitol, 0.75% bacto-yeast extract, 1.5% bacto-yeast peptone, and 1% dextrose for 20 min at 25 °C. The cells were harvested, and cell pellet (6 ml) was resuspended to a A600 of 300/ml in BSS buffer containing 40 mM creatine phosphate (Boehringer Mannheim) and 10 mM DTT. The cells were then quickly frozen with liquid nitrogen and stored at –80 °C until use. A mixture of 0.5 ml of the permeabilized cells with 125 nM [3H]acetyl-Asn-BPhe-Thr-amine (6 × 106 dpm; 260 pmol) contained 1 mM ATP (Sigma), 50 μM GDP-Man (Sigma), and 0.2 mg/ml creatine phosphate kinase (Boehringer Mannheim). Reactions were stopped by adding 0.5 mM of stop buffer (50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM CaCl2, and 1% Nonidet P-40 (Calbiochem) containing 0.25 mM of ConA-agarose), and the glycosylated peptide was recovered on the ConA-agarose beads (24). The fraction that did not bind to ConA-agarose (unglycosylated and deglycosylated peptide) was desalted by using a Sep-Pak Light cartridge, evaporated to dryness, and then passed over a Q-Sepharose HiTrap ion exchange column as described above. Unglycosylated peptide, [3H]acetyl-Asn-BPhe-Thr-amine, was recovered in the flow-through fraction. The column was washed with the equilibration buffer until the elute contained below 100 dpm/0.5 ml. The deglycosylated peptide, [3H]acetyl-Asn-BPhe-Thr-amine, was recovered by elution with 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 6% acetonitrile. Radioactivity was quantitated using a liquid scintillation counter. To study the effect of tunicamycin on product formation, cells were treated with 10 μg/ml of tunicamycin for 60 min just before the preparation of the permeabilized spheroplasts. The same concentration of tunicamycin was also added to the special medium used for preparation of permeabilized cells. At the time of assay for N-glycosylation/de-N-glycosylation, 50 μg/ml tunicamycin was also added in the reaction mixture to prevent the synthesis of any dolichol-PP-oligosaccharide during this time.

RESULTS AND DISCUSSION

Detection of PNGase Activity—Previously, a highly sensitive PNGase assay method was established using [3H]asialofetuin glycopeptide I as a substrate (11). This method is based on a kinetic comparison of glycan release from glycopeptide (detected by paper chromatography) and the introduction of negative charge on the core peptide resulting from conversion of Asn into Asp (confirmed by paper electrophoresis). Only when these two occur at the same rate can we conclude that the observed activity is that of PNGase. Although it was reported that PNGase activity is that of PNGase. Although it was reported that PNGase activity is that of PNGase.
Gase activity could not be detected in yeast (19), using the above mentioned assay method we detected this activity in *Saccharomyces cerevisiae* strain W303-1a, as well as in a variety of other strains. With both methods, the product migrated with a mobility that was identical to the authentic Asp-containing peptide prepared by PNGase F digestion of the starting glycopeptide (not shown). On the other hand, we could detect neither endo-β-N-acetylglucosaminidase activity nor amidase activity, which could act upon GlcNAc-peptide, thereby giving rise to a product identical to deglycosylated product formed PNGase. The absence of detectable activity for these enzymes clearly excludes the possibility that the deglycosylation occurred in two steps as follows: [@Asn(CH0)-XX-Ser-]→@Asn(GlcNAc)-XX-Ser-→@Asp-XX-Ser-)

**Enzymatic Properties of Yeast PNGase**—The pH optimum of the yeast enzyme was determined to be 6.6 using Mes buffer and 7.0 using Hepes buffer, which is similar to that reported for the animal enzyme (10–12, 19, 21). Similar to the animal enzymes (11, 12, 26), the yeast enzyme required DTT for activity and was inhibited by NEM. The yeast enzyme exhibited maximal activity at 37 °C after 1–3 h, but over a longer course of time the enzyme was unstable at temperatures above 30 °C (not shown). Since no decrease in activity was observed at 25 °C for up to 24 h, we routinely used this temperature for assay conditions.

Unlike commercially available bacterial PNGase F and almond PNGase A, the mouse L-929 cell line PNGase does not efficiently cleave sialylated glycopeptides (26). Similar to L-929 PNGase, the yeast enzyme showed lower activity (36%) toward the sialylated fetuin glycopeptide I compared with asialoglycopeptide I. The yeast PNGase showed higher activity (160%) toward high mannose-type glycopeptide, but no activity toward high mannose-type glycosparagamine, suggesting that the peptide length of the substrate is critical for enzyme activity. This property has been described for all PNGases examined (1–3, 27). The *K*-value for the asialofetuin glycopeptide I substrate was 200 μM, which is comparable with that of PNGase from animals (21, 28).

**Subcellular Localization of PNGase Activity in Yeast**—PNGase activity was not detected in spent growth medium. This suggests that either the PNGase is not secreted or upon secretion it is extremely labile. Assays of the cytosol (soluble) and membrane (pellet) fractions prepared by lysis of zymolyase-generated spheroplasts revealed that 94% of the PNGase activity was recovered in the cytosol fraction. In contrast, when the activity of oligosaccharyl transferase, an ER resident enzyme, was assayed in both fractions, 91% of the activity was recovered in the pellet fraction, whereas no activity was detected in the cytosol fraction. These results were consistent with other studies using animal cells (10–12, 21), although we do not exclude the possibility that inside the cells the enzyme is associated with fragile vesicular structures that are lysed during spheroplast lysis. The low level of activity found in the particulate fraction was enhanced 2.0-fold in the presence of 0.8% Tween 80. It is unknown if this apparent activation is due to lysis of a membrane vesicle-associated PNGase of the type reported in rat liver (29) or is the result of the release of soluble PNGase entrapped in the crude membrane pellet. In any case, it is clear that after cell breakage and centrifugation the yeast enzyme exhibits a subcellular distribution similar to that observed in animal cells, although the precise localization of the neutral PNGase within the yeast cell remains to be determined.

**Growth Dependence of PNGase Activity in Yeast**—PNGase activity could barely be detected in the crude lysates of log phase cells but dramatically increased at stationary phase (Fig. 1). This finding leads us to speculate that this enzyme activity may be involved in stabilizing cells in stationary phase, a stress condition (30). Of course, we cannot yet be certain if the enzyme in stationary phase and the low activity detected that in growth phase is due to the same protein. In this connection, it is intriguing that recently a second, soluble neutral PNGase was identified in log phase of L-929 cells (31). This second enzyme had properties that differed from the earlier identified L-929 PNGase purified from confluent cells (21).

**N-Glycosylation and De-N-glycosylation in Lysed Spheroplasts**—In *S. cerevisiae*, like in mammalian cells, glycopeptides do not exit from the ER via a conventional vesicular transport mechanism, but instead are released from the ER into the cytosol (19, 20). Since we detected PNGase in yeast in the soluble fraction, we hypothesized that the glycosylated peptide, once it exited from the ER into the yeast cytosol, might be degraded by the action of this soluble PNGase and thereby be converted to deglycosylated peptide. To investigate this possibility, we first prepared the labeled glycopeptide [3H]Acetyl-Asn(CH0)-BPhe-Thr-amide by incubation of the corresponding peptide with crude lysate. The glycopeptide formed was isolated and tested as a substrate for the PNGase in the yeast-soluble (cytosol) fraction. The peptide formed in the yeast cytosol was shown to be distinct from the unglycosylated peptide, [3H]Acetyl-Asn(BPhe-Thr-amide, and from the Endo H-deglycosylated GlcNAc-peptide, [3H]Acetyl-Asn(GlcNAc)-BPhe-Thr-amide, as judged by paper chromatography (Table I). In contrast, in both paper chromatography systems the reaction product had a mobility virtually identical to that of the authentic PNGase F-deglycosylated peptide, [3H]Acetyl-Asp-BPhe-Thr-amide. This indication that the product was formed by the action of PNGase was further confirmed by its binding to an anion exchange column (Q-Sepharose), indicating that the peptide formed was negatively charged, as expected upon conversion of the Asn into an Asp residue. Neither the starting peptide nor the glycopeptide formed bound to Q-Sepharose. Moreover, the migration position of the reaction product after incubation with yeast cytosol was identical to that purified

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2 Strains used are as follows: PS593 (MATα leu2 ura3 trp1 his3), A364A (MATα ade1 his7 lys2 tyr1 ura1 gal1-1), L40 (MATα ade2-leu2 his3 trp1 lys2:lexAop-HIS3 URA3:lexAop-lacZ), and Y153 (MATα ade2 leu2 his3 trp1 gal4 gal80 lys2:UAS)<sub>G</sub>-HIS3 URA3:UAS)<sub>G</sub>-lacZ).
with authentic PNGase F-deglycosylated peptide (not shown). No deamidation product of the C-terminal amide group was detected on paper electrophoresis when the starting peptide was incubated with cytosol for 24 h. These results established that the glycopeptide is a substrate of yeast PNGase in the cytosol. After 24-h incubation at 25 °C, 79% of the glycopeptide was deglycosylated by yeast PNGase. Under the incubation conditions used, we could not detect any Endo H-like enzyme action on the glycotripeptide judging from paper chromatography in solvent B (not shown).

Next, we asked if sequential glycosylation of peptide in the ER followed by deglycosylation in the cytosol could be detected in permeabilized yeast spheroplasts supplemented with an ATP generating system. After addition of the peptide substrate for glycosylation by oligosaccharyl transferase in the ER, aliquots of the incubation mixture were removed at various times, and the radioactivity in three fractions, glycosylated, unglycosylated, and deglycosylated peptide, was determined after the fractions were separated from each other by using ConA-agarose and Q-Sepharose. As shown in Fig. 2 (solid line), over the 2-h time course the amount of labeled peptide rapidly decreased and the amount of labeled glycopeptide rapidly increased up to 60 min, after which time the level barely changed. The deglycosylated peptide appeared at a slower rate, comparable with that expected from the in vitro deglycosylation (79% conversion at 24-h incubation; see above). When the pool of oligosaccharide-PP-dolichol was depleted by treatment of cells for 24 h. These results established that the glycopeptide is a substrate of yeast PNGase in the cytosol. After 24-h incubation at 25 °C, 79% of the glycopeptide was deglycosylated by yeast PNGase. Under the incubation conditions used, we could not detect any Endo H-like enzyme action on the glycotripeptide judging from paper chromatography in solvent B (not shown).

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N-Glycosylation/de-N-glycosylation in Yeast

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