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Peptides Glycosylated in the Endoplasmic Reticulum of Yeast Are Subsequently Deglycosylated by a Soluble Peptide: *N*-Glycanase Activity*

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Several lines of evidence suggest that soluble peptide: *N*-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-Asn-*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.

Peptide:*N*-glycanase (EC 3.5.1.52, PNGase)[1] cleaves the β-aspartylglucosamine bond of *N*-linked glycans, releasing an intact oligosaccharide, and generating at the site of hydrolysis an aspartic acid residue in the peptide/protein backbone (1–3). This enzyme, first isolated from almonds (4), has been widely used as a tool in studies on *N*-linked glycan chains. Following the demonstration of a deglycosylation reaction catalyzed by PNGase in fish oocytes and embryos (5–7), interest in the biological function of PNGase has grown (3, 6–9). It is now clear that soluble PNGases having in common a neutral pH optimum and a requirement for –SH groups occur widely (10, 11). Recent findings have raised the possibilities that PNGases might modify newly synthesized glycoproteins either by converting overglycosylated proteins into normal *N*-glycosylated isoforms or triggering degradation of aberrant or misfolded glycoproteins (12). In this connection, several studies have established that proteasomes in the cytosol play a central role in the pathway for degradation of aberrant or misfolded (glyco)proteins in organisms ranging from yeast to mammals (13–18). It has been suggested that PNGase may play a role in this degradation pathway in animal cells (13, 14, 16, 18), and, in fact, the action of PNGase in the deglycosylation of glycosylated peptides in the cytosol has been established in mammalian cells (19).

In yeast, like higher eukaryotes, a retrograde transport system for transport of malformed glycoproteins out of the ER to the cytosol exists (15, 17). Furthermore, glycopeptides formed from glycosylation of peptides in the ER of yeast cells are known to be routed to the cytosol, rather than secreted (19, 20). Given these findings we asked if a cytosolic PNGase, like that found in higher eukaryotes, is present in yeast. Our results indicate that such an enzyme is present in yeast and is expressed at high levels in stationary phase cells. Furthermore, the results suggest that the PNGase could function in catabolism of glycopeptides and malformed glycoproteins.

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% Bactopeptone, 2% dextran, and 0.003% adenine sulfate (YPAD). 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 ^{14}C-labeled glycopeptides were used in this study: fetuin glycopeptide I, $([^{14}C]CH_3)_2$Leu-Asn($GlcNAc_5Man_3Gal_3NeuAc_3$)-Asp-Ser-Arg; ovalbumin high mannose glycopeptide, $([^{14}C]CH_3)_2$Glu-Glu-Lys-Tyr-Asn($GlcNAc_2Man_{5.5}$)-Leu-Thr-Ser-Val-Leu-Hse, where Hse represents homoserine; and glycoasparagine GP-IVD, $([^{14}C]CH_3)_2$Asn($GlcNAc_2Man_6$). The methods for preparation of these glycopeptides were reported previously (11, 21, 22). 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 μM 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 μmol of asialofetuin glycopeptide I/h. Endo-β-*N*-acetylglucosaminidase activity and amidase 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 A_{600} of 50–60 (A_{600}/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

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[1] The abbreviations used are: PNGase, peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase (peptide:*N*-glycanase (EC 3.5.1.52)); BPhe, *p*-benzoylphenylalanine; CHO, *N*-linked carbohydrate chain; ConA, concanavalin A; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NEM, *N*-ethylmaleimide; ER, endoplasmic reticulum; Endo H, endo-β-*N*-acetylglucosaminidase H.

TABLE I
Chromatographic characterization of product of incubation of soluble fraction with [^{3}H]acetyl-Asn(CHO)-BPhe-Thr-amide

	(Glyco)peptide structure[a]	R_F value[b]		Anion exchange binding[e]
		Solvent A[c]	Solvent B[d]	
Unglycosylated peptide	Asn-BPhe-Thr	0.63	0.75	No
Glycosylated peptide	Asn(CHO)-BPhe-Thr	0.017	0.01	No
PNGaseF-deglycosylated peptide	Asp-BPhe-Thr	**0.52**	**0.76**	**Yes**
Endo H-deglycosylated peptide	Asn(GlcNAc)-BPhe-Thr	0.48	0.60	No
Reaction product from yeast cytosol		**0.50**	**0.75**	**Yes**

[a] All peptides contained an acetyl group at their N terminus and an amide at their C terminus.
[b] R_F values were determined by cutting 0.5-cm segments from the paper chromatogram extending from the origin to the solvent front (15 cm for Solvent A and 17 cm for Solvent B) and measuring the radioactivity in each.
[c] Solvent A, 1-butanol/ethanol/H_2O = 2/1/1 for 2.5 h.
[d] Solvent B, 1-butanol/acetic acid/H_2O = 5/2/2 for 3.5 h.
[e] The samples were applied to an anion exchange column and eluted with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% acetonitrile.

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 (Pierce) 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 μM) 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 μM each) was added to the reaction mixture and assayed for PNGase activity as described above. The K_m value for PNGase was determined using asialofetuin glycopeptide I as the substrate.

Subcellular Localization of PNGase Activity—Stationary phase yeast cells (total A_{600} of 140) were used. Preparation of spheroplasts was carried out by zymolyase digestion (Zymolyase-100T; ICN) as described (23), and the spheroplasts were lysed in 3 ml of ice-cold 50 mM Tris-HCl buffer (pH 8.0) with 4 mM DTT and various protease inhibitors as described above. The extract thus obtained was clarified by centrifugation at 1,500 × *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 at 4 °C. The membrane pellet was resuspended in B88 buffer, including 4 mM DTT with or without 2.4% Triton X-100 (Sigma). Both the 55,000 rpm supernatant and pellet fractions were assayed for PNGase as described above.

Growth Dependence of PNGase Activity—Cells in YPAD medium (200 ml) were grown in a 1-liter Erlenmeyer flask, and the A_{600} was measured every 2 h from A_{600}/ml of 0.137 to 4.6. Cells (total A_{600} of 5.0) were collected, and cell extract was prepared as described above except that glass beads and buffer added for extraction were one-third of that described above.

Preparation and Analysis of Reference Samples of a Tripeptide Acceptor and Related Compounds—A known tripeptide substrate for oligosaccharyl transferase, [^{3}H]acetyl-Asn-BPhe-Thr-amide (10.6 μCi/nmol), where BPhe represents *p*-benzoylphenylalanine, was kindly provided by Qi Yan, SUNY at Stony Brook. Glycosylation of the peptide by yeast microsomes was carried out as described earlier (24), and the reaction product in 50 mM Tris-HCl buffer (pH 7.5) was applied to a ConA-agarose column (Sigma; 0.9 × 1.2 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. Equilibration buffer was used to elute the fraction that did not bind. Subsequently, the ConA-agarose beads containing bound peptide were mixed with a solution of 0.5 M α-methylmannoside in equilibration buffer stirred well with a Pasteur pipette and eluted with the same buffer. The purified glycopeptide thus obtained was desalted by passage through a Sep-Pak Light cartridge (Waters/Millipore), eluted with 60% acetonitrile, and evaporated to dryness. Authentic deglycosylated peptide was prepared either by digestion of glycopeptide (20,000 dpm) with 1 unit of PNGase F (Boehringer Mannheim) in 20 μl of 50 mM Tris-HCl buffer (pH 8.0) or 5 milliunits of Endo H (Boehringer Mannheim) in 20 μl of 50 mM Mes-NaOH buffer (pH 6.0) at 37 °C for 2 h, respectively. Unglycosylated peptide, glycopeptide, PNGase F-deglycosylated peptide, and Endo H-deglycosylated peptides were subjected to paper chromatography (see Table I). The R_F of each compound was determined after measuring the radioactivity by liquid scintillation counter. For additional characterization, the binding of each reference sample to a 1.0-ml anionic column (HiTrap Q, Amersham Pharmacia Biotech) with 10 mM Tris-HCl (pH 8.0) in 0.1% acetonitrile (v/v) was examined. The isolated purified glycopeptide (20,000 dpm) also was digested with yeast cytosol (100 μg of protein) prepared by zymolyase treatment as described above, incubated for 24 h at 25 °C, 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 A_{600}/ml was 4.7. The cells were then harvested and digested with zymolyase as described (23) and then incubated in 0.7 M sorbitol, 0.75% bacto-yeast extract, 1.5% bacto-peptone, and 1% dextrose for 20 min at 25 °C. The cells were harvested, and cell pellet (6 ml) was resuspended to a A_{600} of 300/ml in B88 buffer containing 40 mM creatine phosphate (Boehringer Mannheim) and 10 mM DTT. The cells were then quickly frozen with liquid N_2, and stored at −80 °C until use. A reaction mixture of 0.5 ml of the permeabilized cells with [^{3}H]acetyl-Asn-BPhe-Thr-amide (6 × 10^6 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 ml of stop buffer (50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1% Nonidet P-40 (Calbiochem) containing 0.25 ml 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, [^{3}H]acetyl-Asn-BPhe-Thr-amide, 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, [^{3}H]acetyl-Asp-BPhe-Thr-amide, 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 ng/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 [^{14}C]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 PN-

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.[2] 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(CHO)-Xaa-Ser- → -Asn(GlcNAc)-Xaa-Ser- → -Asp-Xaa-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 glycoasparagine, 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_m 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.

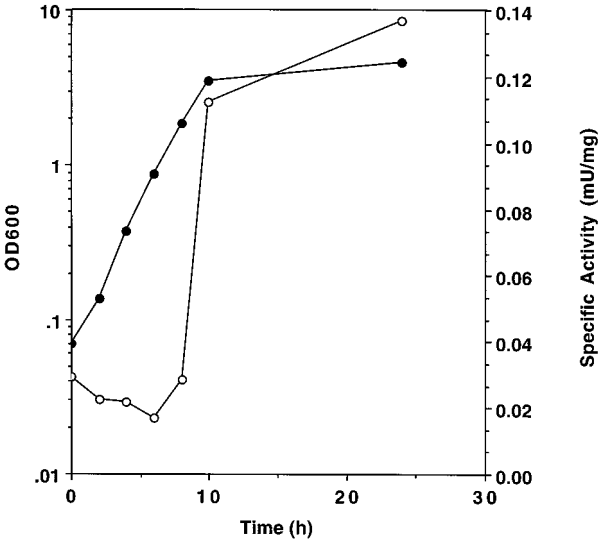


FIG. 1. **PNGase activity as a function of growth phase.** Cells were grown and collected as described under "Materials and Methods," and A_{600} (●)and PNGase activity (○) were measured after preparation of a crude yeast lysate.

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 [^{3}H]acetyl-Asn(CHO)-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, [^{3}H]acetyl-Asn-BPhe-Thr-amide, and from the Endo H-deglycosylated GlcNAc-peptide, [^{3}H]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, [^{3}H]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

[2] Strains used are as follows: PS593 (*MATα leu2 ura3 trp1 his3*), A364A (*MATa ade1 his7 lys2 tyr1 ura1 gal1-1*), L40 (*MATa ade2-leu2 his3 trp1 LYS2::lexAop-HIS3 URA3::lexAop-lacZ*), and Y153 (*MATa ade2 leu2 his3 trp1 gal4 gal80 LYS2::UAS$_G$-HIS3 URA3::UAS$_G$lacZ*).

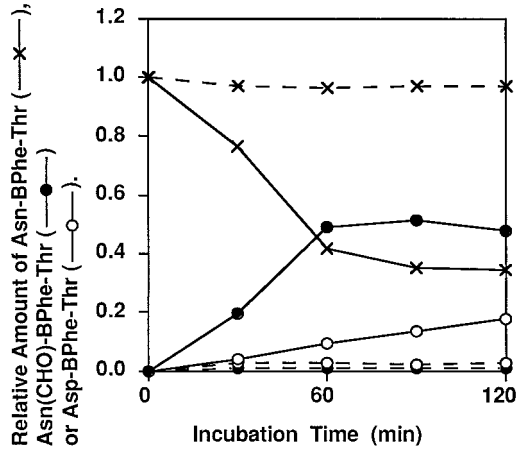
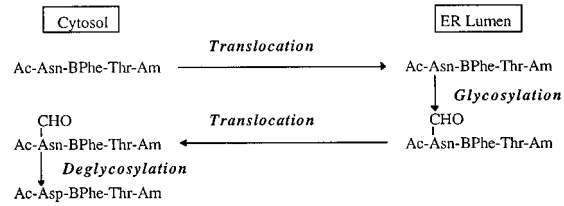


FIG. 2. **Time course of *N*-glycosylation/de-*N*-glycosylation of tripeptide in permeabilized yeast spheroplast.** Permeabilized yeast spheroplasts prepared in the absence (*solid line*) or presence (*dashed line*) of tunicamycin were incubated with [^{3}H]acetyl-Asn-BPhe-Thr-amide as described under "Materials and Methods," and the reaction products at various time points were determined by using ConA-agarose and Q-Sepharose. Values are expressed as the fraction of the total radioactivity recovered.

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 with tunicamycin, the formation of *both* glycosylated and deglycosylated peptide was inhibited (Fig. 2, *dashed line*). When 5 mM NEM was added instead of 10 mM DTT, the formation of deglycosylated peptide was *completely* inhibited. Although the glycosylation was also inhibited to a lesser extent (one-third of control level after incubation for 2 h), the amount of glycosylated peptide increased progressively up to this time point.

General Discussion—PNGase is involved in quality control of newly synthesized glycoproteins and the production of free oligomannosides from both glycoproteins and glycopeptides in mammalian or avian (12, 18, 32) cells. Because of recent evidence in *S. cerevisiae* implicating proteasomes in degradation of glycoproteins (15, 17), we asked if PNGase might participate in this degradation process. Formation of free oligosaccharides has been reported to occur in yeast (33), but this was considered to be the result of hydrolysis of the dolichol-linked biosynthetic intermediates in the *N*-glycosylation of proteins; there has been no evidence reporting the degradation of glycoprotein/glycopeptides as a source of free oligosaccharides in yeast. We have found a soluble neutral PNGase in *S. cerevisiae*, which indicates that this enzyme is distributed over the entire range of eukaryotes from fungi to mammals. So far PNGases from a bacterium and a fungus have been cloned and sequenced (34, 35), but no sequence is available for soluble PNGases in animal cells. Upon examination of the sequences in the *S. cerevisiae* genome data base no homology between these proteins and proteins in yeast could be detected.



SCHEME 1. **Postulated fate of glycosylated peptide.**

This enzyme activity is induced to a high level only in stationary phase cells. In other experiments (data not shown) the level of the activity was shown to be further elevated by various stress conditions, such as incubation with tunicamycin to block glycosylation or with DTT to inhibit disulfide bond formation in proteins. Since it has been shown that these stresses bring about protein unfolding and activation of molecular chaperones both in the cytosol and the ER (30, 36, 37), we speculate that this neutral PNGase may function in a quality control system for newly synthesized glycoproteins in yeast. Presumably it would do this by deglycosylating newly formed glycoproteins that did not fold correctly and were exported from its site of synthesis with ER to the cytosol.

To test this idea we utilized a glycosylatable hydrophobic peptide that is readily taken up into the ER and permeabilized yeast spheroplasts that retain their cytosol, as well as their internal organelles. The results indicated that the glycosylatable peptide was formed and then processed with kinetics in accord with the sequence of events shown in Scheme I.

The reactions actually measured kinetically were the glycosylation step, which requires prior translocation into the ER, the site of action of oligosaccharyl transferase (18), and the de-*N*-glycosylation, which we expect to occur in the cytosol, because we have tentatively identified this to be the site of PNGase. Formation of the deglycosylated peptide was found to require prior glycosylation, since tunicamycin treatment, which inhibits *N*-glycosylation, also abolished formation of the deglycosylated peptide. If these reactions with peptides are a valid model for what happens with unfolded glycoproteins, we speculate that yeast may have a catabolic system analogous to that found in mammalian cells (18). In the future, we plan to study the structure of the yeast PNGase in detail and to test its postulated function in glycoprotein degradation.

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