Site-directed Mutagenesis Study of Yeast Peptide: N-Glycanase

INSIGHT INTO THE REACTION MECHANISM OF DEGLYCOXYLATION*

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Yeast peptide-N-glycanase (Png1p; PNGase), a deglycosylation enzyme involved in the proteasome dependent degradation of proteins, has been reported to be a member of the transglutaminase superfamily based on sequence alignment. In this study we have investigated the structure-function relationship of Png1p by site-directed mutagenesis. Cys-191, His-218, and Asp-235 of Png1p are conserved in the sequence of factor XIIIa, where these amino acids constitute a catalytic triad. Point mutations of these residues in Png1p resulted in complete loss in activity, consistent with a role for each in catalyzing deglycosylation of glycoproteins. Other conserved amino acid residues, Trp-220, Trp-231, Arg-210, and Glu-222, were also vitally important for folding and structure stability of the enzyme as revealed by circular dichroism analysis. The potential effects of the mutations were predicted by mapping the conserved amino acids of Png1p within the known three-dimensional structure of factor XIIIa. Our data suggest that the lack in enzyme activity when any of the catalytic triad residues is mutated is either due to the absence of charge relay in the case of the triad or due to the disruption of the native fold of the enzyme. These findings strongly suggest a common evolutionary lineage for the PNGases and transglutaminases.

Protein synthesis and folding is an essential process in eukaryotes that is accomplished for membrane and secretory proteins in the endoplasmic reticulum (ER). The fate of the newly synthesized protein, whether it is destined for secretion or degradation, is based on its conformation. Proteins with a native conformation enter Golgi transport vesicles and are then sent to other destinations. However, misfolded or non-native proteins cannot enter Golgi transport vesicle and are retained in ER (1). In some cases these misfolded proteins are subjected to degradation by ER-associated degradation mechanism (2–6), in which misfolded protein is transferred from the ER to the cytosol, followed by degradation by the proteasome. In the case of misfolded glycoproteins that are transferred from the lumen of ER to the cytosol for degradation, the cleavage of the N-linked glycan is catalyzed by the enzyme peptide-N-glycanase (PNGase), which hydrolyzes the β-aspartylglycosylamine bond of asparagine-linked glycopeptides and glycoproteins and may act in concert with the proteasomal degradation of proteins that are exported out of the ER to the cytosol (7, 8). The gene PNG1 encoding for the cytoplasmic deglycosylating enzyme has been identified in yeast and was found to be well conserved throughout the eukaryotes (9). More recently, physical interaction between yeast PNGase (Png1p) and the 26 S proteasome was found to be mediated via the protein Rad23 (10), further indicating the functional relationship of the deglycosylating enzyme with the proteasomal degradation. However, up to now knowledge about the structural features of this enzyme has been very limited.

PNGase has been classified as a member of the “transglutaminase superfamily” based on the conservation of amino acid residues surrounding the catalytic triad of transglutaminase (11). Transglutaminases catalyze amide bond formation between the γ-carboxyamide group of glutamine and the ε-amino group of lysine (12). The cross-linking of the two proteins involves deamidation of glutamine. Png1p catalyzes a reaction (cleavage of an amide bond), which is reverse of that in transglutaminases (formation of a amide bond) (Fig. 1). Transglutaminases possess a catalytic triad of three amino acids Cys, His, and Asp. This catalytic triad is similar to that found in thiol proteases (13). We found that this putative catalytic triad is conserved through all of Png1p homologs, suggesting that Png1p might also utilize this triad for its enzymatic reaction. Based on homology with factor XIIIa and with PNGases from other eukaryotes, we examined several mutations of Png1p at putative active site and non-active site residues. The effect on the Png1p-mediated deglycosylation of a glycopeptide (biochemically purified) supports the idea that Png1p utilizes a similar catalytic triad for an enzymatic reaction, which is consistent with its classification as a novel member of the transglutaminase superfamily. We have shown that Png1p requires Cys-191, which is predicted to supply the nucleophilic residue of the catalytic triad. His-218, which is proposed to function as a catalytic base analogous to the invariant histidine residue present in the catalytic triad of serine and cysteine proteases, is also essential (14, 15). Asp-235, the third residue of the catalytic triad, was also found to be crucial. The carboxylic acid of aspartic acid is important to maintain the proper orientation of the histidine residue, facilitating its participation in catalysis (16, 17). Several other residues in Png1p conserved in factor XIIIa have also been identified as essential components for the PNGase activity. The structural and/or functional importance of these mutants in Png1p was examined by comparison with the known three-dimensional structure of factor XIIIa (18).
MATERIALS AND METHODS

Site-directed Mutagenesis—The mutagenic oligonucleotide primers were designed based on the amino acid sequence of yeast Png1p. All residues were mutated to Ala (one at a time) except Cys-129, Cys-132, Cys-165, and Cys-168, which were mutated either to Ala or Ser, and Trp-220 and Thr-231, which were mutated either to Ala or Phe. Mutagenesis was performed by using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's procedure. The primers used are summarized in Table I. All mutations were confirmed by DNA sequencing.

Protein Expression and Extraction—DNA manipulations were performed according to Sambrook et al. (19). Constructs made were transformed into BL21(DE3)pLysS cells and expression of PNG1 and mutants were induced by adding 1 mM isopropylβ-D-thiogalactoside at A600 = 0.8/ml. After 3 h at 37 °C, 5 ml of cells was centrifuged and protein was extracted by adding 400 μl of phosphate-buffered saline, 1% Triton X-100, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride, followed by sonication on ice using a Branson sonicator at level 3 twice for 10 s with a cooling interval of 1 min. The cell extract was centrifuged at 16,000 × g for 10 min at 4 °C, and the supernatant was assayed for PNGase activity. All the protein extracts were analyzed on 10% SDS-PAGE. The protein amount was also checked by Western blot analysis.

Western Blot Analysis—Protein extracts were resolved on SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with 1:1000 dilution of rabbit anti-(his)6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by 1:1000 dilution with anti-rabbit IgG horse radish peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals, Indianapolis, IN). Gels were visualized using chemiluminescence after exposure to medical x-ray film (Fuji photo Film Co., Stamford, CT).

Activity Assay—PNGase activity was assayed using fetuin-derived asialoglycopeptide I ([14C](CH3)2Leu-Asn(GlcNAc5Man3Gal3)-Asp-Ser-Arg) as described previously (20, 21). Radioactivity was monitored on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitated using ImageQuant (version 1.2).

Purification of (His)6-tagged Png1p (Png1(His)6p)—To further explore the kinetic parameters and structural features of the WT and mutant Png1p, the (his)6-tagged WT Png1p and mutant Png1ps (Cys-129Ser, Cys-132Ser, Cys-165Ser, Cys-168Ser, Cys-191Ala, Arg-210Ala, Trp-220Ala, and Tyr-273Ala) were expressed in Escherichia coli as described above. The pellet obtained from a 1-liter culture was suspended with 5–7 ml of buffer containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol (BME), and the cells were lysed using a French press. The extract obtained was subjected to 20% ammonium sulfate precipitation for 2 h before binding the supernatant to a chelating Sepharose column (Amersham Biosciences, Inc., Piscataway, NJ). Chelating Sepharose was charged with Ni2+ according to the manufacturer's instructions and equilibrated with the starting buffer (20 mM sodium phosphate, 0.5 M NaCl, and 10 mM BME). The protein was allowed to bind for 18 h at 4 °C. The entire amount was then transferred to a 20-ml gravity flow column, and the flowthrough was collected. The resin was further washed (3 column volumes) with the starting buffer containing increasing concentrations of imidazole (10 and 100 mM) and then eluted with 3 gel volumes of buffer containing 500 mM imidazole. Column fractions equal to 1 gel volume were collected. After elution DTT and EDTA were added to each fraction to adjust the final concentration to be 1 mM each. Eluted fractions were analyzed by SDS-PAGE and assayed for PNGase activity. The pure fractions were pooled, and imidazole was removed using a Microcon 10 (Amicon Inc., Beverly, MA) with 20 mM Na2HPO4 containing 0.1 M NaCl, 10 mM BME, and 1 mM EDTA for kinetic experiments. For circular dichroism (CD) experiments protein samples were desalted using Microcon 10 and buffer containing 0.1 mM Na2HPO4, 0.1 M NaCl, 10 mM BME, and 1 mM EDTA. All the protein samples were stable for 2–3 days at low salt concentrations. The protein concentration was calculated at 280 nm using an extinction coefficient 69,480 for WT, C191A, C129S, C132S, C165S, C168S, R210A; 63,940 for W220A; and 68,000 for Y273A.
Circular Dichroism Spectroscopy—All CD experiments were done on a Jasco J-715 spectropolarimeter. A 0.1-cm path length cell was used to collect data in the far UV and near UV. All the experiments were done at room temperature. The concentration of all the samples was 5 \mu M for far UV measurements and 75 \mu M for near UV measurements. Spectra were collected with a scan rate of 100 nm/min, 1-nm bandwidth, and 1-s response time. Spectra were measured in buffer containing 0.1 mM NaHPO₄, 1 mM NaCl, and 10 mM BME. The spectrum of a blank containing buffer alone was subtracted from all spectra.

**Determination of V_{max} and K_m—** WT Png1p and four Cys-to-Ser mutants (C129S, C132S, C165S, and C168S) were used to determine the Michaelis constants (K_m and V_{max}) of the enzyme, using asialofetuin glycopeptide I as a substrate. The enzymatic reaction was performed in 120 mM Mes buffer (pH 6.6) containing 1 mM DTT. Because the release of peptide was proportional to a reaction period up to 1 min for WT and up to 40 h for mutants when 0.05 mM (the lowest concentration) of substrate was used, incubation was carried out at various concentrations of the substrate (1, 0.5, 0.25, and 0.1 mM) for 1 min for WT and 40 h for mutants. Initial reaction rates were expressed as nanomoles of peptide released under the assay conditions per minute per milligram of protein.

**RESULTS**

The purpose of the present work was to explore the structurally-functional consequences of mutation of the conserved amino acid residues of Png1p based on the sequence homology with factor XIIIa, a member of the transglutaminase family whose three-dimensional structure is known (18). The protein sequence of Png1p was aligned with six eukaryotic PNGases and blood coagulating factor XIIIa using the ClustalW program (Fig. 2). In the present study Png1p from *Saccharomyces cerevisiae* (ScPng1p) was used; the sequence numbering is shown at the top. Sequence numbering for factor XIIIa is shown at the bottom. The mutations on Png1p studied and the relative activity with WT are listed in Table II. To determine the activity, the mutant proteins were purified as described under “Materials and Methods.” Mutant proteins were well expressed and analyzed by SDS-PAGE. Protein expression for mutants of putative catalytic residues (Cys-191, His-218, and Asp-235), and purified proteins from cysteine mutants in two CXXC motifs (Cys-129, Cys-132, Cys-165, and Cys-168) are shown in Fig. 3 (a and b, respectively). As shown in Fig. 3 (c and d), these proteins were also detected by Western blotting using anti-(his)_{6} antibody.

**Mutation of the Putative Catalytic Residues Resulted in Complete Loss of PNGase Activity—** The Cys-191, His-218, and Asp-235 of Png1p are well conserved residues throughout the Png1p homologs and were aligned with the catalytic triad, Cys-314, His-373, and Asp-396, of factor XIIIa (Fig. 2). Therefore, C191A, H218A, and D235A were prepared separately to determine the role of each of these residues on enzymatic activity. The concentrations of WT and mutant proteins were similar based on SDS-PAGE analysis (Fig. 3a) as well as Western blot using anti-(his)_{6} antibody (Fig. 3c). No deglycosylation of the fetuin-derived [^{14}C]glycopeptide was observed with any of the three mutant proteins even upon extended incubation (24 h), which is in sharp contrast with WT enzyme (Fig. 4; compare lane 2 (WT) and lanes 3–5 (mutants)). Our data thus provide the first biochemical evidence that support the hypothesis that

| Amino acid mutated | PNGase activity relative to WT | Residue alignment with Factor XIIIa |
|--------------------|-------------------------------|-----------------------------------|
| R187A              | 96.5 Conserved                |                                   |
| C191A              | ND                          | Conserved                         |
| C191A              | ND                            | Conserved                         |
| C132S              | 6.7 Not conserved             |                                   |
| C132S              | 7.6 Not conserved             |                                   |
| C165A              | 7.7 Not conserved             |                                   |
| C165S              | 6.2 Not conserved             |                                   |
| F224A              | 99.8 Not conserved            |                                   |
| D235A              | 97.9 Not conserved            |                                   |
| D235A              | 98.6 Conserved                |                                   |
| F261A              | 97.9 Not conserved            |                                   |
| D264A              | 98.6 Not conserved            |                                   |
| V266A              | 98.6 Not conserved            |                                   |
| D268A              | 98.6 Not conserved            |                                   |
| Y354A              | 84.3 Not conserved            |                                   |
| W355A              | 87.5 Not conserved            |                                   |

ND, activity not detected.

With these mutants activity was detected only after 40 h of incubation. Values shown for other mutants were obtained after 30 min of incubation as described under “Materials and Methods.” Activity for WT is set to 100%.

Png1p is a novel member of the transglutaminase superfamily (11), all of which use a similar catalytic triad to carry out their enzymatic reactions. This is a common type of enzymatic catalysis in cysteine proteases (22), serine proteases (23, 24), and \( \alpha/\beta \) hydrolases (25).

**Aromatic Residues Are Essential for the Structural Stability of the Protein—** Other than the aforementioned catalytic residue mutants, several other single-amino acid mutants that lacked enzyme activity were identified by extensive mutant
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Charged Residue Arginine May Form Hydrogen Bonding to the Side Chain of Glutamic Acid—Besides the catalytic triad and hydrophobic residues, other residues, e.g. Arg-210 and Glu-222 of Png1p, which are aligned with Arg-333 and Glu-377 of factor XIIIa, were also found vitally important for the activity of Png1p (Table II). Arg-333 and Glu-377 are involved in the formation of a hydrogen bond in factor XIIIa. Thus, the loss of activity with mutants R210A or E222A is most likely due to the altered conformation of the core structure of the enzyme as confirmed by CD analysis. This result leads us to assume that a similar hydrogen bond might be formed in Png1p.

CXCX Motifs and Other Conserved Residues (Not Conserved in Factor XIIIa) in Png1p Are Essential for the Activity—Among Png1p homologs there are sequence homologies outside the “transglutaminase motif” (9). Among these conserved residues are four cysteine residues that form two CXCX motifs (Cys-129, Cys-132, Cys-165, and Cys-168 in Png1p) (9). The Cys-to-Ala mutations of these residues resulted in loss of the enzymatic activity (Table II). However, mutation to Ser yielded an enzyme with a very low conserved level.

Among the other residues Phe-261, Asp-268, Tyr-273, and Tyr-273 are also highly conserved in PNGases from diverse organisms (but are not conserved in factor XIIIa). These residues spatially located farther from the catalytic residues were found to be crucial for enzyme activity (Table II), because their mutation destroyed activity. CD analysis of Y273A also confirms that this is an essential residue in maintaining the native structure of the enzyme (see below). Besides these residues, none of the other conserved residues in Png1ps had any profound effect on enzyme activity (Table II).

CD Analysis Reveals Conformational Changes in Inactive Png1p Mutants—To further explore the structure-activity relationship of the WT and various inactive mutant Png1ps (C191A, R210A, W220A, and Y273A), far UV and near UV CD spectra were measured. An equal concentration of protein was used for all the experiments. The far UV CD spectra (Fig. 5A) showed negative peaks at 222 and 209 nm and a positive peak at 195 nm, typical of proteins with a high α-helix and low β-sheet content, in agreement with the data available on the secondary structure prediction of Png1p (26). However, loss in α-helicity was observed with the mutant proteins. The far UV CD spectrum of a protein can be deconvoluted to estimate the fraction of secondary structure components (27). An analysis of the secondary structure of WT and mutant Png1p is shown in Table III. Compared with WT, the mutant forms have 7–20% less α-helix structure. Concomitantly, a gain in the percentage of the β-sheet was observed in the mutants. Thus, interconversion of α-helix into β-sheets may have caused the inactivation of the enzyme.

Near UV CD was also used to study the difference in the folded conformation of the WT and mutant forms. The CD spectrum of WT protein showed peaks around 270, 285, and 295, which are attributable to phenylalanine, tyrosine, and tryptophan residues, respectively (Fig. 5B). However, the absorbance pattern changed when mutant proteins (C191A, R210A, W220A, and Y273A) were used (Fig. 5B). These results are consistent with a non-native conformation for the mutant Png1p protein, and the loss in their enzyme activity is most likely due to disruption of the native structure of the enzyme.

Kinetic Analysis of WT and Mutant Png1p—The results in Table II showed that for four Cys-to-Ser mutants (C129S, C132S, C165S, and C168S) no activity was observed after 30 min of incubation. However, after extended incubation (40 h) these Cys-to-Ser mutants revealed very low level of activity, whereas other mutants upon extended time showed no further effect on activity. The differential behavior of these four mutants prompted us to analyze their kinetic parameters and compare the mutant enzymes with WT.
C129S, C132S, C165S, and C168S mutants belong to two CXXC motifs located at the N terminus in all Png1p homologs (9). These mutants were purified as described under "Materials and Methods." The purity of all four protein samples were analyzed by using 10% SDS-PAGE (Fig. 3b). The amount of protein in these Cys to Ser mutants was comparable to WT analyzed by using Western blot (Fig. 3d). Despite the presence of an equal amount of protein in the Cys to Ser mutant, the activity was remarkably low in all the four mutants compared with WT. The kinetic parameters of all four Cys to Ser residues were compared with WT enzyme. As shown in Table IV, more than a 1000-fold difference was found in \( V_{\text{max}} \) and turnover rate of WT compared with Cys-to-Ser mutants. However, the \( K_m \) was similar for the WT and these mutant Png1p. The extremely reduced activity of Cys-to-Ser mutants in the CXXC motifs, therefore, suggest that the hydroxyl bond of the substituted amino acid cannot fully replace the sulfhydryl bond of cysteine residue at that position.

**TABLE III**

Determination of the percentage of \( \alpha \)-helix and \( \beta \)-sheets in WT and mutant Png1p using selcon3 (27) program

| WT or mutant | \( \alpha \)-Helix | \( \beta \)-Sheet |
|--------------|------------------|-----------------|
| WT           | 44.37            | 2.67            |
| C191A        | 37.81            | 6.48            |
| R210A        | 32.77            | 15.01           |
| W220A        | 29.70            | 18.31           |
| Y273A        | 19.02            | 28.64           |

**TABLE IV**

Determination of the kinetic parameters of four Cys to Ser mutants of CXXC motifs

| WT or mutant | \( K_m \) \( \mu \text{M} \) | \( v_{\text{max}} \) \( \mu \text{mol/mg.min} \) | Relative turnover rate* |
|--------------|-----------------|-----------------|----------------------|
| WT           | 210             | 140             | 1                    |
| C129S        | 110             | 0.034           | 4.4 \times 10^{-4}   |
| C132S        | 130             | 0.047           | 5.2 \times 10^{-4}   |
| C165S        | 160             | 0.061           | 5.7 \times 10^{-4}   |
| C168S        | 210             | 0.052           | 3.6 \times 10^{-4}   |

* Turnover rate for WT is set to 1.

**Fig. 6.** The core structure of factor XIIIa. Conserved residues in factor XIIIa were mapped on the core structure (Protein Data Bank code 1fie). The figure was generated by using the Raster3D program (36). All the residues are shown by the ball-and-stick model.

C129S, C132S, C165S, and C168S mutants belong to two CXXC motifs located at the N terminus in all Png1p homologs (9). These mutants were purified as described under "Materials and Methods." The purity of all four protein samples were analyzed by using 10% SDS-PAGE (Fig. 3b). The amount of protein in these Cys to Ser mutants was comparable to WT analyzed by using Western blot (Fig. 3d). Despite the presence of an equal amount of protein in the Cys to Ser mutant, the activity was remarkably low in all the four mutants compared with WT. The kinetic parameters of all four Cys to Ser residues were compared with WT enzyme. As shown in Table IV, more than a 1000-fold difference was found in \( V_{\text{max}} \) and turnover rate of WT compared with Cys-to-Ser mutants. However, the \( K_m \) was similar for the WT and these mutant Png1p. The extremely reduced activity of Cys-to-Ser mutants in the CXXC motifs, therefore, suggest that the hydroxyl bond of the substituted amino acid cannot fully replace the sulfhydryl bond of cysteine residue at that position.
PNGase activity has been implicated in the proteasome-dependent degradation of proteins (7–10, 28–31). Recently it has been reported that yeast Png1p physically associates with the 26 S proteasome through Rad23 protein, a nuclease excision repair protein (10). Furthermore, the mouse homolog of Png1p has been found to bind numerous ubiquitin proteasome pathway-related proteins, supporting the idea that Png1p is indeed involved in proteasomal degradation processes (31). Based on primary structure comparisons, Png1p has been reported to be a member of the transglutaminase superfamily of proteins (11).

In the current study, structure-function relationship of Png1p was further investigated by identifying critical amino acid residues that are essential for enzyme activity.

The reactions catalyzed by these enzymes in transglutaminase superfamily so far involve either the formation of amides by linking alkylamines to the glutamate side chains of proteins (transglutaminase) or hydrolysis of peptide bonds (proteases) (11). Png1p inclusion in the transglutaminase superfamily is consistent with the reaction that involves breakage of the amide bond between N-acetylglucosamine and an asparagine side chains (32).

The three-dimensional structure of Png1p is currently unknown. Thus, the structural effects of the point mutations studied were predicted using the three-dimensional structure of factor XIIa (18). In Fig. 6 is shown the structural location of the catalytic and other conserved residues in factor XIIa. The catalytic residue Cys-314 in factor XIIa, which is shown to be essential for the enzyme's catalytic activity (33, 34), is located at the amino terminus of the longest helix in the structure. The sulfhydryl group of Cys-314 forms a hydrogen bond with cationic His-373 (34, 35), which emanates from a strand in the middle of a β-sheet. The terminal oxygen atom of Asp-396 forms a hydrogen bond with the other nitrogen atom of the His-373. Asp-396 is located on the neighboring strand of the same β-sheet. The hydrogen bond interaction between Cys-314 and His-373 is that of a thiol-imidazolium ion pair, as in the cysteine proteases. The loss in activity of Png1p upon mutation of any one of the three catalytic residues (Cys-191, His-218, and Asp-235) to Ala, therefore, may be due to the breakage of hydrogen bonds between Cys-191, His-218, and Asp-235 thus, affecting the native-like structure which in turn effects charge relay. Loss in hydrogen bond would disrupt the native-like structure, which is consistent with CD analysis of the C191A showing non-native conformation for the enzyme. Our results are consistent with the loss in activity for all the three catalytic residues when mutated to Ala, one at a time, in factor XIIa (34).

Among the conserved residues in Png1p, aromatic residues Trp-220 and Trp-231, which are also conserved in factor XIIa (Fig. 2) showed a loss of enzyme activity on mutation to Ala. A plausible explanation for this can be based on the known structure of factor XIIa. The equivalent aromatic residues in factor XIIa are Trp-375 and Trp-392, respectively. Trp-375 is sequentially and spatially close to the catalytic residue His-373 and has hydrophobic interactions with the aromatic ring of His-373 thus stabilizing the orientation of this catalytic residue (Fig. 6). Another conserved residue, Trp-392, is found in the terminal region of strand 3 and interacts with the hydrophobic part of the side chain of Arg-333. The importance of these hydrophobic interactions between the catalytic His-218 and Trp-220 and the side chain of Arg-210 and Trp-231 in Png1p was elucidated by mutation of Trp-220 and Trp-231 to an equivalent aromatic residue Phe. Interestingly, both of these mutants retained enzyme activity.

In factor XIIa, the side-chain of Arg-333 (strand 1) when mapped on the available core structure, indicates its involvement in the formation of a hydrogen bond with a distance of 2.8 Å to the side chain of Glu-377 located in strand 2 (see Fig. 6). The equivalent residues in Png1p are Arg-210 and Glu-222. In factor XIIa, these residues are likely to be firmly anchored in space to stabilize the structure. Mutation of Arg-210 and/or Glu-222 to Ala would thus tear away this hydrogen bond stabilizing strands 1 and 2 leading to a non-native conformation of the protein. This prediction, based on the sequence mapping in structure of factor XIIa, is consistent with the CD spectra of R210A. These results provide possible evidence for a hydrogen bond between Arg-210 and Glu-222 in Png1p.

The functions of two thioredoxin like motifs (CXXC) and other residues (Y257A, F261A, D268A, and Y273A) in Png1p remain elusive. None of these residues are found to be conserved in factor XIIa. Additional information is needed to
elucidate the biological and structural importance of the CXXC motifs and the other residues.

Taken together all of our results, one can conclude that mutations within the core domain of the enzyme have a profound influence on the structure of protein, because they interfere with proper functioning of the enzyme. The present data indicates that the fold of the core domain of Png1p might be similar with the other members of the transglutaminase superfamily, therefore suggesting a common evolutionary lineage (26). The most significant achievement of this study was the identification of catalytic amino acid residues Cys-191, His-268, and Asp-235 in Png1p, which are involved in the cleavage of amide bond between N-acetylglucosamine and the Asn side chain in a glycopeptide/glycoprotein. These catalytic residues are well conserved in all eukaryotic PNGases, which leads us to propose the catalytic mechanism, similar to the proteolytic mechanism of serine proteases, in the deglycosylation reaction as shown in Fig. 7. The requirement of a nucleophile is fulfilled by Cys-191, which attacks the carbonyl group of the side chain as shown in Fig. 7. The requirement of a nucleophile is fulfilled by Cys-191, which attacks the carbonyl group of the side chain in a glycopeptide/glycoprotein. These catalytic residues are linked to glycan generating a glycosylamine. Water is the attacking nucleophile and ammonia as shown in Fig. 7. The requirement of a nucleophile is fulfilled by Cys-191, which attacks the carbonyl group of the side chain in a glycopeptide/glycoprotein. These catalytic residues are linked to glycan generating a glycosylamine. Water is the attacking nucleophile and ammonia as shown in Fig. 7.

This is the first study on the structure-activity relationships of Png1p. Additional tools, e.g. crystal structure or more structural investigations are needed to further elucidate the structure-function relationship of PNGases, and this line of study is currently underway.

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