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Influence of Substrate Conformation on the Deglycosylation of Ribonuclease B by Recombinant Yeast Peptide:*N*-glycanase

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Abstract Peptide:*N*-glycanase has been thought to be responsible for proteasome-dependent degradation of misfolded glycoproteins translocated from the endoplasmic reticulum (ER) to the cytosol. Therefore, the enzyme was supposed to be able to distinguish between native and non-native glycoproteins. In the present study, a recombinant, yeast peptide:*N*-glycanase, Png1p, was expressed in *Escherichia coli* as inclusion bodies and was purified, refolded and characterized. The results showed that the recombinant enzyme has a broad pH range adaptation, from pH 4.0 to pH 10.0, and has an optimum temperature of 30 ºC. This enzyme is a zinc metalloenzyme. Its activity was abolished with the addition of EDTA and not restored by adding metal ions. Furthermore, the deglycosylation efficiency of recombinant Png1p from *E. coli* was investigated with respect to the substrate conformation *in vitro*. When ribonuclease B (RNase B) was denatured at 60–65 ºC or by 40–60 mM dithiothreitol, indicated by its obvious structural change and sharpest activity change, its deglycosylation by Png1p was most prominent. The deglycosylation efficiency of RNase B by Png1p was found to be related to its structural conformation and enzymatic activity.

Key words glycanase; ribonuclease B; glycoprotein; deglycosylation; circular dichroism

Peptide-N^4-(*N*-acetyl-β-*D*-glucosaminyl) asparagine amidase (EC 3.5.1.52), also known as peptide:*N*-glycanase or PNGase, is a deglycosylating enzyme, which catalyzes the detachment of *N*-linked glycan chains from glycopeptides or glycoproteins by hydrolyzing the β-aspartylglucosaminyl bond. Peptide:*N*-glycanases existing in yeast and other higher eukaryotic cells including plant, worms, insect and mammals are also called Png1p [1]. Png1p has been reported to be present in the endoplasmic reticulum (ER), microsome and cytosol [2], but most is present in cytoplasm [3]. The cytoplasmic Png1p is distributed in two regions: ER-associated and free in the cytosol [4–6]. It was found that the cytoplasmic Png1p was quite distinct in terms of enzymatic properties from the PNGases of plant and bacterial origin because they require an -SH group, a neutral pH for optimal activity and unique carbohydrate binding properties [7]. In yeast, the *Png1p* gene was mapped to the left arm of chromosome XVI by genetic approaches, which encodes a 42.5 kDa soluble protein of 363 amino acids with no apparent signal sequence [8].

Peptide:*N*-glycanase has been thought to be linked to proteasome-dependent degradation for misfolded glycoproteins that are translocated from the ER to the cytosol [9]. Newly synthesized *N*-linked glycoproteins are folded in the ER and enter the secretory pathway, whereas misfolded glycoproteins are initially retained in the ER and subsequently exported to the cytoplasm. In the cytoplasm, they undergo proteasomal mediated ER-associated degradation [10].

Three amino acids, Cys191, His218 and Asp235, are proposed to be involved in the enzyme catalytic activity [11]. This catalytic triad is located in a “transglutaminase” motif, which is the most conserved region in Png1ps from various sources. Therefore, they have been proposed to be part of the transglutaminase-like superfamily. Yeast Png1p is a zinc metalloenzyme, whose Zn-binding domain

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consists of five strands (S1, S2, S3, S4 and S5) and two helices (H7 and H8) [12]. Transglutaminases, which cross-link proteins through an acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine, resulting in an ε-(γ-glutamyl) lysine isopeptide bond, require calcium binding. Reducing reagents, such as dithiothreitol (DTT), are required for *in vitro* enzyme activity of the cytoplasmic Png1p [9].

In yeast, Png1p binds to the 26S proteasome through the interaction with a component of the DNA repair system, Rad23p, which is known to play a pivotal role in nucleotide excision repair [13]. Although Rad23-Png1p binding was observed in yeast Png1p, another interaction was observed in mammalian cells. A previous report indicated that in mammalian cells Png1p also interacts with the C-terminus of Derlin-1 via the N-terminal domain. Derlin-1 is a 22 kDa ER membrane protein consisting of four trans-membrane domains with both the amino and carboxy termini in the cytosol [4–6]. A recent report indicated a complex containing five proteins, mAMFR, mY33K, mp97, mPNGase and mHR23B, is formed in close proximity to the ER membrane [14]. Yeast Png1p has been able to distinguish between native and non-native glycoproteins [15]. However, it is not clear at what status the glyco-protein substrate will be deglycosylated.

The current study set up a number of experiments to test how conformation change of glycoprotein substrate, such as RNase B, affects the deglycosylation activity of peptide:*N*-glycanase *in vitro*.

Materials and Methods

Bacterial expression of *Png1p*

DNA manipulations were carried out according to Sambrook and Russell [16]. The full-length *Png1p* gene sequence was amplified from yeast chromosomal DNA using *pfu* polymerase. The primers are 5′-ATCCATGGGA-GAGGTATACGAAAAAA-3′ and 5′-ATCTCGAGTTTAC-CATCCTCCCCAC-3′. The corresponding *Png1p* gene sequence was cloned into *Nco*I and *Xho*I sites of pET-28a (+) vector (Novagen, Carlsbad, USA). The resulting construct (pET28a/*Png1p*) was transformed into *Escherichia coli* BL21(DE3) pLysS. Expression of *Png1p* gene in *E. coli* was induced by adding 1 mM IPTG at A_{600}=0.8. After 3 h of induction, cells were harvested and disrupted using sonication in buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% glycerol) containing 1% Triton X-100. The mixture was centrifuged at 10,000 *g* for 20 min, and checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the precipitate was collected.

Purification and refolding of Png1p-His6

The precipitate containing Png1p-His6 inclusion bodies were washed with buffer A [50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 100 mM NaCl, 0.5% (*V/V*) Triton X-100, 4 M urea], buffer B (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM NaCl, 3% Triton X-100), buffer C (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 2 M guanidine hydrochloride). Finally, the inclusion bodies were washed with distilled water to remove contaminating salt and detergent, centrifuged at 10,000 *g* for 30 min. The purified Png1p-His6 inclusion bodies were dissolved in buffer D (50 mM HEPEs-NaOH, pH 7.5, 6 M guanidine hydrochloride, 25 mM DTT). Refolding of the purified inclusion bodies was carried out in buffer E (50 mM HEPEs-NaOH, pH 7.5, 0.2 M NaCl, 1 mM DTT, 0.5 M *L*-Arg).

The refolded Png1p was loaded on a Ni^{2+}-NTA column (Qiagen, Hilden, Germany) and equilibrated with buffer F (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% glycerol). The column was washed with five column volumes of buffer F containing 10 mM imidazole and 0.1% Triton X-100, followed by 10 column volumes of buffer F containing 15 mM imidazole. Png1p-His6 was eluted with 5 column volumes of elution buffer (20 mM Tris-HCl, pH 8.0, 5% glycerol, 1 mM DTT, 200 mM imidazole). The imidazole was removed using Sepharose G25 with HEPEs-NaOH buffer (80 mM, pH 7.0).

Png1p activity assay

Fifty micrograms of glycoprotein and ribonuclease B (RNase B) was incubated with 5 μl purified Png1p-His6 in 50 μl HEPEs-NaOH buffer (80 mM, pH 7.0) containing 5 mM DTT at 30 ºC for 15 h. The reaction mixture was analyzed by 15% SDS-PAGE.

Glycoprotein denaturation

Stocks of 1 mg/ml RNase B were prepared in HEPEs-NaOH buffer (80 mM, pH 7.0). For glycoprotein de-naturation in DTT, aliquots from the stock of RNase B was added to solutions with different concentrations of DTT (5, 10, 20, 40, 60, 80, 100 mM), respectively, and incubated for 2 h at 4 ºC. For glycoprotein denaturation by heat, an aliquot from the stock of RNase B was heated at different temperatures (40 ºC to 85 ºC) for 20 min and rapidly frozen in a dry ice-ethanol bath.

Circular dichroism spectroscopy

All circular dichroism (CD) measurements were made on a J-810 Jasco spectropolarimeter (Jasco Co., Tokyo, Japan) equipped with a PTC-348 WI thermostat under a constant nitrogen flow. A 0.1-cm path length cell was used to collect data in the far ultraviolet region (190–240 nm). Spectra were measured in buffer containing 2 mM HEPEs-NaOH (pH 7.0). The spectra of a blank containing buffer alone or containing DTT at different concentrations were subtracted from all spectra. The samples with DTT at different concentrations (5, 10, 20, 40, 60, 80, 100 mM) were measured at room temperature; and the samples without DTT were measured in the melting experiment temperature (40–85 °C), which was increased at a scan rate of 5.0 °C/min, and the CD signals at 222 nm were recorded.

RNase B activity assay

The 0.5 ml reaction mixture, containing 50 mM 3-(N-morpholino)propane-sulfonic acid (MOPS), 5 mM $MgCl_2$, 50 μl yeast RNA and 5 ng RNase B, was incubated at 30 °C. After 15 min, 0.5 ml 10% trichloroacetic acid (TCA) was added. The reaction mixture was then cooled in an ice-water bath for 15 min and centrifuged at 10,000 *g* for 10 min. The increased absorbency at 280 nm over corresponding RNase-free blanks of the supernatant was measured [17].

Results

Enzyme properties of recombinant Png1p

Recombinant Png1p from *E. coli* showed the maximum activity at 30 °C (**Fig. 1**), which is similar to its wild form from yeast, but Png1p was not stable at temperature higher than 30 °C over a long incubation. The pH optimum of the wild type Png1p from yeast was determined to be 6.6 using Mes buffer and 7.0 using HEPEs buffer in a previous experiment using glycopeptides as the substrate [7]. Our experiment indicated that this recombinant enzyme using glycoprotein as a substrate has a broad pH range adaptation, from pH 4.0 to pH 10.0 (**Fig. 2**).

The activity of EDTA treated Png1p was not restored by adding metal ions

Yeast Png1p is a zinc metalloenzyme, whose Zn-binding domain consists of five strands (S1, S2, S3, S4 and S5) and two helices (H7 and H8) [12]. The addition of metal chelating agent, EDTA (20 mM), abolished Png1p deglycosylation activity. However, re-addition of Zn^{2+} could not restore the activity. Enzyme activity was not restored by adding either Ca^{2+} or Co^{2+} in protein solution without Zn^{2+} (**Fig. 3**).

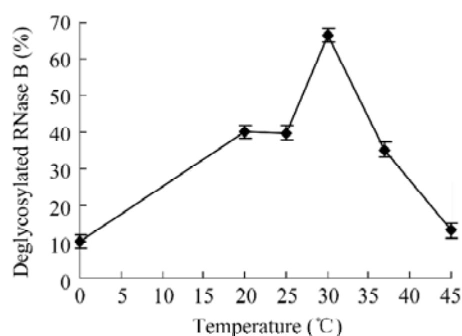

Fig. 1 Influence of temperature on Png1p activity
Ribonuclease B (RNase B) (0.5 mg/ml) was treated in HEPEs (pH 7.0) buffer for 15 h. 1, control; 2, 20 °C; 3, 25 °C; 4, 30 °C; 5, 37 °C; 6, 45 °C.

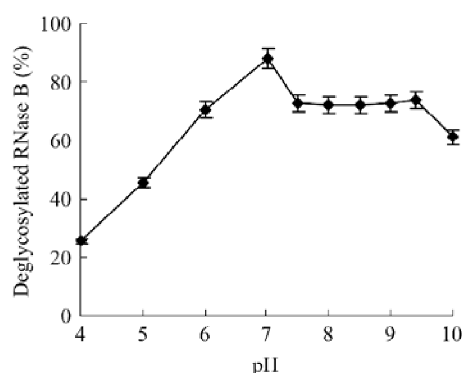

Fig. 2 Influence of pH on Png1p activity
Ribonuclease B (RNase B) (0.5 mg/ml) was treated in different buffers (pH 4.0–6.0, NaOH-HAc; pH 7.0, HEPEs-NaOH; pH 7.5–8.5, Tris-HCl; pH 9.0 and pH 10.0, Gly-NaOH; pH 9.4, Borax) at 30 °C for 15 h. 1, pH 4.0; 2, pH 5.0; 3, pH 6.0; 4, pH 7.0; 5, pH 7.5; 6, pH 8.0; 7, pH 8.5; 8, pH 9.0; 9, pH 9.4; 10, pH 10.0.

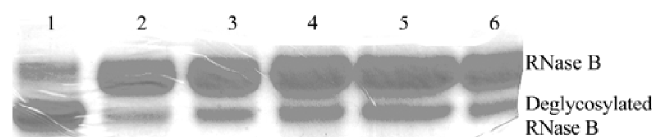

Fig. 3 Effects of the metal chelating agent EDTA and other metal ions on the deglycosylation activity Png1p
Deglycosylation of denatured ribonuclease B (RNase B) (0.5 mg/ml) by different Png1p. 1, wild type-Png1p; 2, control; 3, Png1p treated with EDTA; 4, add Zn^{2+} to Png1p treated with EDTA; 5, add Co^{2+} to Png1p treated with EDTA; 6, add Ca^{2+} to Png1p treated with EDTA.

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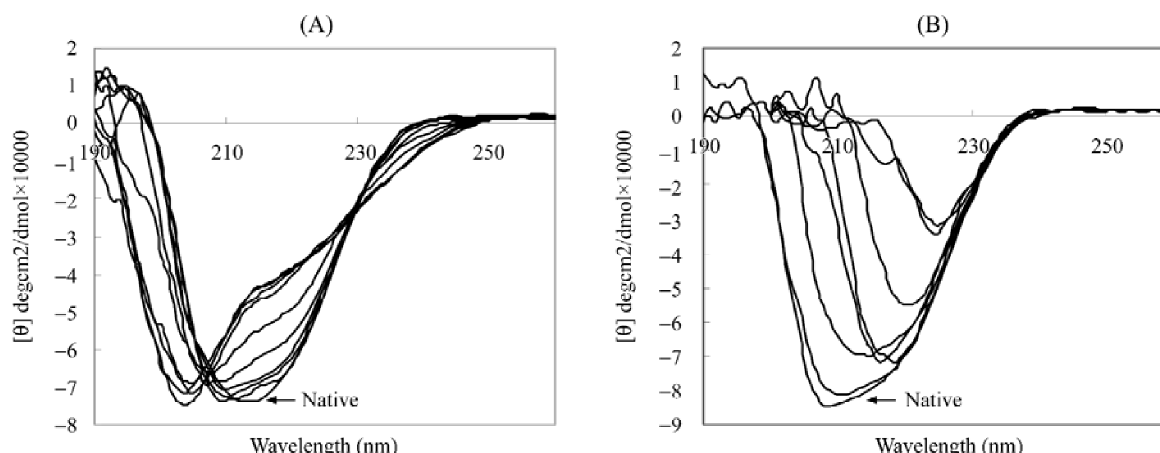

Fig. 4 Far ultraviolet circular dichroism (CD) spectral analysis of ribonuclease B (RNase B)
(A) CD spectrum of RNase B at different temperatures. (B) CD spectrum of RNase B at different DTT concentrations.

Substrate status affects the deglycosylation efficiency

Native RNase B showed a negative peak at 210 nm and a positive peak at 195 nm, which is typical for a protein that has a predominant α-helix conformation. The peak at 215 nm indicated a β-pleated sheet. The spectrum of the denatured RNase B showed decreased α-helix content and β-pleated sheet, confirming the loss in secondary structure after heat treatment (**Fig. 4**). In our experiment, the deglycosylation process of the glycoprotein was monitored with respect to the substrate conformation. Denatured by heating, the change of substrate secondary structure was detected by the CD spectrum [**Fig. 4(A)**]. During the heating denaturing process, the most obvious structural change of RNase B was observed at 60–65 °C and 70–80 °C. At 60–65 °C, α-helix content of RNase B decreased from approximately 11% to 8% and random coil increased from 29% to 34% (**Table 1**). At this point, the RNase B activity decreased suddenly, which indicated the loss of its native structure. This secondary structural change led to the efficient deglycosylating start of the target glycoprotein by Png1p (**Fig. 5**). With the increase of the denaturing temperature, the efficiency of deglycosylation also increased. When the denaturing temperature reached 80 °C, the random coil of RNase B increased to approximately 40%, indicating a complete loss of its native structure. This was confirmed by the complete loss of RNase B activity. The deglycosylation efficiency at this point was the highest.

There are four disulfide bonds in RNase B: Cys26-Cys84, Cys40-Cys95, Cys58-Cys110 and Cys65-Cys72. Treated by DTT with different concentrations, the RNase B

Table 1 Composition of α-helix, β-pleated sheet, β-turn and random coil at different temperatures

Temperature (°C)	α-helix (%)	β-pleated sheet (%)	β-turn (%)	Random coil (%)
0	10.7	52.0	8.2	29.1
40	10.5	53.1	7.6	28.8
45	11.9	48.8	9.7	29.5
50	11.7	47.9	11.3	29.1
55	11.3	50.7	8.6	29.5
60	8.5	50.1	7.1	34.3
65	8.5	50.1	7.1	34.3
70	5.1	51.5	6.0	37.4
75	5.8	47.6	6.4	40.2
80	4.5	50.1	5.2	40.2
85	4.4	50.0	6.5	39.0

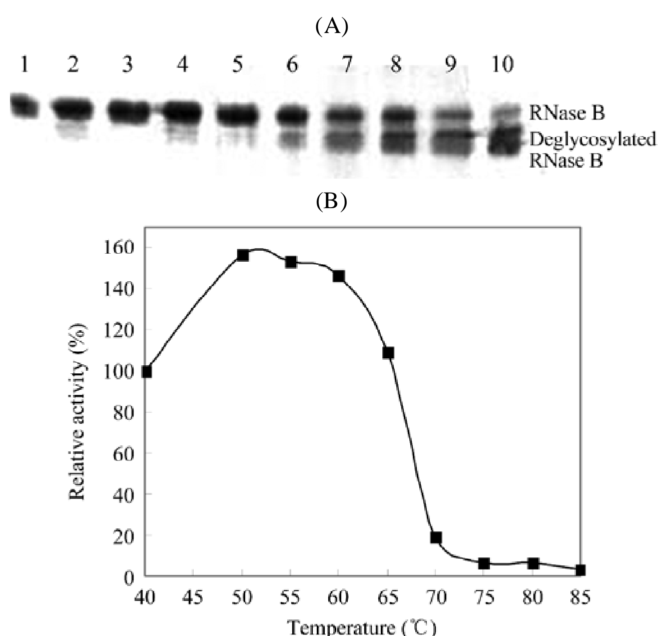

Fig. 5 The activity relations between Png1p and RNase B at different temperatures

(A) Deglycosylation of denatured RNase B (0.5 mg/ml) at different temperatures by Png1p. 1, control; 2, 40 ºC; 3, 50 ºC; 4, 55 ºC; 5, 60 ºC; 6, 65 ºC; 7, 70 ºC; 8, 75 ºC; 9, 80 ºC; 10, 85 ºC. (B) The activity of RNase B at different temperatures.

showed a different secondary conformation [18]. From the CD spectrum, the glycoprotein showed a slight difference even treated with 5 mM DTT [**Fig. 4(B)**]. With the increase of DTT concentration, RNase B gradually lost its native form. This was shown by an increase of α-helix and β-turn from 10% to 20% and a decrease of β-pleated sheet from 50% to 35% (**Table 2**). During this process, RNase B began to be hydrolyzed by Png1p. When the DTT concentration reached 40–60 mM, the content of β-pleated sheet of RNase B decreased to almost zero. At this point, the RNase B activity decreased sharply, although the deglycosylation of the glycoprotein by Png1p became more efficient (**Fig. 6**).

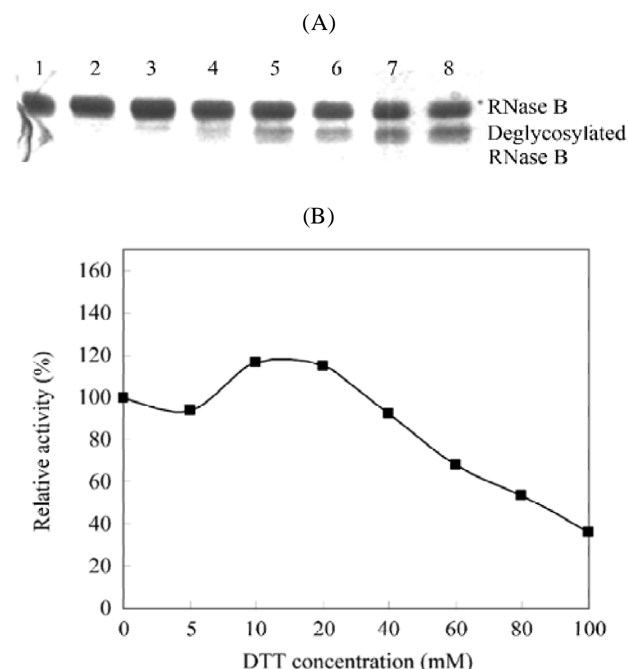

Fig. 6 The activity relations between Png1p and RNase B at different DTT concentrations

(A) Deglycosylation of denatured RNase B (0.5 mg/ml) at different DTT concentrations by Png1p. 1, control; 2, 5 mM; 3, 10 mM; 4, 20 mM; 5, 40 mM; 6, 60 mM; 7, 80 mM; 8, 100 mM. (B) RNase B activity at different DTT concentrations.

Discussion

PNGase is a deglycosylating enzyme that has been thought to be linked to proteasome-dependent degradation for misfolded glycoproteins that are translocated from the

Table 2 Composition of α-helix, β-pleated sheet, β-turn and random coil at different DTT concentration

Concentration of DTT (mM)	α-helix (%)	β-pleated sheet (%)	β-turn (%)	Random coil (%)
0	10.7	52.0	8.2	29.1
5	10.8	50.2	9.8	29.3
10	17.7	32.3	20.2	29.8
20	19.9	27.4	21.4	31.3
40	21.0	23.6	23.1	32.3
60	29.5	0.0	31.0	39.5
80	30.2	0.0	27.2	42.6
100	26.3	0.0	27.2	46.5

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ER to the cytosol [9]. Png1p from yeast was now confirmed to act on both glycopeptides and full length glycoproteins. A retrotranslocated, misfolded glycoprotein is first deglycosylated by Png1p and subsequently degraded by the proteasome [15,19]. However, deglycosylation of glycopeptides derived from proteasomal degradation cannot be ruled out. The earlier finding indicated that this enzyme acted only on glycopeptides, which presumably arises from proteasome-mediated proteolysis of glycoprotein, might be a result of incompletely denatured substrates used in the previous experiments [20]. Furthermore, yeast Png1p has activity only on non-native glycoproteins *in vitro* [15]. Does Png1p act only on the completely denatured glycoprotein? In the present study, the relation between substrate conformation and Png1p deglycosylation efficiency was investigated *in vitro*.

The recombinant Png1p expressed in *E. coil* has an optimum temperature of 30 ºC, which is similar to that reported for the wild type Png1p from yeast. This recombinant enzyme using glycoprotein as substrate has a broad pH range adaptation, from pH 4.0 to pH 10.0, although the wild type one from yeast is pH 6.6 using Mes buffer and pH 7.0 using Hepes buffer in previous experiments using glycopeptides as substrate. Png1p from yeast is a zinc metalloenzyme whose activity was abolished with the addition of EDTA and not restored by adding metal ions. The results indicated the Zn^{2+} plays an important role in the activity of Png1p and is irreplaceable, although it is somewhat distantly located from the active site.

Both reductive unfolding and heat denaturation of substrate caused the conformational changes of the target glycoproteins. However, the conformational change of RNase B caused by the reductive unfolding is different from that caused by heating. The heat denaturation of RNase B decreased α-helix and β-turn content, whereas the reductive unfolding decreased β-pleated sheet content. All these secondary structural changes caused by heating and reductive unfolding led to the formation of extended random conformations, which was indicated as random coil increase in the CD spectrum. Partially unfolded RNase B might also be deglycosylated by Png1p *in vitro*. The critical point for RNase B is 60–65 ºC and 40–60 mM DTT and these characteristics vary from one glycoprotein to another. The deglycosylation efficiency of RNase B by Png1p was confirmed to be linked with its own structural conformation, and was related to enzymatic activity.

We presumed that the native glycoprotein structure shielded substrate access pathway of Png1p, a conformational change of the substrate might overcome this constraint. To access the enzyme active site, substrates must have conformation that they can easily fall into this cleft. However, most glycoproteins are not small enough or soft enough to be positioned correctly into the active site of Png1p. Both reductive unfolded glycoprotein and heat denatured glycoproteins would be highly flexible and would easily access the active site without constraint by the cleft. Recently, it was confirmed that the active site of Png1p is located deep inside the large cleft, where strands S2 and S3 in the Zn-binding domain and two loops between H10 and H11 helices and between H9 and S10 of the core domain located at each side of the cleft [12].

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