Different effects of calnexin deletion in *Saccharomyces cerevisiae* on the secretion of two glycosylated amyloidogenic lysozymes

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Abstract Both glycosylated amyloidogenic lysozymes I55T/G49N and D66H/G49N were expressed in wild-type and calnexin-disrupted *Saccharomyces cerevisiae*. The secretion amounts of mutant I55T/G49N were almost similar in both wild-type and calnexin-disrupted *S. cerevisiae*. In contrast, the secretion of mutant D66H/G49N greatly increased in calnexin-disrupted *S. cerevisiae*, while the secretion was very low in the wild-type strain. In parallel, the induction level of the molecular chaperones BiP and PDI located in the endoplasmic reticulum (ER) was investigated when these glycosylated amyloidogenic lysozymes were expressed in wild-type and calnexin-disrupted *S. cerevisiae*. The mRNA concentrations of BiP and PDI were evidently increased when mutant lysozyme D66H/G49N was expressed in calnexin-disrupted *S. cerevisiae*, while they were not so increased when I55T/G49N mutant was expressed. This observation indicates that the conformation of mutant lysozyme D66H/G49N was less stable in the ER, thus leading to the higher-level expression of ER molecular chaperones via the unfolded protein response pathway. This suggests that glycosylated amyloidogenic lysozyme I55T/G49N may have a relatively stable conformation in the ER, thus releasing it from the quality control of calnexin compared with mutant lysozyme D66H/G49N.

Key words: Calnexin; Quality control; *N*-Glycosylation; Amyloidogenic lysozyme; *Saccharomyces cerevisiae*

1. Introduction

The endoplasmic reticulum (ER) quality control system is extremely important to the eukaryotic organism which retains misfolded intermediates in the ER until these substrates fold properly or until the misfolded proteins are degraded [1–3]. It seems likely that the stable conformation of the nascent protein in the ER leads it to the secretion pathway and the unstable conformation of the nascent protein leads it to the degradation pathway [4]. The lowering of the function of the ER quality control system usually leads the organism to suffering the over-expression of misfolded proteins which may result in serious diseases. Calnexin is a component of the ER quality control system through its oligosaccharide moieties for glycoproteins [3]. Interestingly, disruption of the calnexin gene leads to the deletion of ER quality control for glycoproteins but does not affect the normal growth of *Saccharomyces cerevisiae* [4,5]. Thus, wild-type and calnexin-disrupted *S. cerevisiae* were used as model strains with and without ER quality control, respectively.

It has been reported that the two naturally occurring human lysozyme variants were both amyloidogenic, causing autosomal dominant hereditary amyloidosis [6]. The two natural mutations are heterozygous for the single base changes which encode non-conservative amino acid substitutions, Ile56Thr and Asp67His, respectively [6]. Ile56 is the pivotal residue for the structural integrity of the lysozyme fold in that it links the two domains. Its importance is emphasized by its high conservation in the lysozyme sequences [7,8]. Substitution of Asp67 with histidine destroys the network of hydrogen bonds that stabilizes the β domain, resulting in a large and concerted movement of the β sheet and the long loop within the β domain away from each other. Interestingly, the crystal structure of variant I56T is similar to that of wild-type lysozymes [6]. This is presumably due to the fact that the hydrogen-bonding potential of the hydrophilic side chain is partly satisfied by a hydrogen bond to one of the water molecules found in both the wild-type and the variant structures, even though its hydrophilic side chain is in an unfavorable hydrophobic environment [6]. We have reported in a previous paper that the mutant hen lysozymes Ile55Thr and Asp66His, corresponding to human amyloidogenic mutant lysozymes Ile56Thr and Asp67His, respectively, were secreted in the yeast *S. cerevisiae* and that glycosylated amyloidogenic hen egg white lysozymes I55T/G49N and D66H/G49N which have the *N*-glycosylation signal sequence (Asn-X-Ser) by the substitution of glycine with asparagine at position 49 could suppress the aggregation of amyloidogenic lysozymes expressed in yeast [9]. Both of the glycosylated amyloidogenic lysozymes had similar secondary structures with non-glycosylated types (I55T and D66H) and can be secreted in soluble form to the medium [9]. To investigate the effect of the quality control system on the glycosylated amyloidogenic lysozymes, we attempted to secrete glycosylated amyloidogenic lysozymes I55T/G49N and D66H/G49N in wild-type and calnexin-disrupted *S. cerevisiae*.

Recently Mori et al. [10] reported that, when unfolded proteins are accumulated in the ER, an intracellular signaling pathway termed the unfolded protein response (UPR) is activated to induce transcription of ER-localized molecular chaperones. It seems likely that we can estimate the accumulation unfolding level of nascent protein from the induced concentration of molecular chaperones. Therefore, this paper also compares the concentration of molecular chaperones (BiP

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and PDI) in the ER when glycosylated amyloidogenic lysozymes (I55T/G49N, D66H/G49N) are expressed in wild-type and calnexin-disrupted *S. cerevisiae*.

2. Materials and methods

2.1. Materials

Restriction endonuclease, T4 DNA ligase, DNA sequencing kit and DNA blunting kit were purchased from Takara Shuzo (Kyoto, Japan). The Quick Change™ site-directed mutagenesis kit was purchased from Stratagene (Cambridge, MA, USA). Synthetic oligonucleotides were purchased from Kurabo (Osaka, Japan). CM-Toyopearl resin was a product of Toso (Tokyo, Japan).

2.2. Bacterial strains and plasmids

Escherichia coli XL-1 blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac (F′ proAB, lacI^{q}Z ΔM15, Tn10 (tet^r)^c), which was used as a host cell in all cloning experiments, was supplied by Amersham Japan. The *S. cerevisiae* diploid strain W303-1b (Mat a ade2-1 can1-100 ura3-1 leu2-3, 112 trp1-1 his3-11, 15) and W303-1b Δ(*cne1::Leu2*) were provided by Dr. Parlati, McGill University, Canada. AH22 (Mat α, Leu, His4, Cir$^{+/-}$), used as another yeast expression strain of mutant lysozymes, was provided by Dr. I. Kumagai, Tohoku University. *S. cerevisiae* strain AH22 Δ(*cne1::Leu2*) was constructed in our previous study [5]. The recombinant plasmid pKK-1, which contains a full-length hen egg white lysozyme cDNA, was provided by Dr. I. Kumagai, Tohoku University [11]. pYG100, an *E. coli*-yeast shuttle vector, was provided by Dr. K. Matsubara of Osaka University. The yeast episomal vectors pRS423 and pRS426 were provided by Dr. R. Akada, Department of Applied Chemistry and Chemical Engineering, Yamaguchi University.

2.3. Construction of S. cerevisiae W303-1b and AH22 expression plasmids of the mutant hen lysozymes

The cDNAs of mutant lysozymes were inserted into the *Sal*I site in the pYG100 vector between the GPD promoter and terminator. The pYG100 carrying mutant lysozyme genes was treated with *Hin*dIII. Thus, a fragment (1.7 kb) was obtained from the promoter to the terminator region containing the mutant hen lysozyme cDNA. By using the blunt end approach, these fragments were inserted into the multiclonal site of pRS426, the expression plasmid of yeast *S. cerevisiae* W303-1b, and the multiclonal site of pRS423, the expression plasmid of yeast *S. cerevisiae* AH22.

2.4. Expression of the mutant lysozymes in S. cerevisiae W303-1b and AH22

The expression vector was introduced into *S. cerevisiae* W303-1b and AH22 by the lithium acetate procedure. The over-expressing subclones with the highest levels of lysozyme activity were screened and propagated from single colonies inoculated into 3 ml of yeast minimal medium and incubated for 2 days at 30°C with shaking. This preculture was subcultured to 100 ml of the same medium in a flask (500 ml) and incubated another 2 days at 30°C with shaking, and then 10 ml of the second preculture was transferred to a fresh 1 l of the same medium in a 3 l flask and cultured under the same conditions [5].

2.5. Purification of the mutant hen lysozymes

The growth medium of the host cell was centrifuged at 7000 rpm for 10 min at 4°C. The supernatant was applied to a CM-Toyopearl 650M column (1.5×5.0 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5), and then adsorbed proteins were step-eluted using the same buffer containing 0.5 M sodium chloride [12]. The fraction containing the protein was collected, and then the samples which were desalted and concentrated by Centricon centrifugal concentrators (Amicon, USA) were used in the experiments.

2.6. Analysis of mRNA by competitive reverse transcriptase-polymerase chain reaction

Analysis of BiP and PDI expression was performed using competitive reverse transcriptase-polymerase chain reaction (RT-PCR) as described by Siebert and Larrick [13]. A BiP-specific primer pair (5′-GCCCGCTGTAGAAGTAAGTG-3′ and 5′-CTCTTGGTGCTG-GTGGAATG-3′), yielding RT-PCR products of 1099 bp, and a PDI-specific primer pair (5′-ACGTTAAAGCCGCCGAGACT-3′ and 5′-CGTTGGCGTAGGTATCAGCT-3′), yielding RT-PCR products of 1064 bp, were prepared based on the BiP and PDI cDNA sequences of *S. cerevisiae* [14,15]. The internal standard RNA was constructed as follows. 913 bp (for BiP) and 876 bp (for PDI) homologous competitor mRNA fragments (a competitor that has the same nucleotide sequences as the target RNA but contains a deletion of about 200 bp), competing for the same set of primers, were obtained as described using a competitive DNA construction kit and a competitive RNA transcription kit [5].

Yeast transformants were cultured at 30°C in 50 ml of selective medium to the mid-log phase (OD_{600} = 1.5) and the harvest was collected by centrifugation at 1000×*g* for 5 min at 4°C. Total yeast RNA was isolated using RNeasy Mini Kits (Qiagen). 500 ng of total RNA and 2×10^7 copies of competitive mRNA were co-converted into first-strand cDNA using antisense-specific primers. Subsequently, equal portions of cDNA (a mixture of the target and a competitor) were co-amplified by PCR with the BiP- or PDI-specific primer pair. The products were then resolved on a 1.5% agarose gel stained with ethidium bromide. Because of the competition, the ratio of the amounts of the two amplified products reflects the ratio of the target mRNA and RNA competitor. The amount of target mRNA is directly proportional to the log (A_t/A_c), where A_t is the amount of amplified product from the target mRNA, and A_c is the amount of amplified product from the competitor RNA. The densities of the target and competitor band in the gel were determined with a Molecular Imager (Bio-Rad, Japan) [5].

3. Results and discussion

3.1. Amyloidogenic mutant lysozymes I55T, D66H secreted at a similar level in wild-type and calnexin-disrupted S. cerevisiae

It was reported in our previous study that the disruption of the calnexin gene did not lead to gross effects at the level of growth but led the yeast to decrease the quality control to nascent glycoproteins in the ER [5]. The secretion amount of mutant lysozyme I55T is much higher than that of mutant D66H, although the secretion of amyloidogenic lysozymes is extremely small in wild-type and calnexin-disrupted *S. cerevisiae* (Fig. 1). The disruption of calnexin has no effect on the secretion of non-glycosylated amyloidogenic mutant lysozymes. The difference between the secretion of mutants I55T and D66H suggests that I55T mutant lysozyme may have a more stable conformation than D66H so that it is difficult to

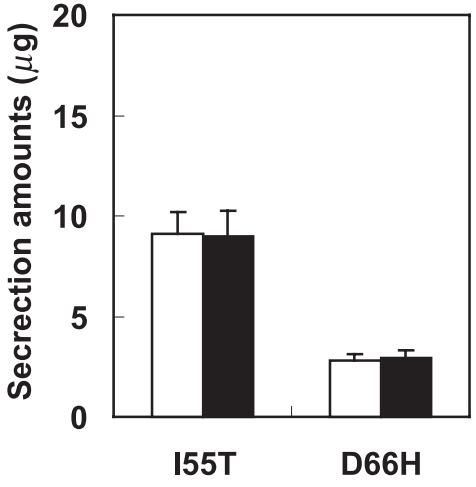

Fig. 1. Secretion amounts of the amyloidogenic mutant lysozymes I55T and D66H expressed in the wild-type (white columns) and calnexin-disrupted (black columns) *S. cerevisiae* AH22. The vertical bars indicate the standard deviation (*n* = 3).

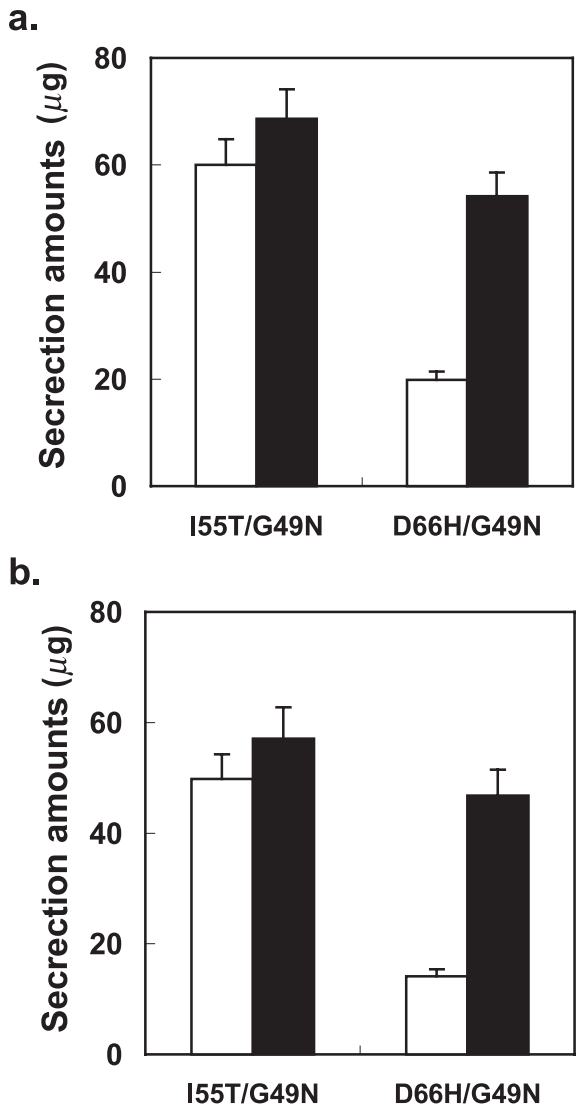

Fig. 2. Secretion amounts of the glycosylated amyloidogenic mutant lysozymes I55T/G49N and D66H/G49N expressed in (a) the wild-type (white columns) and calnexin-disrupted (black columns) *S. cerevisiae* AH22 and (b) wild-type (white columns) and calnexin-disrupted (black columns) *S. cerevisiae* W303-1b. The vertical bars indicate the standard deviation ($n = 3$).

be aggregated after the secretion or to be degraded by the quality control system in yeast.

3.2. *Glycosylated amyloidogenic lysozyme I55T/G49N is secreted at a similar level in the wild-type and calnexin-deleted S. cerevisiae while mutant D66H/G49N is secreted in calnexin-deleted strain much more than in the wild-type strain*

The secretion amounts of glycosylated amyloidogenic lysozymes I55T/G49N and D66H/G49N were compared in the expression system of wild-type and calnexin-deleted *S. cerevisiae*. Fig. 2 shows the secretion amounts of I55T/G49N and D66H/G49N mutants in *S. cerevisiae*. The secretion amounts of glycosylated amyloidogenic lysozymes were much higher than those of the non-glycosylated amyloidogenic mutants for the glycosylated chains masking the β-strand of amyloidogenic lysozymes from the intermolecular cross-β-sheet association and then improving the solubility of amyloidogenic lysozymes [9]. Interestingly, the secretion amounts of mutant I55T/G49N were almost similar in both wild-type and calnexin-disrupted *S. cerevisiae*. In contrast, the secretion of mutant D66H/G49N was greatly increased in calnexin-disrupted *S. cerevisiae*, although the secretion was very low in the wild-type strain. This was also confirmed by SDS–PAGE stained for protein and carbohydrate with Coomassie brilliant blue and 0.5% periodic acid–fuchsin solution, respectively (data not shown). This strongly suggests that mutant lysozyme I55T/G49N can release from the quality control of calnexin in the ER while mutant D66H/G49N cannot.

3.3. *BiP and PDI were induced at higher concentrations when the glycosylated amyloidogenic lysozyme D66H/G49N are secreted by wild-type and calnexin-disrupted S. cerevisiae compared with mutant I55T/G49N*

To investigate how the molecular chaperones located in the ER are induced as a result of the accumulation of glycosylated amyloidogenic lysozymes I55T/G49N and D66H/G49N in the ER, the mRNA concentrations of both molecular chaperones BiP and PDI located in the ER were determined when these lysozymes were expressed in wild-type and calnexin-disrupted *S. cerevisiae* W303-1b. The results of the induction of BiP and PDI are shown in Figs. 3 and 4, respectively. As a control, the induction of BiP and PDI was investigated when the stable glycosylated mutant lysozyme G49N was expressed in *S. cerevisiae* W303-1b. There was only a small difference in the concentrations of BiP and PDI induced between that of

Table 1
Comparison between glycosylated amyloidogenic mutants and other mutant lysozymes

	T_d[a] (°C)	Secretion[b]		BiP[c]		PDI[c]	
		[wild] (μg)	[Δcne] (μg)	[wild]	[Δcne]	[wild]	[Δcne]
G49N[d]	67.1	308.3	317.9	0.52	0.77	0.60	0.90
I55T/G49N[e]	53.5	49.8	57.1	0.56	0.81	0.79	1.04
K13D/G49N[d]	52.9	24.1	97.5	0.62	0.98	0.91	1.41
D66H/G49N[e]	46.9	14.1	46.8	0.64	1.00	1.05	1.58
C76A/G49N[d]	47.4	19.4	76.2	0.64	1.05	1.02	1.64

[a] T_d (transition point of denaturation) was determined by the denaturation curves drawn by following changes in the ellipticity at 222 nm during heating at 40°C to 90°C at pH 3.0.
[b] The secretion amounts of mutant lysozymes in wild *S. cerevisiae* W303-1b [wild] or calnexin-disrupted *S. cerevisiae* W303-1b [Δcne].
[c] The relative mRNA levels of BiP/PDI by using quantitative analysis when the mutant lysozymes were secreted in wild-type and calnexin-disrupted *S. cerevisiae* W303-1b.
[d] Data from [5].
[e] Part of the data from [9].

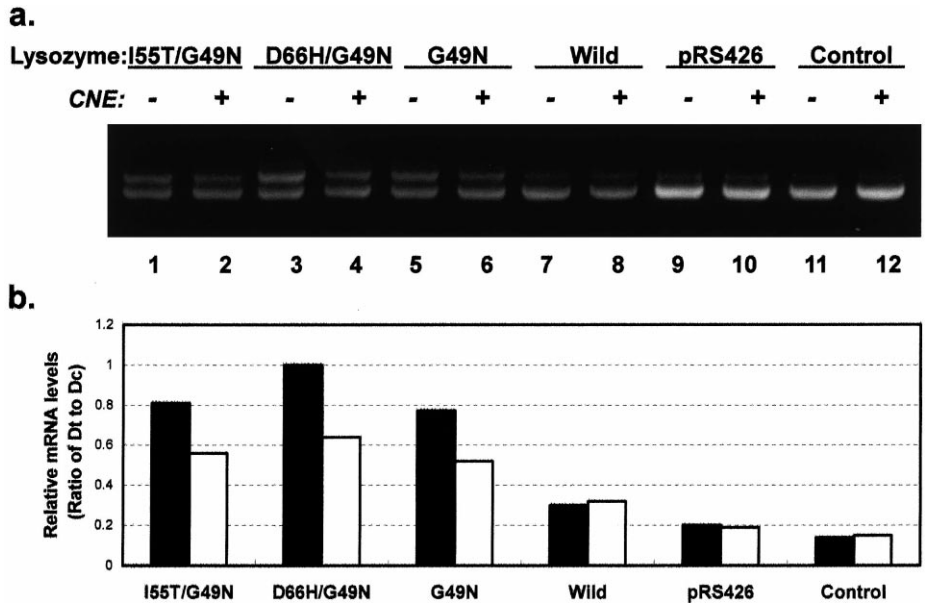

Fig. 3. Quantitative analysis of BiP mRNA level when the glycosylated amyloidogenic mutant lysozymes were secreted by wild-type (CNE^+) and calnexin-disrupted (CNE^-) *S. cerevisiae* W303-1b. a: Electrophoresis of RT-PCR products separated in a 1.5% agarose gel was stained with ethidium bromide. The 1099 bp products (upper) from yeast RNA and the 913 bp products (below) from BiP competitor RNA are indicated. b: In order to quantitatively determine the mRNA levels, the intensities of the RT-PCR patterns were measured with a densitometer. The graph shows the ratios of the density of the target band (upper) to that of the competitor band (lower) for calnexin-disrupted *S. cerevisiae* (black columns) and wild-type *S. cerevisiae* (white columns). D_t, density of the band from target mRNA; D_c, density of the band from competitor RNA.

mutant lysozymes I55T/49N and G49N. In contrast, BiP and PDI were induced in higher concentrations when the glycosylated amyloidogenic lysozyme D66H/G49N was secreted in wild-type and calnexin-disrupted *S. cerevisiae* W303-1b. This unfolded protein response was markedly observed in the secretion in the calnexin-disrupted strain. In a different yeast strain, *S. cerevisiae* AH22, a similar observation was made to that for *S. cerevisiae* W303-1b (data not shown). This observation strongly indicates that the conformation of mutant lysozyme D66H/G49N was very unstable in the ER thus leading to the higher-level induction of ER molecular chaperones via the UPR pathway. On the other hand, glycosylated amy-

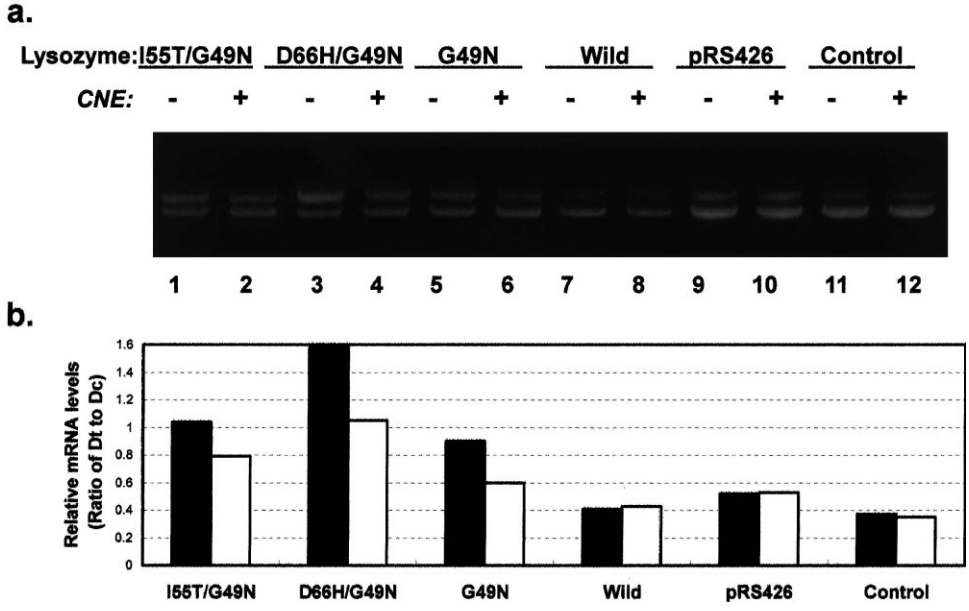

Fig. 4. Quantitative analysis of PDI mRNA level when the glycosylated amyloidogenic mutant lysozymes were secreted by wild-type (CNE^+) and calnexin-disrupted (CNE^-) *S. cerevisiae* W303-1b. a: Electrophoresis of RT-PCR products separated in a 1.5% agarose gel was stained with ethidium bromide. The 1064 bp products (upper) from yeast RNA and the 876 bp products (below) from PDI competitor RNA are indicated. b: In order to quantitatively determine the mRNA levels, the intensities of the RT-PCR patterns were measured with a densitometer. The graph shows the ratios of the density of the target band (upper) to that of the competitor band (lower) for calnexin-disrupted *S. cerevisiae* (black columns) and wild-type *S. cerevisiae* (white columns). D_t, density of the band from target mRNA; D_c, density of the band from competitor RNA.

loidogenic lysozyme I55T/G49N seems to have a relatively stable conformation which is not so different from that of stable mutant G49N in the ER. Therefore mutant I55T/G49N can release from the quality control of calnexin. This is also supported by the similar secretion amounts of the mutant lysozyme I55T/G49N between wild-type and calnexin-disrupted *S. cerevisiae*.

3.4. Comparison of the secretion and induction of BiP and PDI between glycosylated amyloidogenic mutants and other mutant lysozymes

The unfolding transition midpoints for I55T/G49N and D66H/G49N are 53.5°C and 46.9°C, which are similar to those of mutant lysozymes K13D/G49N (52.9°C, which destabilizes α-helix 5–15) and C76A/G49N (47.4°C, which lacks a disulfide bridge between cysteine 76 and 94), respectively. As shown in Table 1, both the secretion amounts and induction of BiP and PDI of mutant lysozymes K13D/G49N and C76A/G49N were evidently enhanced in calnexin-disrupted *S. cerevisiae* compared with those in wild-type strain [5]. This tendency is similar to that of mutant D66H/G49N but different from that of I55T/G49N. This suggested that mutant I55T/G49N may have a specific stable conformation in the ER, which cannot be explained from the stability obtained by thermal denaturation curves. Therefore, glycosylated mutant amyloidogenic lysozyme I55T/G49N seems likely to release from the quality control of calnexin with its stable conformation in the ER in wild-type *S. cerevisiae*, thus resulting in a similar secretion amount as that in calnexin-disrupted strain. On the other hand, the mutant D66H/G49N with its unstable conformation in the ER must undergo the digestion pathway via the quality control of calnexin, resulting in a very low secretion amount, while the secretion was recovered in the calnexin-disrupted strain without the quality control of calnexin.

This paper proposes the possibility of the release of quality control in the ER for glycosylated amyloidogenic lysozymes. In *S. cerevisiae*, although the secreted form is polymannosylated lysozyme with a large carbohydrate chain, it is sure that the protein is glycosylated with a $Glc_{1-3}Man_{8-9}GlcNAc_2$ oligosaccharide chain in the ER [16,17]. It was recently reported that this oligosaccharide chain functions as an anchor which binds with the oligosaccharide moieties of calnexin and the removal of the terminal mannose from the central branch of oligosaccharide chain determines the fortune of nascent glycoproteins [18–20]. The detailed mechanism was not clear but the mannose trimming seems likely to be dependent on the folding manner of the polypeptide. Thus, it seems likely that the glycosylated mutant amyloidogenic lysozyme I55T/G49N can release from the quality control of calnexin in the ER with the folded structure close to native lysozyme.

In our previous study, it was clear that both of the glycosylated amyloidogenic lysozymes I55T/G49N and D66H/G49N had similar secondary structures with non-glycosylated types (I55T and D66H) [9]. It seems likely that amyloidogenic mutant lysozyme I55T also has a relatively stable conformation in the ER. This suggests that mutant I55T may also release from the normal quality control system in the ER and then lead to amyloid disease. On the other hand, the other amyloidogenic lysozyme, D66H, seems to be hard to lead to amyloid disease in a cell with a normal ER quality control system, because most of this mutant is degraded by the quality control system in the ER due to its less stable conformation. Therefore, this observation might give new ideas on the mechanism of amyloidosis disease that the normal quality control system in the ER can rescue the cell from molecular disease for mutant lysozyme D66H, but not for mutant lysozyme I55T, despite similar amyloidogenic mutant lysozymes.

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