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Expression and Characterization of *Saccharomyces cerevisiae* Cne1p, a Calnexin Homologue

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The calnexin homologue (Cne1p) of *Saccharomyces cerevisiae* was expressed in *Escherichia coli* to evaluate its chaperone function. The chaperone function was examined as to the effects on the suppression of thermal denaturation and the enhancement of refolding, using citrate synthase (CS) as a nonspecific chaperone substrate. Cne1p effectively suppressed the thermal denaturation of CS and enhanced the refolding of thermally or chemically denatured CS in a concentration-dependent manner. In addition, the chaperone function of Cne1p was greatly affected in the presence of monoglucosylated oligosaccharides (G1M9) that specifically bind to the lectin site. These results indicated that Cne1p functions as a molecular chaperone in *Saccharomyces cerevisiae*.

Key words: calnexin homologue, chaperone function, *Saccharomyces cerevisiae*.

Abbreviations: Cne1p, *S. cerevisiae* calnexin homologue; ER, endoplasmic reticulum; GST, glutathione S-transferase; CS, citrate synthase; G1M9, monoglucosylated oligosaccharides ($Glc_1Man_9GlcNAc_2$).

Calnexin is an important component of the endoplasmic reticulum (ER) quality control mechanism that retains nascent glycoproteins in the ER through an oligosaccharide moiety, $Glc_1Man_9GlcNAc_2$, until these substrates are properly folded or until misfolded proteins have been degraded (*1*). The sequence of a gene in *Saccharomyces cerevisiae* (*S. cerevisiae*) exhibiting similarity to that of mammalian calnexins has been reported (*2–4*). The calnexin homologue (Cne1p) in *S. cerevisiae* exhibits the sequence similarity to that in mammalian cells, but it lacks a cytoplasmic tail and has no calcium-binding capacity (*3*). Calnexin gene–disrupted *Schizosaccharomyces pombe* cells (*5*) or mammalian cells (*6*) were lethal, while disruption of the *CNE1* gene in *S. cerevisiae* did not affect cell growth (*7*). In addition, calreticulin, with the same function as calnexin in mammalian cells, is not found in yeast *S. cerevisiae*. This raises the question of whether *S. cerevisiae* Cne1p functions in a similar manner to those in mammalian cells or not. The lack of calreticulin in yeast and the viability of *CNE1*-disrupted *S. cerevisiae* raise the question of whether Cne1p functions as a chaperone and as a component of the quality control mechanism for of glycoproteins or not. In order to elucidate the quality control function of *S. cerevisiae* Cne1p, we investigated the expression of stable and unstable mutant glycosylated lysozyme in *CNE1*-disrupted *S. cerevisiaes* cells, and found that secreted amount of the unstable mutant was greatly increased, and that the expression level of associated chaperones involved in the quality control was increased in the *CNE1*-disrupted strain (*7*). On the other hand, the chaperone function of Cne1p remains to be determined. In particular, it should be determined whether *S. cerevisiae* Cne1p is positively involved in the folding of glycoprotein or not. This paper describes the characterization of Cne1p expressed in *E. coli*.

MATERIALS AND METHODS

Materials—Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Tokyo). The pT7 Blue T-vector, GST binding resin, and buffer kit were purchased from Novagen. Citrate synthase (CS) was from Sigma (St Louis, MO). Monoglucosylated oligosaccharides (Glc1Man9GlcNAc2, G1M9) prepared from chicken egg yolk immunoglobulin (IgY) were kindly provided by Dr. Kato (Nagoya City University). All other chemicals were of analytic grade for biochemical use.

Construction of Expression Plasmids of GST-Cne1p—A full-length calnexin homologue cDNA containing a transmembrane region was amplified from genomic DNA of *S. cerevisiae* W303–1b (wild-type) by PCR using two primers designed according to the sequence of the *CNE1* gene. The sequence of the sense primer was 5′-GGCCATGGGGACTTCATTGCTATCCAACGTTA-3′, containing a *Nco*I site (CCATGG). The sequence of the antisense primer was 5′-GGGAAGCTTTGTGGTGCAATTATTGAGACC-3′, containing a *Hin*III site (AAGCTT). PCR was performed with *ExTaq* polymerase in a GeneAmp® PCR system 2400 under the standard PCR conditions with a hot start at 94°C for 5 min, and then 25 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 7 min of elongation at 72°C. The amplification product was purified by electrophoresis on a 1.0% low-melting agarose gel and then subcloned into the pT7 BlueT vector (Novagen). DNA sequencing of the subcloned cDNA was performed on an ABI 310 Genetic Analyzer with a dye-terminator cycle sequencing kit. After that, the cDNA was excised and ligated into GST fusion expression vector pET-42b(+) (Novagen) with *Nco*I and *Hin*dIII sites. The resulting plasmid was designated as pET/CNE1.

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Expression and Purification of GST-Cne1p—The constructed pET/*CNE1* expression plasmid was transformed into the *E. coli* BL21 (DE3) strain, followed by selection on LB agar plates containing kanamycin (50 ng/ml). An overexpressing colony was inoculated into 4 ml LB medium and then incubated at 37°C overnight. This preculture product was transferred to 400 ml fresh LB medium and then incubated at 37°C for 2 h. Protein expression was induced with 0.5 mM IPTG at 30°C for 4 h. Harvested cells were collected by centrifugation at 10,000 ×*g* for 5 min at 4°C. The cell pellet was resuspended in 1× GST binding buffer (Novagen) containing 0.1% triton X-100 and 1 mM PMSF, sonicated and then centrifuged at 15,000 ×*g* for 20 min. The supernatant was filtered through a 0.45 μm membrane and applied to the GST binding column. The GST-Cne1p fusion protein was eluted with 10 mM reduced glutathione, according to the manufacturer's protocol (Novagen). The GST-Cne1p was further purified by Sephadex G-75 gel filtration (Pharmacia) and concentrated using a Centriplus YM-50 concentrator (Millipore). Protein concentrations were determined by the Lowry method (*8*).

SDS-Polyacrylamide Gel Electrophoresis—SDS–polyacrylamide gel electrophoresis was conducted according to Laemmli (*9*) using a 12% acrylamide separating gel and a 5% stacking gel containing 1% SDS. Electrophoresis was carried out at a constant current of 20 mA using Tris-Glycine buffer containing 0.1% SDS. After electrophoresis, the gel sheet was stained for protein with a 0.025% Coomassie Brilliant Blue R-250 solution.

Citrate Synthase Activity Assay—The enzymatic activity of citrate synthase (CS) was monitored as described (*10*). CS was mixed with a reaction solution (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM Oxaloacetate, 20 mM DTNB, 5mM acetyl-CoA) in a cuvette and then the increase in absorbance at 412 nm was measured for 60 s using a spectrophotometer U-2001 (Hitachi). The enzymatic activity was calculated from the increase in the absorbance.

Thermal Aggregation of Citrate Synthase—Thermal aggregation of citrate synthase (CS) was followed by adding 0.2 μM CS to a solution of 40 mM HEPES-KOH, pH 7.5 in the absence and presence of GST-Cne1p, followed by heating to 45°C. Aggregation was monitored with a fluorescence spectrophotometer, 650-10-S (Hitachi). The excitation and emission wavelengths were both set to 500 nm, and both slits were set to 2 nm.

Thermal Inactivation and Reactivation of Citrate Synthase—Thermal inactivation of CS was monitored by incubating 0.2 μM CS in 50 mM Tris-HCl buffer (pH 8.0) in the absence and presence of GST, GST-Cne1p and monoglucosylated oligosaccharides (G1M9) at 43°C. The remaining activity of the CS was assayed spectrophotometrically at various time points. Reactivation of thermally inactivated CS was performed as follows: 0.8 μM CS in 50 mM Tris-HCl buffer (pH 8.0) was inactivated at 43°C for 30 min, and then reactivation was initiated by 4-fold dilution with the same buffer in the absence and presence of GST-Cne1p at 25°C. The activity of the CS was then assayed spectrophotometrically at various time points.

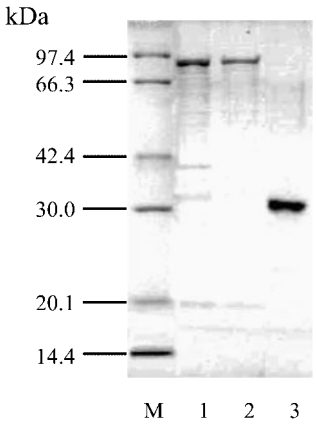

Fig. 1. **SDS-PAGE patterns of GST-Cne1p purified from the soluble cell extract.** The gel sheet was stained with Coomassie Brilliant Blue R250. Lane M, molecular weight marker proteins; lane 1, GST-Cne1p (eluted from GST binding resin); lane 2, GST-Cne1p (purified by gel filtration); lane 3, GST [prepared by expression of the pET-42b(+) vector].

Reactivation of Chemically Denatured Citrate Synthase—Twenty micromolar CS was denatured in 6 M guanidine-HCl in 50 mM Tris-HCl buffer (pH8.0) containing 20 mM dithiothreitol for 1 h at room temperature. Reactivation of the denatured CS was initiated by 100-fold dilution with reactivation buffer (50 mM Tris-HCl, pH 8.0) in the absence and presence of GST-Cne1p at 25°C. The activity of the reactivated CS was then assayed spectrophotometrically at various time points.

RESULTS AND DISCUSSION

Expression and Purification of GST-Cne1p—GST-Cne1p was successfully expressed in *E. coli* BL21 (DE3) cells harboring an expression plasmid, pET/CNE1. This recombinant protein was purified from the soluble cell extract using a GST binding column (Fig. 1, lane 1), and further purified by Sephadex G-75 gel filtration (Fig. 1, lane 2). GST was purified from *E. coli* BL21 (DE3) cells transformed with the pET-42b(+) expression vector (Novagen) only (Fig. 1, lane 3). GST was used as a control.

GST-Cne1p Suppresses the Thermal Aggregation of Citrate Synthase—To assess the aggregation-suppressing function of GST-Cne1p, citrate synthase, a nonspecific substrate of molecular chaperones, was used for the aggregation assay. As shown in Fig. 2, CS formed large aggregates when heated alone or in the presence of equimolar GST at 45°C, while the thermal aggregation of CS was greatly suppressed in the presence of equimolar GST-Cne1p. The suppression of the thermal aggregation of CS with GST-Cne1p was slightly affected by the addition of five-fold monoglucosylated oligosaccharides (G1M9). These monoglucosylated oligosaccharides (G1M9) are known to bind to the lectin site of mammalian calnexin. Cne1p may also bind to G1M9, which results in the conformational changes causing the slight recovery of the suppression of the thermal aggregation of CS. However, the main domain responsible for the suppression of thermal aggregation of CS is not the lectin site domain,

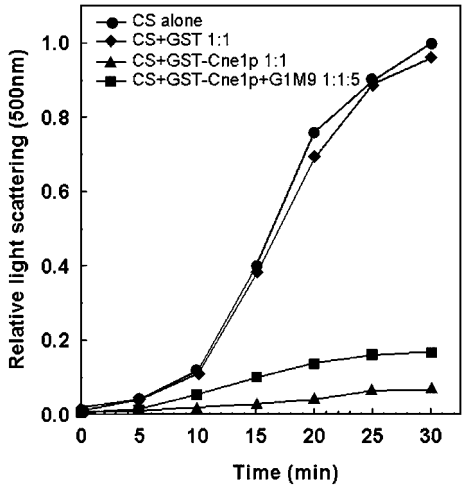

Fig. 2. **Effect of GST-Cne1p on the thermal aggregation of citrate synthase.** CS (0.2 μM) was incubated at 45°C in the absence (circles) or presence of 0.2 μM GST (diamonds), 0.2 μM GST-Cne1p (triangles), or 0.2 μM GST-Cne1p + 1.0 μM G1M9 (squares). Aggregation was measured by monitoring light scattering at 500 nm.

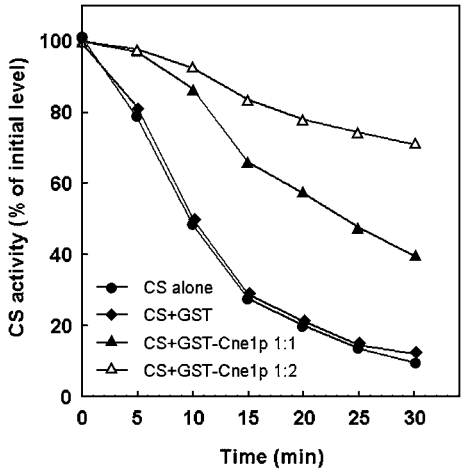

Fig. 3. **Effect of GST-Cne1p on the thermal inactivation of citrate synthase.** CS (0.2 μM) was incubated at 43°C in the absence (circles) or presence of 0.2 μM GST (diamonds), 0.2 μM GST-Cne1p (solid triangles), or 0.4 μM GST-Cne1p (open triangles). CS activity was assayed at various time points up to 30 min.

but another domain. The effective suppression of the thermal aggregation of CS through the polypeptide-binding sites of mammalian calnexin in vitro has been reported (*11*, *12*). It seems likely that Cne1p binds to the partially unfolded proteins through a polypeptide-binding domain in a similar manner to in the case of mammalian calnexin.

Effect of GST-Cne1p on the Thermal Inactivation and Reactivation of Thermally Denatured Citrate Synthase—The results in Fig. 2 demonstrate that GST-Cne1p suppresses the aggregation of CS during heat stress. Therefore, it is expected that GST-Cne1p is capable of protecting the CS activity against thermal denaturation. To confirm this possibility, the CS activity was monitored during heating at 43°C in the absence and presence of

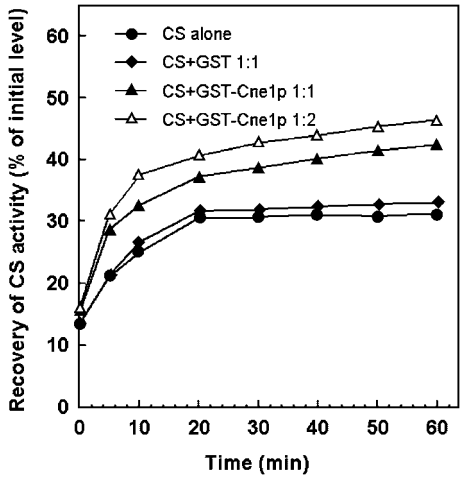

Fig. 4. **Effect of GST-Cne1p on the reactivation of thermally denatured CS.** CS (0.8 μM) in 50 mM Tris- HCl buffer (pH 8.0) was thermally inactivated by incubation at 43°C for 30 min. Reactivation of inactivated CS was initiated by 4-fold dilution with the same buffer in the absence (circles), or presence of 0.2 μM GST (diamonds), 0.2 μM GST-Cne1p (solid triangles), or 0.4 μM GST-Cne1p (open triangles) at 25°C. CS activity was assayed at various time points up to 60 min. Values are the means for three independent experiments.

GST-Cne1p. As shown in Fig. 3, on heating alone or with GST, the CS activity decreased quickly, an about 90% decrease in the initial activity being observed after heating at 43°C for 30 min. In contrast, the decrease in CS activity was suppressed in the presence of GST-Cne1p in a molar ratio-dependent manner as to CS/Cne1p. It has been reported that calnexin-CS complexes are formed between mammalian calnexin and CS on heating conditions (*11*). It seems that the formation of Cne1p-CS complexes during heating stabilizes the conformation of CS and suppresses the formation of large aggregates, thus keeping the CS in an activate state.

The effect of Cne1p on the refolding of thermally denatured CS was also investigated to elucidate its function as a molecular chaperone. After CS had been inactivated at 43°C for 30 min, reactivation was initiated by diluting the heated sample with buffer alone and buffer containing GST or GST-Cne1p at 25°C. As shown in Fig. 4, the reactivation of CS was not observed in the presence of GST, while GST-Cne1p significantly enhanced the reactivation of CS in a molar ratio-dependent manner as to CS/Cne1p. This suggests that GST-Cne1p is involved in the refolding of the thermally denatured protein.

Effect of GST-Cne1p on the Reactivation of Chemically Denatured Citrate Synthase—Chemically denatured CS was obtained by incubating CS in 6 M guanidine-HCl at room temperature for 1 h. Unlike thermally denatured CS, which readily takes on an aggregated form at an elevated temperature, chemically denatured CS remained in a monomeric unfolding state, leading to a higher refolding yield (*10*). The refolding of chemically denatured CS was initiated by 100-fold dilution with reactivation buffer (50 mM Tris-HCl, pH 8.0) in the absence and presence of GST or GST-Cne1p at 25°C. As shown in Fig. 5, the reactivation yield of CS with the spontaneous refolding reaction was about 15%, and little change was

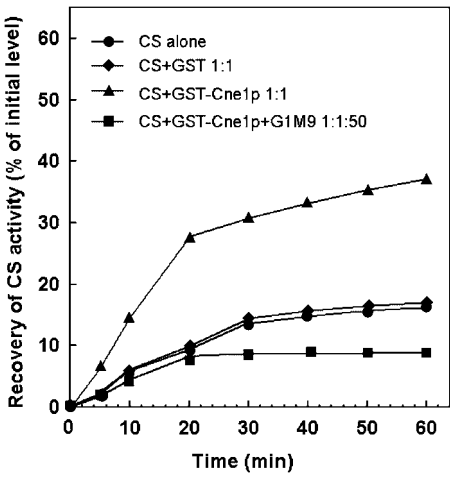

Fig. 5. **Reactivation of chemically denatured citrate synthase.** CS (20 μM) was denatured in 6 M guanidine-HCl containing 20 mM DTT. Reactivation was initiated by 100-fold dilution (0.2 μM final concentration) with 50 mM Tris buffer (pH 8.0) (circles), and in the presence of 0.2 μM GST, 0.2 μM GST-Cne1p (triangles), or 0.2 μM GST-Cne1p + 25 μM G1M9 (squares). CS activity was assayed at various time points up to 60 min.

observed in the presence of GST, while a great increase (about 39%) was observed in the presence of equimolar GST-Cne1p at 60 min. Therefore, the results support the chaperone function of GST-Cne1p in the refolding of the unfolded protein.

Effect of Monoglucosylated Oligosaccharides on the Reactivation of Chemically Denatured Citrate Synthase by GST-Cne1p—The effect of monoglucosylated oligosaccharides on the reactivation of chemically denatured citrate synthase by GST-Cne1p was investigated. Calnexin functions as a component of the glycoporein quality control system in the endoplasmic reticulum through its lectin site specific for monoglucosylated oligosaccharides (*13*). The lectin-oligosaccharide interaction acts as an initial step in the folding and assembly of unfolded glycoproteins *in vivo* (*14*). To determine whether the lectin site was related to the chaperone function of GST-Cne1p, the role of monogulcosylated oligosaccharides (G1M9) in the suppression of thermal aggregation was examined. As shown in Fig. 2, oligosaccharides slightly affected the ability of GST-Cne1p to suppress the thermal aggregation of CS. In addition, the oliosaccharides effectively decreased the ability of GST-Cne1p as to the refolding of chemically denatured CS, as shown in Fig. 5. Ihara *et al.* (*11*) reported that conformational changes in calnexin were induced on binding of oligosaccharides to its lectin sites. Thus, the decreased refolding ability of GST-Cne1p may be due to conformational changes induced by oligosaccharide binding. Judging from these results, *S. cerevisiae* Cne1p is also consistent with the model of mammalian calnexin in the ER, functioning both as a lectin and as a molecular chaperone (*14*, *15*).

In conclusion, we constructed GST-Cne1p and investigated its relevant function in quality control *in vitro*. Our experiments demonstrated that GST-Cne1p was capable of suppressing the thermal aggregation of CS and enhancing the refolding of thermally or chemically denatured CS in a concentration-dependent manner, like other molecular chaperones. Furthermore, the lectin site of Cne1p specifically binds to monoglucosylated oligosaccharides, thereby affecting the suppression of thermal aggregation of CS and the refolding of chemically denatured CS.

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