P-domain and lectin site are involved in the chaperone function of *Saccharomyces cerevisiae* calnexin homologue

Xiaohua Xu, Hiroyuki Azakami, Akio Kato*

Department of Biological Chemistry, Yamaguchi University, Yamaguchi 753-8515, Japan

Received 13 May 2004; revised 3 June 2004; accepted 3 June 2004

Available online 26 June 2004

Edited by Gianni Cesareni

Abstract Cne1p, a calnexin homologue from *Saccharomyces cerevisiae*, has been shown to possess a conserved P-domain and lectin site as mammalian calnexin. The effect of P-domain and lectin site on the function of Cne1p was investigated in vitro using recombinant P-domain, P-domain deletion mutant of Cne1p, and lectin site mutant of Cne1ps (E181A and E398A) The binding of monoglucosylated oligosaccharide (G1M9) with Cne1p was clearly demonstrated using lectin site mutants. The P-domain deletion mutant and the letcin site mutants partially decreased the ability to suppress the aggregation of citrate synthase (CS) and chicken egg yolk immunoglobulin at levels different from Cne1p. Furthermore, the P-domain deletion mutant and the lectin site mutants decreased the ability to enhance the refolding of CS. These results suggest that the cooperation between the P-domain and the lectin site are important for the complete function of Cne1p. Thus, we conclude that P-domain in cooperation with the lectin site of Cne1p functions as a chaperone.

Keywords: Calnexin homologue; Chaperone; *Saccharomyces cerevisiae*

1. Introduction

Calnexin is a molecular chaperone in the endoplasmic reticulum (ER) involved in the folding and quality control of nascent glycoproteins through an oligosaccharide moiety, $Glc_1Man_9GlcNAc_2$, until these substrates are properly folded or misfolded proteins have been degraded [1]. The isolation and sequencing of calnexin cDNA for genomic clones of eukaryotic cells have revealed that the general structural organization of calnexin has been conserved through evolution [2,3]. Cne1p, a homologue of calnexin in *Sacchromyces cerevisiae*, is about 23% identical at amino acid level with mammalian calnexin, and lacks a cytoplasmic tail and calcium-binding capacity [2]. In addition, there is no calreticulin in *S. cerevisiae*, which is a calnexin homologue and fulfills the same function as calnexin in mammalian ER lumen. To better understand the function of the calnexin family in yeast, we constructed recombinant Cne1p from *S. cerevisiae* and found that Cne1p functions as a molecular chaperone in a manner similar to mammalian calnexin [4].

The crystal structure of canine calnexin (CNX) has shown that the protein consists of a P-domain and a globular lectin domain [3]. The function of lectin domain is involved in the binding of oligosaccharides, while P-domain is involved in the binding of the thiol oxidoreductase ERp57 [5,6]. Recently, two conflicting models, lectin-only model and dual-binding model, have been proposed for mammalian calnexin. In the lectin-only model, calnexin functions solely as a lectin with cycles of glycoprotein release and rebinding through monoglucosylated oligosaccharide [7,8]. In the dual-binding model, calnexin functions both as a lectin and as a classical molecular chaperone [9,10]. This model proposes that calnexin possesses a second site that binds to polypeptide-segments of unfolded glycoproteins in addition to lectin binding. The functional elucidation of P-domain and lectin site of Cne1p may help to understand the molecular mechanism of calnexin family. In the present study, we engineered the Cne1p P-domain, the P-domain deletion mutant of Cne1p (ΔP), and point mutants of Cne1p lectin site. These mutants were tested for the ability in aggregation–suppression and refolding of non-glycosylated or glycosylated substrates to elucidate its chaperone function.

2. Materials and methods

2.1. Materials

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto). Citrate synthase (CS), trypsin, chymotrypsin and actinase were obtained from Sigma. Glycoamidase A was from Seikagaku Kogyo (Tokyo). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was purchased from Wako (Osaka). All other chemicals were of analytical grade for biochemical use.

2.2. Construction of Cne1p and mutants

The construction of recombinant Cne1p was described previously [4]. cDNAs for all other mutants were constructed by PCR using a cDNA encoding Cne1p in pT7 BlueT vector as template. The following primers were used to prepare mutants of Cne1p.

Primer A, 5′-GCCATGGGGACTTCATTGCTATCCAACGTTA-3′;

* Corresponding author: Fax: +81-83-933-5820.
E-mail address: akiokato@yamaguchi-u.ac.jp (A. Kato).

Abbreviations: Cne1p, *S. cerevisiae* calnexin homologue; CNX, canine calnexin; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; CS, citrate synthase; IgY, chicken egg yolk immunoglobulin; SEC, size exclusion chromatography; ΔP, P-domain deletion mutant of Cne1p; E181A, point mutant of Cne1p at Glu181; E398A, point mutant of Cne1p at Glu398

0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.039

primer B, 5′-GGCAGCGGGAGCGGCGAAGAGCAGCACCC-ATTGC-3′;
primer C, 5′-GCCGCTCCCGCTGCCTAAAGGCGGTGTAA-TGGGTG-3′;
primer D, 5′-GGGAAGCTTTGTGGTGCAATTATTGAGACC-3′;
Primer E, 5′-CCATGGGGATGATTCCTGATGTTTCA- G-3′;
Primer F, 5′-GGGAAGCTTGTAGTATAGCGGGTTT- TC-3′;
primer G, 5′-GACAAGATCACACATGCATCTAAACTAAG-3′;
primer H, 5′-TGTGATCCTCGCGTTTTGGAGTGGAT-3′;
primer I, 5′-CTTAGTTTAGATGCATGTGTGATCTTGTC-3′;
and primer J, 5′-ATCCACTCCAAAACGCGAGGATCACA-3′.

The ΔP, Cne1p 20–247/381–502, was prepared by a two-step sequence overlap extension method [11], using primers A, B, C, and D. Primers A and D contain a *Nco*I site (underline) and a *Hin*dIII site (underline), respectively. Primers B and C contain a sequence encode for a GSGSG linker (underline). The first two cDNA fragments (Cne1p 20–247 and Cne1p 381–502) were prepared by PCR using 5′-primers A and B and 3′-primers C and D, respectively. Then, these two cDNA fragments were mixed and subjected to PCR in the presence of flanking primers A and D. The P-domain, Cne1p 247–380, was prepared using primer E as the 5′-primer and primer F as the 3′-primer. The lectin sites mutants, in which $Glu^{181} \rightarrow Ala$ (E181A) $Glu^{398} \rightarrow Ala$ (E398A), were prepared by site-directed mutagenesis methods using 5′-primers G and H and 3′-primers I and J, respectively. All of cDNA constructs were subcloned into pT7 BlueT vector (Novagen) and sequenced on ABI 310 Genetic Analyzer. After digestion with *Nco*I and *Hin*dIII, cDNAs were ligated into pET-42b(+) (Novagen) expression vector.

2.3. Expression and purification of recombinant proteins

These constructed expression plasmids were transformed into *Escherichia coli* BL21 (DE3) cells for protein expression. Overexpression of recombinant proteins was induced with 0.5 mM IPTG at 30 °C for 4 h. Cell pellets were harvested by centrifugation at 10 000 × *g* for 5 min at 4 °C and resuspended in 1× glutathione *S*-transferase (GST) binding buffer (Novagen) containing 0.1% Triton X-100 and 1 mM PMSF, and then centrifuged at 15 000 × *g* for 20 min after sonication. Supernatants were passed through 0.45 μm filter before being applied to GST binding column (Novagen). Proteins were eluted with 10 mM reduced glutathione, according to the manufacturer's protocol (Novagen). Samples were 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 [12].

2.4. Preparation of monoglucosylated oligosaccharide (G1M9)

Fluorescently labeled G1M9 was prepared from chicken egg immunoglobulin (IgY) with a method provided by Dr. Kato (Nagoya City University). IgY was dissolved in 10 mM Tris–HCl buffer (pH 8.0) and heated at 100 °C for 10 min. Oligosaccharides were released from IgY by sequential digestion with trypsin, chymotrypsin, glycoamidase A and actinase. The oligosaccharide fractions were collected after Dowex 50W-X8(H+) (Sigma) and Amberlite (Sigma) column. Pyridylamino derivatization of oligosaccharides was reductively aminated with 2-aminopyridine and dimethylamine-borane and purified by gel filtration on a Sephadex G-15 column. G1M9 was further purified from pyridylamino-oligosaccharides with a TSK-gel Amide-80 column (0.46 × 25 cm) using high-performance liquid chromatography system (Hitachi), equipped with two pumps (L-6000) and a fluorescence detector (L-7485).

2.5. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis was conducted according to Laemmli [13] 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 sheets were stained for protein with a 0.025% Coomassie Brilliant Blue R-250 solution.

2.6. Oligosaccharide binding assay

Oligosaccharide binding to GST-fused Cne1p proteins was performed by incubation of 1 μM Cne1ps with 5 μM G1M9 for 30 min at

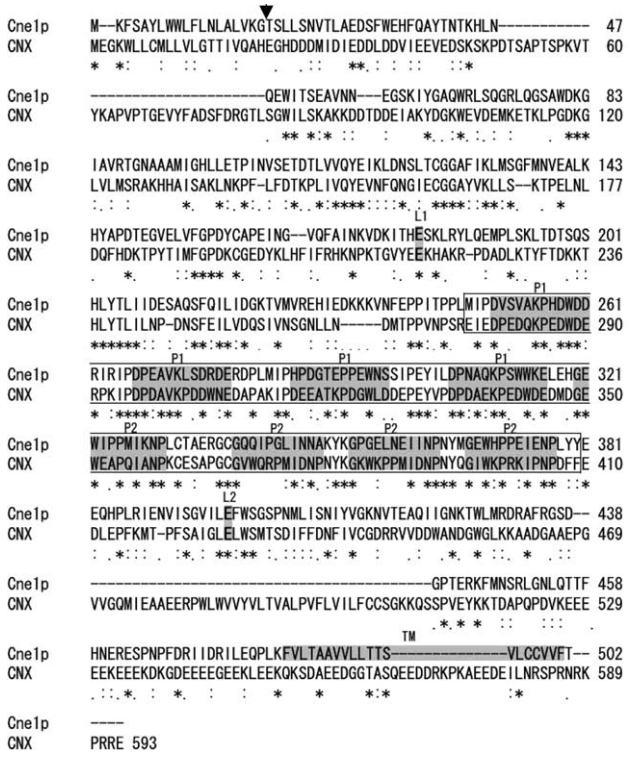

Fig. 1. Alignment of amino acid sequences of calnexins from *S. cerevisiae and* canine (Dog). Alignment was generated by ClustalW program. Identical residues are denoted by "*", conserved substitutions are denoted by ":", and semi-conserved substitutions are denoted by ".". Predict signal peptide cleavage site of Cne1p is shown by arrow. P-domain is shown in large open boxes, whereas P1 represent repeat motif 1 (shaded in gray), and P2 represent repeat motif 2 (shaded in gray). Two conserved residues L1 and L2 were also shaded in gray according to CNX lectin site (3). TM, transmembrane region.

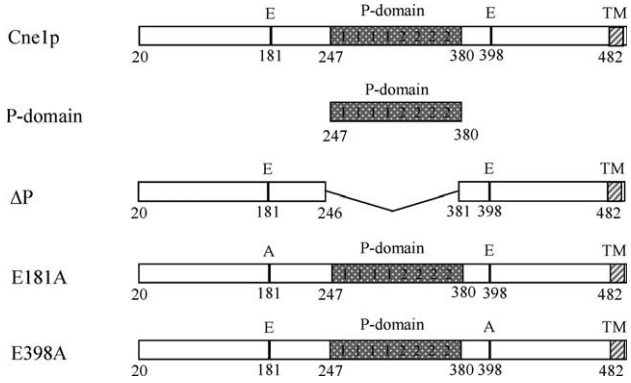

Fig. 2. A schematic representation of the Cne1p constructs in this study. Cne1p is the full-length construct; P-domain is P-domain alone construct; ΔP is P-domain deletion mutation construct; E181A and E398A are two of point mutation constructs in putative lectin site.

25 °C in 50 mM Tris–HCl buffer (pH 8.0). The interaction of Cne1p with oligosaccharide was analyzed by size-exclusion chromatography (SEC) using a TSK-gel G3000SW column in TS buffer (50 mM Tris–HCl, pH 8.0 and 0.2 M NaCl). The eluate was detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. Oligosaccharide binding is expressed as a percentage of the

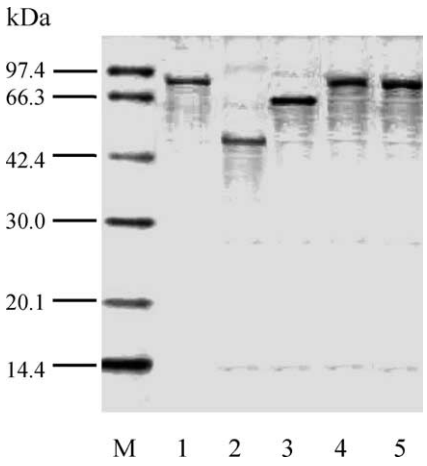

Fig. 3. SDS-PAGE patterns of Cne1ps purified from the soluble cell extract. The gel sheet was stained with Coomassie brilliant blue R250. Lane M, molecular weight marker proteins; lane 1, GST-Cne1p; lane 2, GST-P-domain; lane 3, GST-ΔP; lane 4, GST-E181A; lane 5, GST-E398A.

specific binding observed for GST-Cne1p after subtraction of control binding for GST.

2.7. Citrate synthase activity assay

The enzymatic activity of CS was monitored as described [14]. CS was mixed with a reaction solution (50 mM Tris–HCl, pH 8.0, 2 mM EDTA, 10 mM oxaloacetate, 20 mM DTNB and 5 mM 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, Japan). The enzymatic activity was calculated from the increase in the absorbance.

2.8. Aggregation assay

0.2 μM CS in 40 mM HEPES-KOH, pH 7.5, was mixed with GST or GST fusion proteins in a total volume of 1 ml and heated to 45 °C. Aggregation was monitored with a fluorescence spectrophotometer 650-10-S (Hitachi, Japan). The excitation and emission wavelengths were both set to 500 nm, and both slits were set to 2 nm.

IgY (10 mg/ml) was denatured with 0.1 M Tris–HCl (pH8.0), 6 M guanidine–HCl and 40 mM dithiothreitol for 2 h at room temperature. The denatured proteins were diluted 200-fold into 10 mM Tris–HCl amd 0.15 M NaCl in the absence and presence of GST or GST fusion proteins in a total volume of 1 ml. Protein aggregation was initiated by incubating the sample at 44 °C. Aggregation was monitored by a fluorescence spectrophotometer 650-10-S (Hitachi, Japan). The excitation and emission wavelengths were both set to 360 nm, and both slits were set to 2 nm.

2.9. Reactivation of thermally inactivated citrate synthase

Reactivation experiment for CS was carried out as described [4]. 0.8 μM CS was thermally inactivated in 50 mM Tris–HCl buffer (pH 8.0) at 43 °C for 30 min. Reactivation was initiated by 4-fold dilution with the same buffer in the absence and presence of GST or GST fusion proteins at 25 °C. The activity of the reactivated CS was assayed spectrophotometrically at various time points.

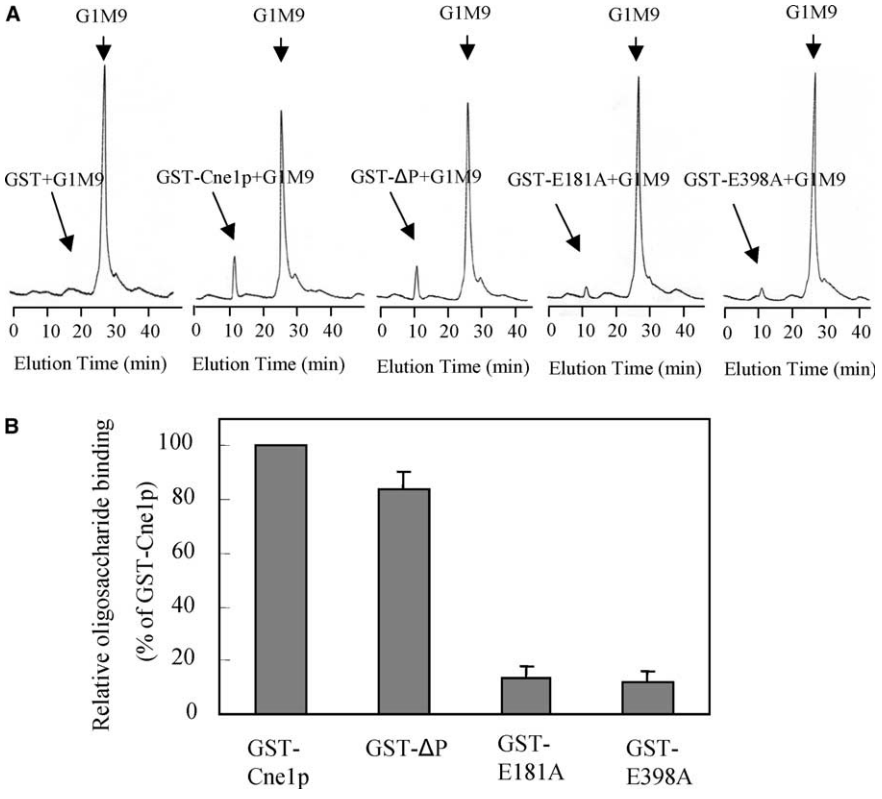

Fig. 4. Estimation of oligosaccharide binding capacity with Cne1ps by size exclusion chromatography. (A) 1 μM GST or Cne1ps was incubated with 5 μM G1M9 for 30 min at 25 °C in 50 mM Tris–HCl buffer (pH 8.0). Eluates separated by SEC using a TSK-gel G3000SW column in TS buffer were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. (B) Oligosaccharide binding is expressed as a percentage of the specific binding observed for GST-Cne1p after subtraction of control binding for GST. Error bars represent the standard error in three replicate experiments.

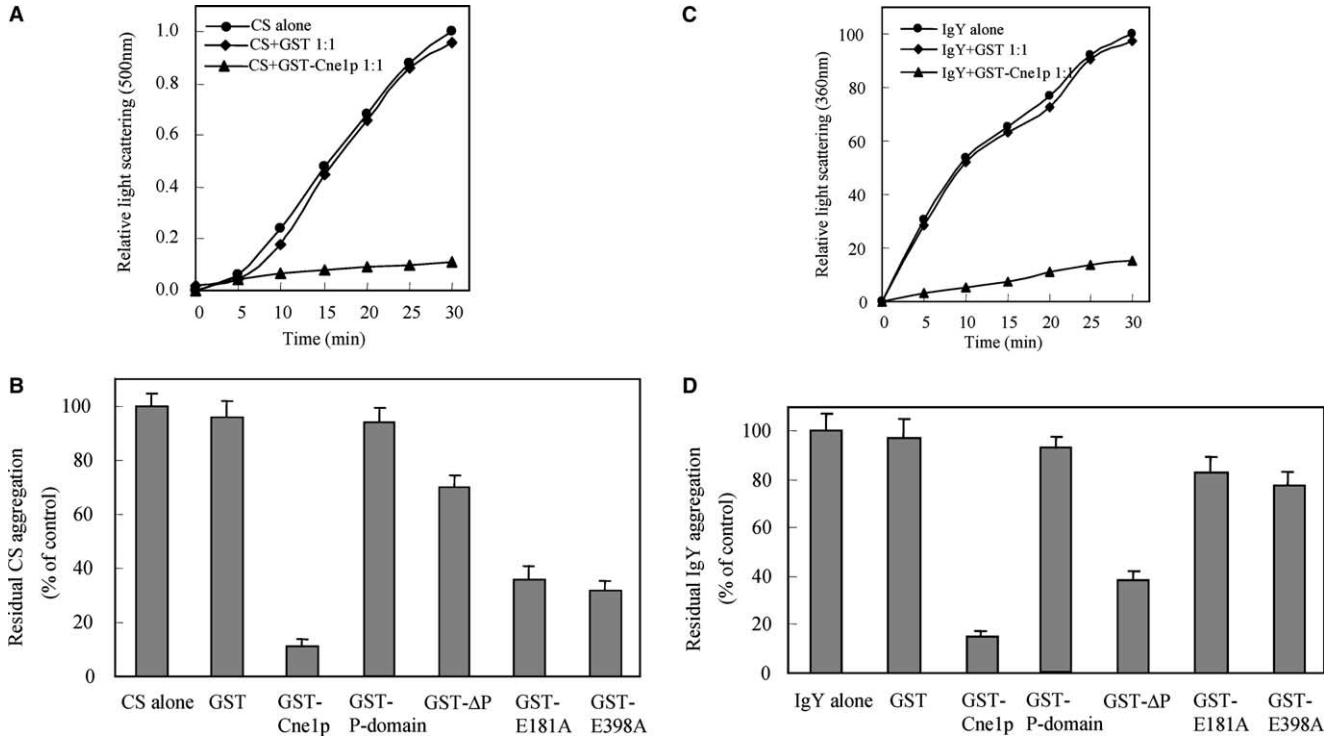

Fig. 5. Aggregation–suppression of a non-glycosylated and a glycosylated substrate by Cne1ps. (A) CS (0.2 μM) was incubated at 45 °C in the absence (●), or presence of 0.2 μM GST (◆), or 0.2 μM Cne1p (▲). Aggregation was measured by monitoring light scattering at 500 nm for 30 min. (B) The values of light scattering at 30 min for 0.2 μM CS incubated at 45 °C in the presence of 0.2 μM GST-Cne1p or GST-fused mutants were compared to that obtained in the absence of GST-Cne1p. Error bars represent the standard error in three replicate experiments. (C) Chemically denatured IgY was rapidly diluted to a 0.3 μM with buffer in the absence (●), or presence of 0.3 μM GST (◆), or 0.3 μM Cne1p (▲). Aggregation was monitored by measuring light scattering at 360 nm for 30 min at 44 °C. D, The values of light scattering at 30 min for 0.3 μM IgY incubated at 44 °C in the presence of 0.3 M GST-Cne1p or GST-fused mutants were compared to that obtained in the absence of GST-Cne1p. Error bars represent the standard error in three replicate experiments.

3. Results

3.1. Construction, expression, and purification of GST fusion proteins

To address the position of P-domain and lectin site of Cne1p, the amino acid sequence alignment of Cne1p and CNX was shown in Fig. 1. The P-domain of CNX comprises residues 276–409 containing four copies each of two different proline-rich sequence motifs (P1 and P2). The residues 247–380 in Cne1p are highly conserved when compared with that of P-domain in CNX, containing four copies each of two different proline-rich sequence motifs (P1 and P2). Thus, the putative P-domain of Cne1p was identified as the residues 247–380. On the other hand, the putative lectin site of CNX is composed of six residues, including Y165, K167, Y186, M189, E217 and E426. The last two residues are strictly conserved in the calnexin family [3,15]. We found that residues E181 and E398 in Cne1p correspond to E217 and E426 in CNX, respectively. On the basis of this alignment search, the P-domain, the ΔP, and two of the point mutants (E181A and E398A) at the Cne1p lectin site were constructed to evaluate the contribution of P-domain and the lectin site to molecular chaperone function of Cne1p. The schematic representation is shown in Fig. 2. These mutants were expressed as a GST fusion form in *E. coli* and purified from the soluble cell extract by GST binding affinity column followed by Sephadex G-75 gel filtration. All recombinant proteins exhibited purity greater than 90% by SDS–PAGE (Fig. 3).

3.2. Lectin site mutants of Cne1p are impaired in oligosaccharide binding

To examine whether the lectin–oligosaccharide interaction was impaired in lectin site mutants, the binding capacity of Cne1ps to oligosaccharide (G1M9) was estimated by SEC (Fig. 4). The peak of GST-Cne1p-G1M9 complex was greatly decreased by lectin site mutants, E181A and E398A. Thus, it was confirmed that point mutation of residues at the lectin site of Cne1p dramatically lowered the binding to oligosaccharides, indicating that these mutants impaired the lectin–oligosaccharides interaction. These findings are consistent with previous studies on the point mutation of the lectin site in CNX that dropped the oligosaccharide binding [15].

3.3. P-domain mutants and lectin site mutants exhibit distinct ability to suppress the aggregation of non-glycosylated citrate synthase and glycosylated IgY

To assess whether various mutants affect the aggregation–suppression of proteins, we tested the effects on non-glycosylated CS and glycosylated IgY. CS is a common

substrate in thermal aggregation and refolding experiments with molecular chaperones [16,17]. IgY containing 27.1% monoglucosylated oligosaccharides ($Glc_1Man_{7-9}GlcNAC_2$) [18], has been used as a glycosylated substrate in aggregation–suppression experiments of calreticulin [19]. As shown in Fig. 5, panels A and C, GST had no effect on both CS and IgY aggregation, whereas the aggregation was effectively suppressed in the presence of equimolar GST-Cne1p. When tested at a Cne1p:substrate ratio of 1:1, P-domain had little effect on suppressing the aggregation of CS and IgY, but the P-domain deletion mutant and the lectin site mutants exhibited a partially decreased aggregation–suppression of CS and IgY. The residual aggregation percentage of P-domain deletion mutant is 70 for CS and 40 for IgY. This suggests that the P-domain deletion mutant was more effective at suppressing the aggregation of CS than IgY. On the other hand, the residual aggregation percentage of lectin site mutants is about 35 for CS and 80 for IgY. This suggests that the lectin site mutants, E181A and E398A, were more effective in suppressing the aggregation of IgY than CS. Additionally, the ability of the P-domain mutant and lectin site mutants to aggregation-suppression of CS and IgY was increased at a Cne1p:substrate ratio of 2:1 (data not shown). These results suggest that the P-domain of Cne1p cooperates with the lectin site in the polypeptide-based interaction and lectin–oligosaccharide interaction.

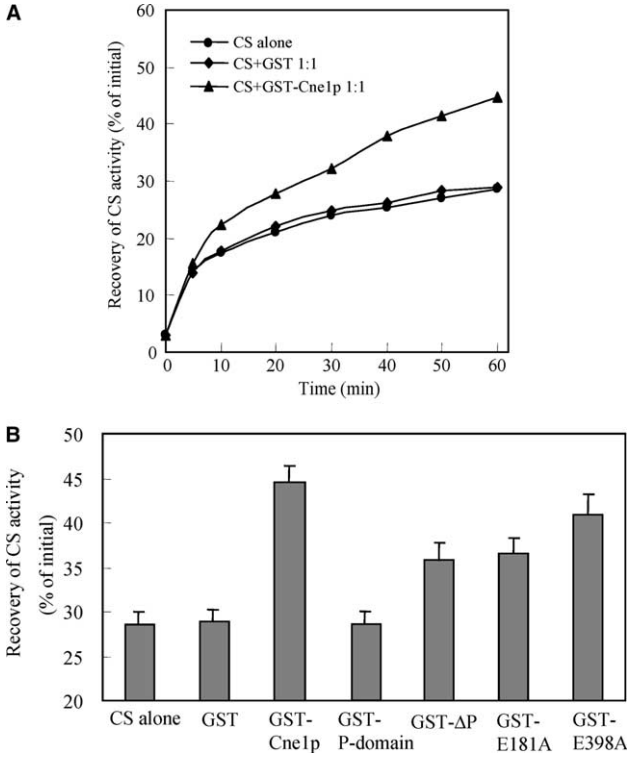

Fig. 6. Reactivation of thermally denatured CS by Cne1ps. (A) CS (0.8 μM) in 50 mM Tris–HCl buffer (pH 8.0) was thermally inactivated by incubation at 43 °C for 30 min. Reactivation of inactivated CS was initiated by 4-fold dilution with the same buffer in the absence (●), or presence of 0.2 μM GST (◆), or 0.2 μM Cne1p (▲) at 25 °C. CS activity was assayed at various time points up to 60 min. (B) The values of CS activity at 60 min for reactivation of inactivated CS at 25 °C in the presence of 0.2 μM GST-Cne1p or GST-fused mutants were compared to that obtained in the absence of GST-Cne1p. Error bars represent the standard error in three replicate experiments.

3.4. *P-domain mutants and lectin site mutants retain distinct ability to enhance the refolding of thermally denatured citrate synthase*

To investigate whether various mutants retain the additional molecular chaperone function, we tested the ability to enhance the refolding of unfolded proteins. We have shown that Cne1p effectively enhanced the reactivation of thermal denatured CS in a molar ratio-dependent manner [4]. As shown in Fig. 6, panel A, the reactivation of CS was enhanced in the presence of equimolar GST-Cne1p after 60 min. All mutants partially retained the ability to enhance the refolding of thermally denatured CS at levels different from Cne1p except P-domain (panel B). Although P-domain alone was unable to enhance the refolding of thermally denatured CS, the P-domain deletion mutant apparently decreased the ability to enhance the refolding of thermally denatured CS. In addition, the lectin site mutants also partially decreased the ability to enhance the refolding of denatured CS. Thus, these results suggest that the cooperation between the P-domain and the lectin site are important for the complete function of Cne1p.

4. Discussion

At the present stage of calnexin research, there has been ongoing debate concerning the molecular mechanism of chaperone function. We have reported the molecular chaperone function of Cne1p by estimating the ability to prevent the aggregation and to enhance the refolding of protein substrate in vitro [4]. The present study demonstrated that Cne1p consists of a structurally conserved P-domain and lectin site in a manner similar to mammalian calnexin. The elucidation of individual function of P-domain and lectin site in Cne1p may help to understand the molecular chaperone function of the calnexin family.

The P-domain of Cne1p was incapable of suppressing the aggregation of non-glycosylated CS and glycosylated IgY. This finding is consistent in that the P-domain of CNX was unable to suppress aggregation of non-glycosylated CS and MDH [20]. This demonstrates that the function of P-domain was evolutionarily conserved. It is probable that the P-domain of Cne1p is involved in the interaction with an ERp57 homologue in yeast as the P-domain of CNX. In addition, we found that the lectin site is functionally conserved. Cne1p showed the ability to bind oligosaccharide and this ability was impaired in lectin site mutants. Therefore, the P-domain and the lectin site are important to the chaperone function of Cne1p. Not only did the P-domain deletion mutant of Cne1p decrease the ability to suppress the aggregation and to enhance the refolding of CS and IgY, but also point mutants of the lectin site (E181A and E398A) decreased the ability to suppress the aggregation and to enhance the refolding of CS and IgY. Thus, we conclude that the P-domain and the lectin site of Cne1p cooperatively function as a molecular chaperone. These results also support the dual model of interaction between calnexin and substrate.

References

[1] Ellgaard, L., Molinari, M. and Helenius, A. (1999) Science 286, 1882–1888.
[2] Parlati, F., Dominguez, M., Bergeron, J.J. and Thomas, D.Y. (1995) J. Biol. Chem. 270, 244–253.
[3] Schrag, J.D., Bergeron, J.J., Li, Y., Borisova, S., Hahn, M., Thomas, D.Y. and Cygler, M. (2001) Mol. Cell. 8, 633–644.
[4] Xu, X., Kanbara, K., Azakami, H. and Kato, A. (2004) J. Biochem. (Tokyo) 135, 615–618.
[5] Oliver, J.D., van der Wal, F.J., Bulleid, N.J. and High, S. (1997) Science 275, 86–88.
[6] Frickel, E.M., Riek, R., Jelesarov, I., Helenius, A., Wuthrich, K. and Ellgaard, L. (2002) Proc. Natl. Acad. Sci. USA 19, 1954–1959.
[7] Hammond, C., Braakman, I. and Helenius, A. (1994) Proc. Natl. Acad. Sci. USA 91, 913–917.
[8] Hebert, D.N., Foellmer, B. and Helenius, A. (1995) Cell 81, 425–433.
[9] Ware, T.E., Vassilakos, A., Peterson, P.A., Jackson, M.R., Lehrman, M.A. and Williams, D.B. (1995) J. Biol. Chem. 270, 4697–4704.
[10] Williams, D.B. (1995) Biochem. Cell Biol. 73, 123–132.
[11] Vallejp, A.N., Pogulis, R.J. and Pease, L.R. (1995) in: PCR Primer: A Laboratory Manual (Dieffenbach, C.W. and Devksler, G.S., Eds.), pp. 603–612, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[12] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[13] Laemmli, U.K. (1980) Nature 277, 680–685.
[14] Buchner, J., Grallert, H. and Jakob, U. (1998) Methods Enzymol. 290, 323–338.
[15] Leach, M.R. and Willams, D.B. (2004) J. Biol. Chem. 279, 9072–9079.
[16] Buchner, J., Schmidt, M., Fuchs, M., Jaenicke, R., Rudolph, R., Schmid, F.X. and Kiefhaber, T. (1991) Biochemistry 30, 1586–1591.
[17] Jakob, U., Lilie, H., Meyer, I. and Buchner, J. (1995) J. Biol. Chem. 270, 7288–7294.
[18] Ohta, M., Hamako, J., Yamamoto, S., Hatta, H., Kim, M., Yamamoto, T., Oka, S., Mizuochi, T. and Matsuura, F. (1991) Glycoconj. J. 8, 400–413.
[19] Saito, Y., Ihara, Y., Leach, M.R., Cohen-Doyle, M.F. and Williams, D.B. (1999) EMBO J. 18, 6718–6729.
[20] Leach, M.R., Cohen-Doyle, M.F., Thomas, D.Y. and Williams, D.B. (2002) J. Biol. Chem. 277, 29686–29697.