Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 323 (2004) 281–287
BBRC
www.elsevier.com/locate/ybbrc

Gentamicin binds to the lectin site of calreticulin and inhibits its chaperone activity☆

Tomohisa Horibe[a], Hiroaki Matsui[a], Mami Tanaka[a], Hiroaki Nagai[a], Yoshiki Yamaguchi[b,c], Koichi Kato[b,c], Masakazu Kikuchi[a,*,1]

[a] *Department of Bioscience and Technology, Faculty of Science and Engineering, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Mizuho-ku, Nagoya 467-8603, Japan.*
[c] *CREST, JST, Kawaguchi 332-1102, Japan*

Received 4 August 2004
Available online 28 August 2004

Abstract

Recently, it became clear that aminoglycoside antibiotics affect protein–protein interactions involving protein disulfide isomerase as well as protein synthesis in the endoplasmic reticulum. In this study, we used affinity column chromatography to screen gentamicin-binding proteins in microsomes derived from bovine kidney in order to learn about the possible mechanisms of gentamicin-associated nephrotoxicity. One of the gentamicin-binding proteins was identified as calreticulin (CRT) by N-terminal amino acid sequence analysis. Interestingly, gentamicin inhibited the chaperone and oxidative refolding activities of CRT when *N*-glycosylated substrates such as α1-antitrypsin and α-mannosidase were used as substrates, but it did not inhibit the chaperone activity of CRT when unglycosylated citrate synthase was used. Moreover, CRT suppressed the aggregation of deglycosylated and denatured α-mannosidase, but gentamicin did not inhibit its chaperone activity. Experiments with domain mutants suggest that the lectin site of CRT is the main target for gentamicin binding and that binding of gentamicin to this site inhibits the chaperone activity of CRT.

Keywords: Gentamicin; Calreticulin; Molecular chaperone; Oxidative refolding; N-linked oligosaccharide

Calreticulin (CRT) is expressed in the endoplasmic reticulum (ER) and assists in the folding and assembly of newly synthesized glycoproteins. CRT functions as a lectin-like chaperone and works as part of the ER quality control system that couples the processing of N-linked glycans with protein folding [1]. CRT also associates with ERp57, an ER thiol oxidoreductase, and they cooperate in the proper formation of disulfide bonds in newly synthesized glycoproteins [2]. CRT is a soluble ER-resident protein, and shares considerable sequence and structural homology with the lumenal domain of calnexin, an ER transmembrane protein. Both chaperones are predicted to possess a globular domain with an extended arm comprised of proline-rich repeats known as the P-domain [3–7]. The globular N-domain possesses the lectin site with specificity for monoglycosylated glycans [3–7], and the P-domain has been mapped as the site of interaction with ERp57 [3–7].

☆ *Abbreviations:* hCRT, human calreticulin; bCRT, bovine calreticulin; ER, endoplasmic reticulum; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CBB-R250, Coomassie brilliant blue R250; PBS, phosphate-buffered saline; RU, resonance unit; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; PNGase, glycopeptidase F (peptide *N*-glycosidase F); PVDF, polyvinylidene difluoride.

* Corresponding author. Fax: +81 77 561 2659.
E-mail address: kikuchi@is.ritsumei.ac.jp (M. Kikuchi).
[1] Present address: Department of Bioscience and Bioinformatics, College of Information Science and Engineering, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, Japan.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.08.099

Gentamicin is widely used as a bactericidal agent for the treatment of several gram-negative bacterial infections; however, its clinical use is limited by its nephrotoxicity. There are many reports on the clinical effects of gentamicin, but only a few reports detail the mechanism of this side effect [8,9]. Recently, Sandoval and Molitoris showed that gentamicin is trafficked retrogradely through the secretory pathway and is released into the cytosol via the ER. They also reported that the rapid retrograde transport of gentamicin to the ER decreases protein synthesis [10].

We previously reported that aminoglycoside antibiotics such as gentamicin and ribostamycin bind to protein disulfide isomerase (PDI) and inhibit its chaperone activity [11,12]. PDI is known to catalyze the formation, reduction, and isomerization of disulfide bonds [13], and is essential for yeast viability [14–16]. PDI was suggested to be involved in the quality control system, which degrades misfolded proteins in the cell [17,18]. Another function of PDI is to bind other proteins, such as the prolyl 4-hydroxylase β-subunit [19] and microsomal triglyceride transfer protein large subunit [20], in order to stabilize them. These observations suggest that PDI is a multifunctional and indispensable protein in the cell.

Since it is suggested that interference with chaperone activity and protein synthesis in the kidney by gentamicin could contribute to renal side effects, we focused on finding gentamicin-binding proteins in bovine kidney microsomes using affinity column chromatography. Subsequent SDS–PAGE analysis showed that several proteins bound to gentamicin. Here we describe that gentamicin binds to the lectin site of CRT to inhibit its chaperone activity.

Materials and methods

Materials. CNBr-activated Sepharose 4B was purchased from Amersham–Pharmacia Biotech (Uppsala, Sweden). Bovine kidney was purchased from Nippon Ham (Osaka, Japan). α1-Antitrypsin, jack bean α-mannosidase, and citrate synthase were purchased from Sigma Chemical (St. Louis, USA). Glycopeptidase F [peptide *N*-glycosidase F (PNGase)] was purchased from Takara (Shiga, Japan). PVDF membrane was purchased from Bio-Rad (California, USA). Gentamicin, α-chymotrypsin, and other reagents were mostly from Wako Pure Chemical Industries (Osaka, Japan). All reagents were of reagent grade quality.

Strains and plasmids. *Escherichia coli* AD494 (DE3) {Δ*ara*, *leu*7697, Δ*lac*X74, Δ*pho*A, *Pvu*II, *Pho*R, Δ*mal*F3, F′ [*lac*$^{-}$, (*lacI*^{q}), *pro*], *trx*B::*kan* (DE3)} and pET-15b (Novagen, Madison, USA) were used for the expression of human CRT and several domain deletants.

Preparation of the gentamicin–Sepharose 4B affinity column. A gentamicin–Sepharose 4B affinity column was prepared according to the method of Horibe et al. [11].

Preparation of microsomes from bovine kidney and screening of gentamicin-binding proteins. Microsomes were prepared from 300*g* of bovine kidney by the differential centrifugation method of Tangen et al. [21] using homogenizing buffer (0.25 M sucrose, 5 mM EDTA, and 0.05 M Tris–HCl [pH 7.5]). The microsomes were solubilized in solubilization buffer (0.1 M sodium phosphate buffer [pH 7.5], 5mM EDTA, and 1% [v/v] Triton X-100) with a Teflon homogenizer (RIKAGAKUGARASU SEISAKUJYO, Japan). The supernatant obtained was dialyzed against 0.05 M sodium phosphate buffer (0.05 M NaH_2PO_4, and 0.05 M Na_2HPO_4 [pH 7.4]) containing 0.15 M NaCl, and was applied to the gentamicin–Sepharose affinity column. After washing out unbound proteins with the same buffer, bound proteins were eluted with elution buffer (0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, and 1 M NaCl [pH 7.4]).

SDS–PAGE. SDS–PAGE was carried out according to the method of Laemmli [22] using 12.5% (w/v) gels. Gels were stained with CBB-R 250.

Analysis of the N-terminal amino acid sequence. After SDS–PAGE, the protein bands were transferred to PVDF membranes. Proteins were extracted from the membrane, and the N-terminal amino acid sequence was determined according to the method of Edman [23] using the peptide sequencer PPSQ-10 (SHIMADZU, Kyoto, Japan).

Preparation of bovine CRT from bovine kidney. Bovine CRT was prepared from bovine kidney according to the method of Rowling et al. [24]. Briefly, microsomes were prepared from bovine kidney, and the 55–95% ammonium sulfate-saturated fraction was retained. After dialysis against 0.05 M sodium phosphate buffer (0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4 [pH 7.4]), the extract was applied to a gentamicin–Sepharose affinity column and eluted as described above. Eluted proteins were dialyzed against 0.02 M Hepes buffer (0.02 M Hepes, 2.5 mM KCl, and 0.5 mM $MgCl_2$ [pH 7.5]) and applied to a ConA–Sepharose column. After washing out unbound proteins with the same buffer, bound proteins were eluted with an elution buffer (0.02 M Hepes, 2.5 mM KCl, 0.5 mM $MgCl_2$ [pH 7.5], and 1 M NaCl). The extract was dialyzed against 0.05 M sodium phosphate buffer (0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4 [pH 6.3]), applied to a HiTrap Q FPLC column, and then eluted with linear gradient of 0–1 M NaCl.

Expression and purification of recombinant human CRT and its domain deletants. The human CRT (hCRT) cDNA [25] was inserted downstream from the His-tag coding region of pET-15b using the *Xho*I and *Bam*HI sites. PCR was used to construct domain deletant cDNAs that lacked either the N domain (hCRT-PC) or the N and P domains (hCRT-C). These cDNAs were inserted into pET-15b as described above. Expression and purification of hCRT, hCRT-PC, and hCRT-C were carried out as described previously, using a nickel-chelating resin column [26–28]. Further purification of hCRT-PC and hCRT-C was performed using a HiTrap Q FPLC column.

Interaction of CRT with aminoglycoside antibiotics. Surface plasmon resonance experiments were performed with a BIACORE 3000 biosensor system (BIACORE, Uppsala, Sweden) according to the method of Horibe et al. [11,12,27–29] at a flow rate of 20 μl/min at 25 °C. HBS buffer (0.01 M Hepes, 0.15 M NaCl, 0.005% Tween 20, and 3 mM EDTA [pH 7.4]) was used as the running buffer during the assay to prohibit non-specific binding. Data analysis was performed using BIA evaluation software version 3.1.

Deglycosylation of α1-antitrypsin and jack bean α-mannosidase. α1-Antitrypsin and jack bean α-mannosidase were deglycosylated with glycopeptidase F [peptide *N*-glycosidase F (PNGase)] according to the manufacturer's protocol. Briefly, both proteins were dissolved to a concentration of 10 mg/ml in 0.1 M Tris–HCl (pH 8.6) containing 0.1% SDS and 0.15% (v/v) of 2-mercaptoethanol. After the samples were boiled for 3 min at 100 °C, Nonidet P-40 was added to a final concentration of 0.5% (v/v), and then the sample was digested in the presence of 0.5 mU/μl PNGase. The proteins were precipitated with 95% ethanol and dried by evaporation.

Aggregation assay. Native and deglycosylated jack bean α-mannosidase (33.33 μM) were dissolved in 20 mM sodium phosphate buffer (pH 7.4) containing 6 M guanidinium–HCl and 0.15 M dithiothreitol. After denaturation for 2 h at room temperature, samples were diluted out of the denaturant to 0.67 μM in 20 mM sodium phosphate buffer (pH 7.4) containing various concentrations

of bCRT, hCRT, or CRT domain deletants (hCRT-PC and hCRT-C) and antibiotics. Aggregation was monitored over a period of 15 min at 25 °C by monitoring the increase in absorbance at 360 nm. The aggregation of citrate synthase upon thermal denaturation was assayed by the method of Shao et al. [30]. Briefly, citrate synthase was diluted in prewarmed Hepes buffer (0.04 mM Hepes [pH 7.4]) at 43 °C. Protein aggregation was monitored by measuring light scattering with a Hitachi Spectrofluorometer F4010. The excitation and emission wavelengths were set to 500 nm. The excitation and emission slit widths were set to 3 nm.

Oxidative refolding of α1-antitrypsin. Oxidative refolding of native and deglycosylated α1-antitrypsin was performed as described previously [27,28].

Results

Screening of proteins binding to gentamicin

A protein extract prepared from bovine kidney microsomes was applied to a gentamicin–Sepharose column. Unbound materials were thoroughly washed out, and the proteins eluted from the gentamicin–Sepharose column were analyzed using SDS–PAGE. As shown in Fig. 1, several bands were observed. Analysis of the N-terminal amino acid sequences revealed that these proteins were 94-kDa glucose-regulated protein (GRP 94), calreticulin (CRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), apolipoprotein A1, and proteasome subunit β type 9 (PSB9) (data not shown). GRP 94 and CRT are ER-resident proteins [1,2,31], and apolipoprotein A1 is a secreted protein [32]. In contrast, GAPDH and PSB9 are not ER-resident proteins [33,34].

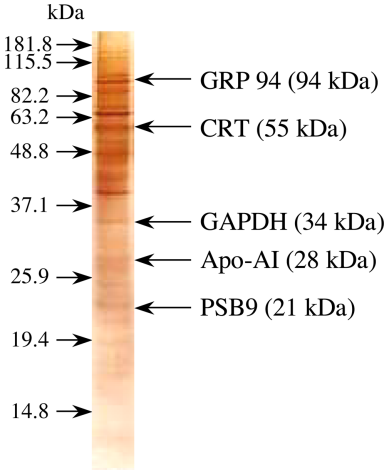

Fig. 1. SDS–PAGE of gentamicin-binding proteins. Electrophoresis was carried out in a 12.5% gel as described in the Materials and methods. The arrows on the left indicate the molecular weight markers. The arrows on the right indicate 94-kDa glucose-regulated protein (GRP 94), calreticulin (CRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), apolipoprotein A1 (Apo-A1), and proteasome subunit β type 9 (PSB9), which were identified using analyses of their N-terminal amino acid sequences and their molecular weight.

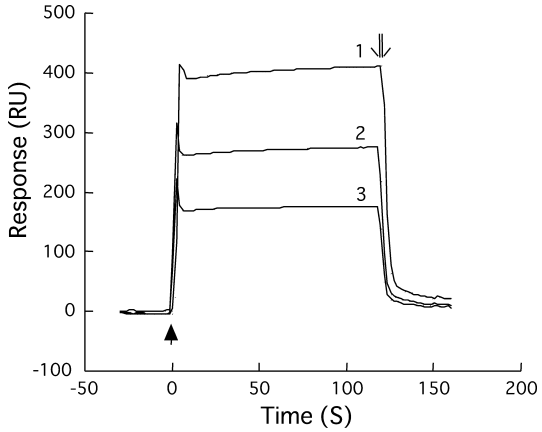

Fig. 2. Sensorgrams of gentamicin bound to immobilized bCRT on the BIACORE biosensor. All analytes [1 (361.42 μM), 2 (180.71 μM), and 3 (90.36 μM)] were injected over the bCRT immobilized on a sensor chip. The progress of binding to immobilized bCRT was monitored by following the increase in the signal [resonance units (RU)] induced by gentamicin. The arrow and double arrow indicate the beginning and end of the injection, respectively.

Interaction of CRT with gentamicin

Since CRT is a soluble and abundant ER-resident chaperone protein, we selected CRT as a first target for investigating the effects of gentamicin. To investigate the interaction of CRT with gentamicin, we purified bovine CRT (bCRT) from bovine kidney. Purified bCRT was limited to a single band in SDS–PAGE (data not shown). Purified bCRT was immobilized on the surface of a CM5 sensor chip, and the interaction of bCRT with gentamicin was analyzed using BIACORE 3000. Fig. 2 shows that gentamicin easily associated and dissociated with bCRT immobilized on the sensor chip. The K_D of bCRT to gentamicin was determined to be 3.85×10^{-4} M using BIA evaluation software.

Effect of gentamicin on CRT activity

We next studied the effect of gentamicin on the activity of bCRT. As shown in Fig. 3, gentamicin did not inhibit the chaperone activity of bCRT when citrate synthase (CS), which naturally lacks N-linked oligosaccharides, was used as a substrate. An excess amount of gentamicin had no effect on the chaperone activity of bCRT (data not shown). However, when α1-antitrypsin and α-mannosidase, which have N-linked oligosaccharides, were used as substrates, gentamicin inhibited the oxidative refolding and chaperone activities of bCRT in a manner dependent on the concentration of gentamicin (Figs. 4A and B). We then deglycosylated α1-antitrypsin and α-mannosidase with glycopeptidase F (PNGase), and confirmed the digested products using SDS–PAGE (data not shown). Interestingly, when deglycosylated α1-antitrypsin was used as a substrate, bCRT was unable to oxidatively

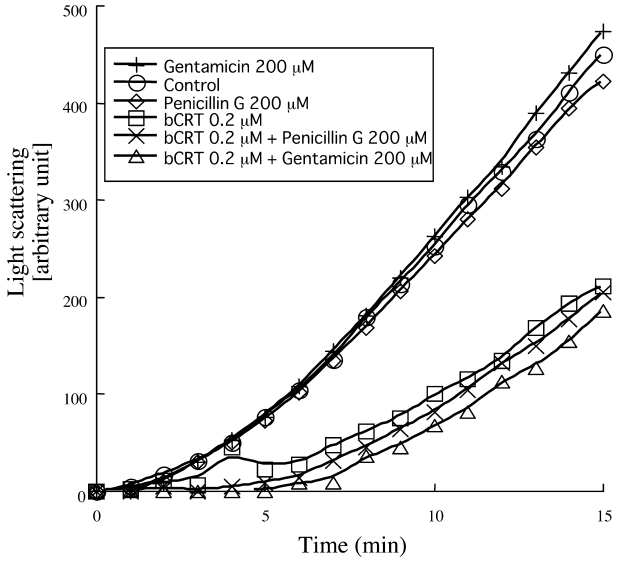

Fig. 3. Effect of gentamicin on the chaperone activity of bCRT using thermally denatured citrate synthase as a substrate. The suppression of thermally denatured citrate synthase aggregation by bCRT was investigated. Citrate synthase (30 μM) was diluted 200-fold into prewarmed Hepes buffer (0.15 μM final concentration) in the absence of both gentamicin and bCRT as a control (○). The inset indicates the concentration of bCRT and antibiotics tested. Penicillin G was used as a negative control.

refold denatured α1-antitrypsin (Fig. 4A). In contrast, bCRT suppressed aggregation of the deglycosylated and denatured α-mannosidase, but gentamicin did not inhibit its chaperone activity (Fig. 4B). These observations suggest that N-linked oligosaccharides are important for the oxidative refolding activity of bCRT, and that gentamicin competes for the lectin site of bCRT and inhibits its chaperone activity.

Role of each domain of CRT in the oxidative refolding and chaperone activities, and effect of gentamicin on these activities

It has been reported that CRT has a domain structure of N-P-C [3–7], and the domains have been suggested to function as a lectin, an ERp57-binding site, and a peptide-binding site, respectively [3–7]. To investigate the role of each CRT domain in the oxidative refolding and chaperone activities and the effect of gentamicin on these activities, we constructed two domain deletants of wild-type human CRT (hCRT), hCRT-PC and hCRT-C. As shown in Figs. 5A–C, all of the purified proteins migrated as single bands on SDS–PAGE. Since it is known that native CRT has N-linked oligosaccharides [1,2], we investigated whether gentamicin would have different effects on the oxidative refolding and chaperone activities of recombinant hCRT and bCRT purified from bovine kidney, but no differences were found (data not shown). hCRT-PC and hCRT-C, which lack the lectin site in the N domain, were unable to refold the denatured α1-antitrypsin and their activity was not affected by gentamicin (data not shown). Both hCRT-PC and hCRT-C suppressed the aggregation of α-mannosidase and its deglycosylated product, although their activities were less than that of wild-type CRT (Figs. 6A and B). However, gentamicin had little effect on the activity of the domain deletants (Figs. 6A and B). These observations suggest that the lectin site of CRT is the main target for gentamicin-binding and that this binding inhibits the chaperone activity of CRT.

Discussion

Although gentamicin has been widely used clinically, its clinical use is partially limited by its renal toxicity. Although there are many reports on the clinical effects of gentamicin, there have been few reports detailing the mechanisms of this side effect or identifying gentamicin-specific-binding proteins [8,9]. Recently, Sandoval and Molitoris [10] showed that gentamicin is retrogradely trafficked through the secretory pathway and is released into the cytosol via the ER. They also reported that the rapid retrograde transport of gentamicin to the ER decreases protein synthesis [10]. Moreover, we previously reported that aminoglycoside antibiotics such as gentamicin and ribostamycin bind to PDI and inhibit its chaperone activity [11,12]. The effect of gentamicin on protein synthesis and the chaperone activity of PDI might be one of the causes of the renal side effect of gentamicin. To learn more about the effects of gentamicin on cellular function in the kidney, we screened gentamicin-binding proteins in bovine kidney microsomes and identified several gentamicin-binding proteins, including 94-kDa glucose-regulated protein (GRP 94), CRT, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), apolipoprotein A1, and proteasome subunit β type 9 (PSB9) using N-terminal amino acid sequence analysis (Fig. 1). Of these identified proteins, GRP 94 [31] and CRT [1,2] are ER-resident proteins, and apolipoprotein A1 is a secreted protein [32]. However, GAPDH [33] and PSB9 [34] are not ER-resident proteins, suggesting that these proteins might bind to gentamicin-binding proteins or could have been introduced as contaminants during the cell fractionation step. Gentamicin did not inhibit the activity of GAPDH (data not shown). Therefore, we focused on the effect of gentamicin on the activity of CRT, a known chaperone protein [1,2].

We found a specific, but not strong, interaction between purified bCRT and gentamicin (Fig. 2) by BIACORE analysis, and the interaction had a K_D value of 3.85×10^{-4} M. We then investigated the effect of gentamicin on bCRT activities by assaying bCRT's chaperone and oxidative refolding activities. Interestingly, although gentamicin inhibited the chaperone and oxidative refolding activities of bCRT when *N*-glycosylated

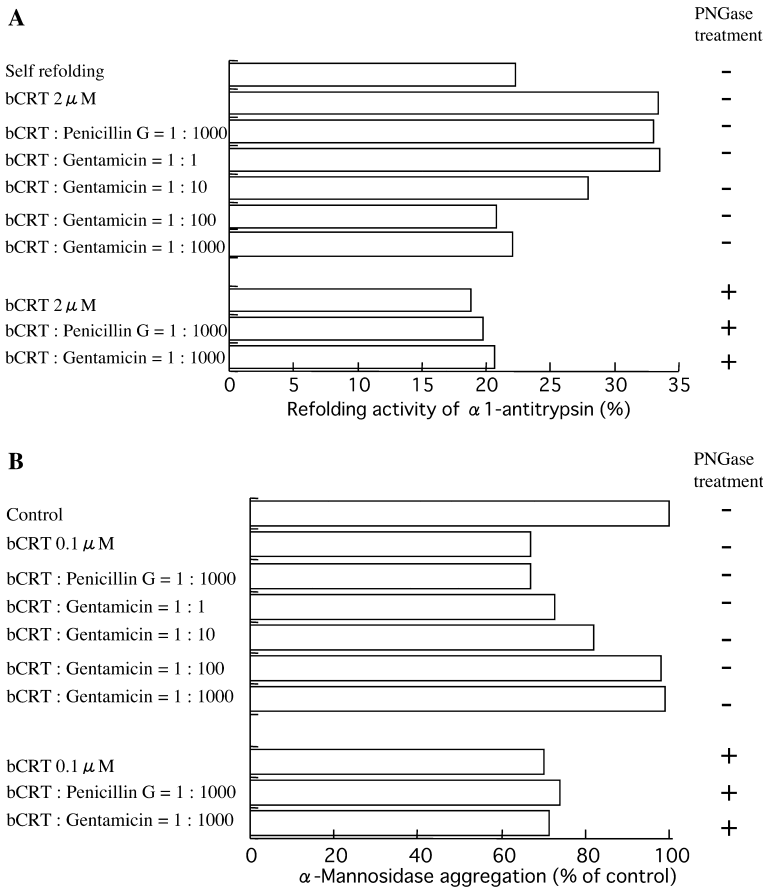

Fig. 4. Effect of gentamicin on the oxidative refolding (A) and chaperone (B) activities of bCRT. The left side of the bar graphs indicates molar ratios of bCRT to gentamicin. The presence (+) or absence (−) of glycopeptidase F (PNGase) treatment is shown in the right of the figure. Penicillin G was used as a negative control. (A) As a control, denaturation and reactivation of α1-antitrypsin and the product of its deglycosylation with glycopeptidase F (0.25 mg/ml) were carried out in the absence of both gentamicin and bCRT (self-refolding). (B) As a control, the suppression of denatured α-mannosidase aggregation by bCRT was investigated. Jack bean α-mannosidase and the product of its deglycosylation with glycopeptidase F were denatured and diluted (the final concentration was 0.67 μM) in the absence of both gentamicin and CRT as a control; this value was set to 100%.

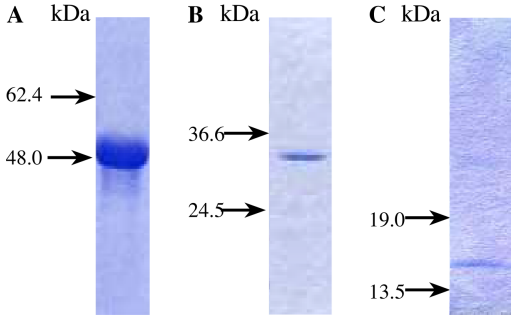

Fig. 5. SDS–PAGE of purified wild-type hCRT (A), hCRT-PC (B), and hCRT-C (C). Electrophoresis was carried out in 12.5% (A) and 15% (B,C) gels as described in the Materials and methods. The arrows indicate the locations of molecular weight markers.

substrates such as α1-antitrypsin and α-mannosidase were used, it did not inhibit the chaperone activity of bCRT when non-glycosylated citrate synthase was used as a substrate. These results suggest that the N-linked oligosaccharide is critical for the inhibition of chaperone activity of CRT by gentamicin. In the assay using α1-antitrypsin and α-mannosidase, a 100:1 molar ratio of gentamicin to bCRT almost completely inhibited the chaperone and oxidative refolding activities of bCRT. Other antibiotics such as aminobenzylpenicillin, penicillin G, and cefoxitin did not show any effect on either the chaperone or the oxidative refolding activities of bCRT (data, except for penicillin G, not shown). Saito et al. [35] reported that calreticuin inhibits the aggregation of both glycosylated and non-glycosylated proteins in vitro. However, bCRT did not show the oxidative refolding activity when deglycosylated α1-antitrypsin was used as a substrate. This observation suggests that the sugar chain is necessary for the refolding activity of CRT. Next we constructed a wild-type hCRT and the domain deletants hCRT-PC and hCRT-C in order to investigate the relationship between the role of each CRT domain in oxidative refolding and chaperone activities and the effect of gentamicin on these activities. hCRT-PC and hCRT-C, which lack the lectin site, did not refold the denatured α1-antitrypsin and their activity was not affected by gentamicin. Together, these re-

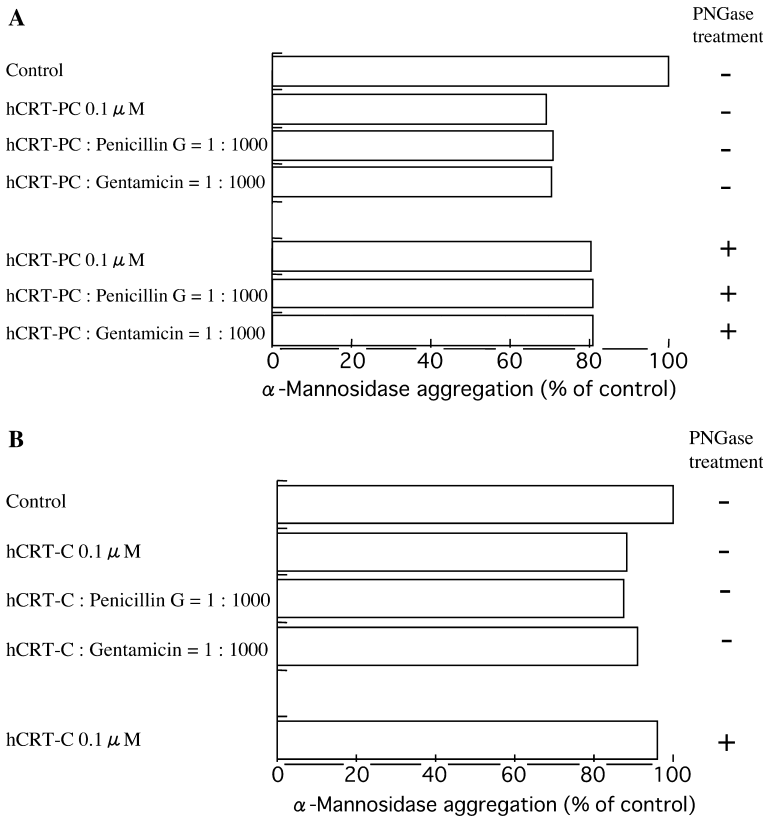

Fig. 6. Effect of gentamicin on the chaperone activity of hCRT-PC (A) and hCRT-C (B) using α-mannosidase as a substrate. The left sides of the bar graphs indicate molar ratios of hCRT-PC (A) and hCRT-C (B) to gentamicin. The presence (+) or absence (−) of glycopeptidase F (PNGase) treatment is shown in the right sides of the figures. Penicillin G was used as a negative control.

sults indicate that both the lectin site and N-linked oligosaccharides are critical for the oxidative refolding activity of CRT, and that gentamicin competes for the lectin site, thereby inhibiting CRT's chaperone activity.

Recently, Miyazaki et al. [9] reported that gentamicin binds to HSP 73 and inhibits its chaperone activity, which may be associated with gentamicin-induced acute tubular injury in the kidney. We also reported previously that aminoglycoside antibiotics bind to PDI and inhibit its chaperone activity [11,12]. Together with the retrograde trafficking of gentamicin through the secretory pathway [10], the inhibition of the chaperone activity of proteins in the cell by gentamicin might underlie the nephrotoxicity caused by this antibiotic.

Acknowledgment

We thank Dr. Sunji Natori (Natori Special Laboratory, The Institute of Physical and Chemical Research [RIKEN]), for providing human calreticulin cDNA.

References

[1] K.H. Krause, M. Michalak, Calreticulin, Cell 88 (1997) 439–443.

[2] J.D. Oliver, H.L. Roderick, D.H. Llewellyn, S. High, ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin, Mol. Biol. Cell 10 (1999) 2573–2582.

[3] M. Michalak, E.F. Corbett, N. Mesaeli, K. Nakamura, M. Opas, Calreticulin: one protein, one gene, many functions, Biochem. J. 344 (1999) 281–292.

[4] L. Ellgaard, R. Riek, T. Herrmann, P. Gunter, D. Braun, A. Helenius, K. Wuthrich, NMR structure of the calreticulin P-domain, Proc. Natl. Acad. Sci. USA 98 (2001) 3133–3138.

[5] M. Michalak, J.M. Robert Parker, M. Opas, Ca^{2+} signaling and calcium binding chaperones of the endoplasmic reticulum, Cell Calcium 32 (2002) 269–278.

[6] K. Nakamura, A. Zuppini, S. Arnaudeau, J. Lynch, I. Ahsan, R. Krause, S. Papp, H. De Smedt, J.B. Parys, W. Müller-Esterl, D.P. Lew,, K.H. Krause, N. Demaurex, M. Opas, M. Michalak, Functional specialization of calreticulin domains, J. Cell Biol. 154 (2001) 961–972.

[7] M.R. Leach, M.F. Cohen-Doyle, D.Y. Thomas, D.B. Williams, Localization of the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin, J. Biol. Chem. 277 (2002) 29686–29697.

[8] H. Ohtani, H. Wakui, A. Komatsuda, K. Satoh, A.B. Miura, H. Itoh, Y. Tashima, Induction and intracellular localization of 90-kilodalton heat-shock protein in rat kidneys with acute gentamicin nephropathy, Lab Invest. 72 (1995) 161–165.

[9] T. Miyazaki, R. Sagawa, T. Honma, S. Noguchi, T. Harada, A. Komatsuda, H. Ohtani, H. Wakui, K. Sawada, M. Otaka, S. Watanabe, M. Jikei, N. Ogawa, F. Hamada, H. Itoh, 73-kDa molecular chaperone HSP73 is a direct target of antibiotic gentamicin, J. Biol. Chem. 279 (2004) 17295–17300.

[10] R.M. Sandoval, B.A. Molitoris, Gentamicin traffics retrograde through the secretory pathway and is released in the cytosol via the endoplasmic reticulum, Am. J. Physiol. Renal. Physiol. 286 (2004) F617–624.
[11] T. Horibe, H. Nagai, K. Sakakibara, Y. Hagiwara, M. Kikuchi, Ribostamycin inhibits the chaperone activity of protein disulfide isomerase, Biochem. Biophys. Res. Commun. 289 (2001) 967–972.
[12] T. Horibe, H. Nagai, H. Matsui, Y. Hagiwara, M. Kikuchi, Aminoglycoside antibiotics bind to protein disulfide isomerase and inhibit its chaperone activity, J. Antibiot. 55 (2002) 528–530.
[13] R.B. Freedman, in: T.E. Creighton (Ed.), Protein Folding, W.H. Freeman, New York, 1992, pp. 455–539.
[14] R. Farquhar, N. Honey, S.J. Murant, P. Bossier, L. Schultz, D. Montgomery, R.W. Ellis, R.B. Freedman, M.F. Tuite, Protein disulfide isomerase is essential for viability in *Saccharomyces cerevisiae*, Gene 108 (1991) 81–89.
[15] R. Gunther, C. Brauer, B. Janetzky, H.H. Forster, E.M. Ehbrecht, L. Lehle, H. Kuntzel, The *Saccharomyces cerevisiae* TRG1 gene is essential for growth and encodes a lumenal endoplasmic reticulum glycoprotein involved in the maturation of vacuolar carboxypeptidase, J. Biol. Chem. 266 (1991) 24557–24586.
[16] M.L. LaMantia, T. Miura, H. Tachikawa, H.A. Kaplan, W.J. Lennarz, T. Mizunaga, Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast, Proc. Natl. Acad. Sci. USA 88 (1991) 4453–4457.
[17] M. Otsu, F. Omura, T. Yoshimori, M. Kikuchi, Protein disulfide isomerase associates with misfolded human lysozyme in vivo, J. Biol. Chem. 269 (1994) 6874–6877.
[18] M. Otsu, R. Urade, M. Kito, F. Omura, M. Kikuchi, A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum, J. Biol. Chem. 270 (1995) 14958–14961.
[19] T. Pihlajaniemi, T. Helaakoski, K. Tasanen, R. Myllylä, M.L. Huhtala, J. Koivu, K.I. Kivirikko, Molecular cloning of the β-subunit of human prolyl 4-hydroxylase: this subunit and protein disulphide isomerase are products of the same gene, EMBO J. 6 (1987) 643–649.
[20] J.R. Wetterau, K.A. Combs, S.N. Spinner, B.J. Joiner, Protein disulfide isomerase is a component of the microsomal triglyceride–transfer protein complex, J. Biol. Chem. 265 (1990) 9800–9807.
[21] O. Tangen, J. Jonsson, S. Orrenius, Isolation of rat liver microsomes by gel filtration, Anal. Biochem. 54 (1973) 597–603.
[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[23] P. Edman, On the mechanism of the phenyl isothiocyanate degradation of peptides, Acta Chem. Scand. 10 (1956) 761–768.
[24] P.J. Rowling, S.H. McLaughlin, G.S. Pollock, R.B. Freedman, A single purification procedure for the major resident proteins of the ER lumen: endoplasmin, BiP, calreticulin and protein disulfide isomerase, Protein Expr. Purif. 5 (1994) 331–336.
[25] Y. Okuyama, J.H. Cho, Y. Nakajima, K. Homma, K. Sekimizu, S. Natori, Binding between azurocidin and calreticulin: its involvement in the activation of peripheral monocytes, J. Biochem. 135 (2004) 171–177.
[26] M. Kikuchi, E. Doi, I. Tsujimoto, T. Horibe, Y. Tsujimoto, Functional analysis of human P5, a protein disulfide isomerase homologue, J. Biochem. 132 (2002) 451–455.
[27] T. Horibe, M. Gomi, D. Iguchi, H. Ito, Y. Kitamura, T. Masuoka, I. Tsujimoto, T. Kimura, M. Kikuchi, Different contributions of the three CXXC motifs of human protein-disulfide isomerase-related protein to isomerase activity and oxidative refolding, J. Biol. Chem. 279 (2004) 4604–4611.
[28] T. Horibe, D. Iguchi, T. Masuoka, M. Gomi, T. Kimura, M. Kikuchi, Replacement of domain b of human protein disulfide isomerase-related protein with domain b' of human protein disulfide isomerase dramatically increases its chaperone activity, FEBS Lett. 566 (2004) 311–315.
[29] T. Horibe, C. Yosho, S. Okada, M. Tsukamoto, H. Nagai, Y. Hagiwara, Y. Tsujimoto, M. Kikuchi, The chaperone activity of protein disulfide isomerase is affected by cyclophilin B and cyclosporin A in vitro, J. Biochem. 132 (2002) 401–407.
[30] F. Shao, W.M. Bader, U. Jakob, J.C.A. Bardwell, DsbG, a protein disulfide isomerase with chaperone activity, J. Biol. Chem. 275 (2000) 13349–13352.
[31] G. Koch, M. Smith, D. Macer, P. Webster, R. Mortara, Endoplasmic reticulum contains a common, abundant calcium-binding glycoprotein, endoplasmin, J Cell Sci. 86 (1986) 217–232.
[32] L.K. Curtiss, R.S. Smith, Localization of two epitopes of apolipoprotein A-I that are exposed on human high density lipoproteins using monoclonal antibodies and synthetic peptides, J. Biol. Chem. 263 (1988) 13779–13785.
[33] J.B. Fox, W.B. Dandliker, A study of some of the physical properties of glyceraldehyde-3-phosphate dehydrogenase, J. Biol. Chem. 218 (1956) 53–57.
[34] D.P. Singal, M. Ye, S.A. Quadri, Major histocompatibility-encoded human proteasome LMP2. Genomic organization and a new form of mRNA, J. Biol. Chem. 270 (1995) 1966–1970.
[35] Y. Saito, Y. Ihara, M.R. Leach, M.F. Cohen-Dovle, D.B. Williams, Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins, EMBO J. 18 (1999) 6718–6729.