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Effects of divalent cations on encapsulation and release in the GroEL-assisted folding

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Abstract Chaperonin GroEL assists protein folding in the presence of ATP and magnesium. Recent studies have shown that several divalent cations other than magnesium induce conformational changes of GroEL, thereby influencing chaperonin-assisted protein folding, but little is known about the detailed mechanism for such actions. Thus, the effects of divalent cations on protein encapsulation by GroEL/ES complexes were investigated. Of the divalent cations, not only magnesium, but also manganese ions enabled the functional refolding and release of 5,10-methylenetetrahydroforate reductase (METF) by GroEL. Neither ATP hydrolysis nor METF refolding was observed in the presence of zinc ion, whereas only ATP hydrolysis was induced by cobalt and nickel ions. SDS-PAGE and gel filtration analyses revealed that cobalt, nickel and zinc ions permit the formation of stable substrate-GroEL-GroES *cis*-ternary complexes, but prevent the release of METF from GroEL.

Keywords GroEL · Chaperonin · Divalent cation · Manganese · Cobalt · Nickel · Zinc · Refolding · Encapsulation

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Introduction

Chaperonin GroEL is a heptameric double-ring protein that assists protein folding in cooperation with its cofactor GroES (Ellis 1996; Xu et al. 1997; Braig 1998). GroEL/ES-assisted protein folding involves capsulation and the subsequent release of substrate proteins (Martin et al. 1993; Rye et al. 1997; Farr et al. 2003), in which several components (i.e., potassium, magnesium and ATP) are essential (Goloubinoff et al. 1989; Todd et al. 1993). Also, metal divalent cations other than magnesium ion have been shown to influence protein folding as well as the GroEL conformation (Azem et al. 1994). For example, manganese ion reduced the rate of ATP hydrolysis but increased the affinity of GroES for GroEL (Diamant et al. 1995), while zinc ion modulated the exposure of hydrophobic surfaces for GroEL (Brazil et al. 1998). However, it has not yet been clarified how the divalent cations affect the GroEL/ES-induced protein capsulation in the protein folding function.

Recent proteomic analysis has revealed that about 250 proteins interact with the GroEL (Kerner et al. 2005). Among them, only ~85 proteins were obligate GroEL-dependent substrates that fold through the complete chaperonin pathway coupled with ATP hydrolysis, whereas other identified proteins partially interacted with GroEL and folded with no requirement for

GroES. Moreover, not only proteins from *E. coli* but also proteins from various organisms were refolded by assist of GroEL (Badcoe et al. 1991; Holl-Neugebauer et al. 1991; Fisher 1992; Mizobata et al. 1992; Hartman et al. 1993; Makino et al. 1997; Chaudhuri et al. 2001).

In this study, we have studied the effects of divalent cations on protein folding by the GroEL-GroES complex using 5,10-methylenetetrahydroforate reductase (METF) [EC 1.5.1.20] from *E. coli*. METF is a homo-tetrameric protein with a subunit molecular mass of 33,100 that catalyzes the reduction of 5,10-methylenetetrahydroforate to 5-methyltetrahydroforate using NAD(P)H and FAD (Sheppard et al. 1999), and is an obligate substrate for GroEL-assisted folding (Kerner et al. 2005). We here report that, of the divalent cations tested, only magnesium and manganese ions can be used for METF refolding, while cobalt, nickel and zinc ions prevent the release of METF from the GroEL-GroES complex.

Materials and methods

Reagents

Guanidine hydrochloride (Gdn-HCl) was obtained from Nacalai Tesque (Kyoto, Japan). Divalent cation salts were obtained from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque and Sigma-Aldrich Japan, whereas NADH was purchased from Oriental Yeast Co. (Osaka, Japan). ATP-trisodium salt, *trans*-1, 2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra acetic acid (CDTA), FAD, menadione, proteinase K, PMSF and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Japan. $BeCl_2$ was obtained from Aldrich. Ammonium molybdate, triton X-100 and NaF were obtained from Wako Pure Chemical Industries. Malachite green oxalate was obtained from Chroma-Gesellschaft, mbH & Co. (Münster, Germany).

Gene cloning

Escherichia coli genome DNA was prepared from *E. coli* strain K-12 provided by the National Institute of Genetics (Shizuoka, Japan) (Blattner et al. 1997). The region of DNA encoding the *groEL* gene was amplified from genomic DNA by PCR. The primers used for amplification were groEL-F (5′-GGGAATTCCATATGGCAGCT AAAGACGTAAAATTCGG-3′) and groEL-R (5′-CCGCTCGAGTTACATCATGCCGCCCA TGC-3′). PCR was carried out using 1 U KOD-plus DNA polymerase (Toyobo, Osaka, Japan), 20 ng of *E. coli* K-12 genomic DNA as a template, 0.2 μM primer pair and 0.2 mM dNTP mixture in 50 μl according to the following protocol: 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 53°C for 30 s, and 68°C for 90 s. After gel purification, the isolated fragment was cloned into *Nde*I and *Xho*I sites of pET-22b (+) vector (Novagen, WI, USA); similarly, the *groES* gene was cloned into the pET-22b (+) vector. The primers used for PCR were groES-F (5′-GGGAATT CCATATGAATATTCGTCCA TTGC-3′) and groES-R (5′-CCGCTCGAGTTA-CGCTTCAA CAATTGCC-3′). Moreover, the *metf* gene fused with the His-tag sequence at the 3′-end was also cloned into pET-22b (+) vector. The primers used for PCR were metf-F (5′-GGGAATTCCATAT GAGCTTTTTTCAC-GCCAGCC-3′) and metf-R (5′-CCGCTCGAG-TAAACCAGGTCGAACC CC-3′). These *groEL*, *groES* and *metf* genes were entirely sequenced by the dye-terminator method using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences Co., Piscataway, NJ, USA) in combination with an ABI3100 genetic analyzer (Applied Biosystems Japan, Tokyo, Japan).

Purification of GroEL

Recombinant GroEL was expressed in *E. coli* strain BL21 (DE3) (Novagen). The plasmid construct was used to transform *E. coli* BL21 (DE3), and the transformants were grown at 37°C in LB broth containing ampicillin (100 μg/ml). The protein expression was then induced by the addition of 0.5 mM IPTG. After incubation at 25°C for 12 h, the protein-overexpressing bacterial cells were harvested and resuspended in buffer A (50 mM Tris–HCl, pH 7.5, containing 1 mM EDTA and 50 mM NaCl). Lysozyme (0.5 mg/ml, Nacalai Tesque) and benzonase

nuclease (5 units/ml, Novagen) were added to the cell suspension, which was subsequently disrupted by ultrasonication (Sonifire 450, Branson, CT, USA). The supernatant was fractionated by ammonium sulfate precipitation by a stepwise gradient from 30% (w/v) to 65% (w/v) saturation. The protein precipitate obtained at 65% ammonium sulfate saturation was collected and dissolved in buffer A. The protein solution was dialyzed overnight with 100-fold volume of buffer A with two buffer changes. Dialyzed protein was loaded onto a HiPrep Q anion exchange column (Amersham Biosciences, NJ, USA) with buffer A and eluted by a linear gradient of NaCl from 50 mM to 1 M. Fractions containing GroEL, as determined by SDS-PAGE analysis, were pooled and the protein was concentrated by ultrafiltration using a 100-kDa cutoff filter (Millipore, MA, USA). The concentrated GroEL was purified by gel filtration using a TSK-GEL G4000SW column (column size: 21.5 × 600 mm, TOSOH, Tokyo, Japan) with buffer B (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 250 mM NaCl, 20% (v/v) ethanol). Fractions containing 14 meric GroEL were pooled and concentrated using a 100-kDa cutoff filter. The concentrated GroEL was loaded onto a Mono Q anion exchange column (Amersham Biosciences) with buffer A and eluted with a linear gradient of NaCl from 145 mM to 1 M. The GroEL-containing fractions were pooled and dialyzed with refolding buffer (50 mM MOPS-KOH, pH 7.0, 10 mM KCl). The concentrations of purified GroEL were determined by the Bradford method using bovine serum albumin as the standard, and their purity was checked by SDS-PAGE and native PAGE analysis using Coomassie brilliant blue R-250 (Nacalai Tesque) for protein staining.

Purification of GroES

Recombinant GroES was expressed in *E. coli* and extracted from cells by the same procedure as for GroEL. The supernatant obtained by the lysis of *E. coli* over-expressing recombinant GroES was fractionated by ammonium sulfate precipitation by a stepwise gradient of ammonium sulfate from 35% (w/v) to 55% (w/v) saturation. The precipitates resulting from 55% ammonium sulfate saturation were harvested and dissolved in buffer A. The protein solution was then dialyzed overnight with 100-fold volume of buffer A with two buffer changes. The dialyzed proteins were loaded onto a HiPrep Q anion exchange column with buffer A and eluted by a linear gradient of NaCl from 50 mM to 1 M. Fractions containing GroES were pooled and concentrated by ultrafiltration using a 10-kDa cutoff filter (Millipore). Concentrated GroES was dialyzed overnight with 100-fold volumes of buffer C (50 mM acetic acid buffer, pH 5.3) with two buffer changes and the insoluble matter was removed from the dialyzed proteins by centrifugation. The resultant supernatants were loaded onto a Mono Q anion exchange column with buffer C and eluted with a linear gradient of NaCl from 0 mM to 400 mM. Moreover, the fraction containing GroES were purified using a Mono Q anion exchange column with 4-column volume of buffer A. The proteins were eluted with a linear gradient of NaCl from 50 mM to 1 M. Finally, GroES-containing fractions were purified by gel filtration using a Superdex 200 GL column (Amersham Biosciences) with buffer B. The fractions containing 7 meric GroES proteins were pooled and dialyzed with refolding buffer. The purity of the proteins was checked by SDS and native PAGE as described for GroEL.

Purification of METF

Recombinant METF protein fused with additional amino residues (LEHHHHHH) from pET22b (+) vector at the C terminus was expressed in *E. coli* strain BL21 (DE3) and extracted from the cells by the same procedure as for GroEL with buffer D (50 mM Tris–HCl, pH 7.6, 500 mM NaCl and 5 mM imidazole). The crude extract was purified by Ni-NTA affinity resin (Novagen) followed by gel filtration using a Superdex 200 GL column with refolding buffer. The purity of the proteins was checked by SDS and native PAGE as described for GroEL.

METF refolding assay

Unless otherwise stated, the molar concentrations of METF, GroEL and GroES are expressed as

those of 4, 14 and 7 mer, respectively. METF (10 μM) was denatured with 4 M Gdn–HCl for 1 h at 25°C. Denatured METF was diluted 100-fold at 37°C into refolding buffer containing 0.5 μM GroEL, 1 μM GroES, 2 mM ATP-Na_3, 10 mM divalent cation, 1 mg/ml BSA and 0.5 μM FAD. The refolding reaction was stopped by the addition of 40 mM CDTA. METF activity was assayed at 25°C for 15 min by measuring absorbance at 340 nm in METF assay solution (50 mM Tris–HCl, pH 7.2, 200 μM NADH, 180 μM menadione, 2 mM EDTA and 1 mg/ml BSA). The refolding yield was determined as the percentage of activity of the refolding enzyme relative to that of the native enzyme.

ATP hydrolysis assay

Inorganic phosphate produced during ATP hydrolysis by GroEL was quantified colormetrically. 0.1 μM denatured METF, 0.5 μM GroEL and 1.0 μM GroES were added to the refolding buffer containing 2 mM ATP-Na_3 and 10 mM divalent cation. After incubation, the reaction was stopped by addition of 1 M perchloric acid. The released phosphate was quantified by absorbance at 650 nm with the color reagent (0.034% malachite green oxalate, 1.05% ammonium molybdate, 0.1% triton X-100, and 1.5 M HCl) (Lanzetta et al. 1979). The enzyme reaction was corrected for buffer as well as increases in absorbance caused by the non-enzymatic hydrolysis of nucleotides. Concentrations of inorganic phosphates were determined using a standard solution of potassium dihydrogen phosphate in the color reagent.

Protease sensitivity assay

METF saturated-GroEL was prepared according to published protocols (Motojima and Yoshida 2003; Taguchi et al. 2004). The reaction mixtures contained 1 μM METF saturated GroEL, 2 μM GroES, 1 mM ATP-Na_3, 10 mM divalent cation, 10 mM NaF and 2 mM $BeCl_2$ in the refolding buffer. Subsequently, proteinase K (final concentration, 1 μg/ml) was added to the mixture. Following incubation for 30 min at 25°C, PMSF (final concentration, 1 mM) was added to the solution to stop protease digestion. The resulting solution was analyzed by 13% SDS-PAGE.

Gel filtration assay

The solution containing 1 μM METF saturated GroEL, 2 μM GroES, 2.5 mM ATP-Na_3, 10 mM divalent cation and 5 μM FAD in refolding buffer was incubated at 25°C for 10 min, and then applied to a gel filtration column (TSK-GEL G4000SW, column size: 6 × 600 mm, TOSOH, Tokyo, Japan) equilibrated with refolding buffer. METF elution was monitored by absorbance at 450 nm for the maximum absorbance of FAD at a flow rate of 0.5 ml/min.

Results

Refolding of denatured enzymes by GroEL in the presence of various divalent cations

Purified GroEL was assembled to yield 800 kDa as revealed by gel filtration chromatography using a TSK-GEL G4000SW column. Consistent with this observation, GroEL showed low mobility in native PAGE (data not shown). Both SDS and native PAGE analyses indicated that GroEL, GroES and METF tested in this study were of high purity. METF denatured by 4M of Gdn-HCl failed to refold spontaneously in the refolding solution; however, METF recovered by about 40% relative to the native enzyme in few minutes in the presence of the GroEL, GroES, ATP and magnesium, but not without GroES. Guanidine hydrochloride (40 mM) had no effect on native METF activity. Moreover, various divalent cations and EDTA also had no effect on the native METF activity. As shown in Fig. 1, manganese ion induced the GroEL-assisted refolding of METF as was the case for magnesium; however, alkaline earth metal ions such as calcium, strontium and barium ions, and transition metal ions such as cobalt and nickel and zinc ions did not induce such protein refolding. The refolding yield of METF in the presence of manganese was about 70% of that in the presence of magnesium.

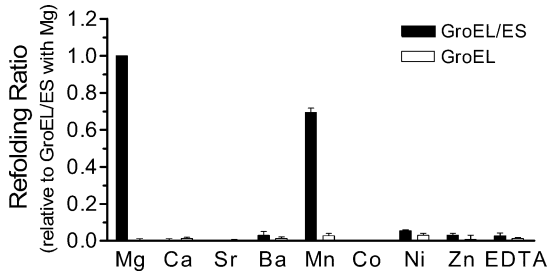

Fig. 1 Effects of divalent cation on GroEL-assisted METF refolding. Refolding assays were conducted as described in ''Materials and methods.'' Each refolding ratio is shown as the relative value to that observed in the presence of magnesium. Data represent the mean ± SEM of four independent experiments

Effects of divalent cations on ATP hydrolysis by GroEL/ES

ATP hydrolysis by GroEL was investigated in the presence of various cations under refolding conditions. ATP hydrolysis occurred time-dependently in the presence of either GroEL, or GroEL/ES complexes (data not shown). ATP hydrolysis by GroEL was also observed in the presence of manganese ion at a rate of about 15% of that observed in the presence of magnesium ion (Fig. 2A). Regardless of the absence (Fig. 2A) or presence (Fig. 2B) of GroES, nickel and cobalt ions induced ATP hydrolysis by GroEL, but alkaline earth metal ions and zinc ion did not.

It has been shown that fluoroberyllate (BeFx) mimics the phosphate part of the enzyme-bound nucleotide (Taguchi et al. 2004), thereby inhibiting ATP hydrolysis and forming a GroEL-GroES complex in which the GroEL ring encapsulates a folding component substrate protein. Addition of BeFx resulted in a block of ATP hydrolysis by GroEL in the presence of not only magnesium, but also manganese, cobalt and nickel ions.

Effects of divalent cations on the formation of substrate-GroEL-GroES *cis*-ternary complexes

To investigate whether various divalent cations permit the formation of the *cis*-ternary complex with ATP, a protease sensitivity assay was carried out as reported elsewhere (Motojima and Yoshida 2003). In this assay, the substrate protein band is

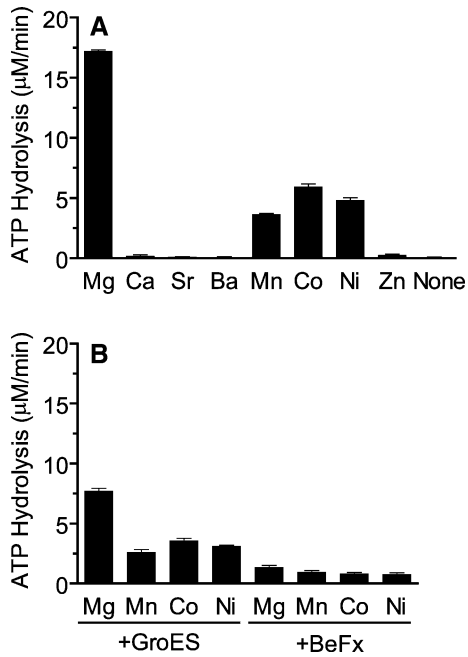

Fig. 2 Hydrolysis of ATP by GroEL in the presence of various divalent cations. (**A**) Hydrolysis of ATP by GroEL alone. (**B**) Hydrolysis of ATP by GroEL/ES complex and GroEL in the presence of BeFx. The concentrations of ATP-Na_3, divalent cation, $BeCl_2$ and NaF were 2, 10, 10 and 50 mM, respectively. ''None'' represents the ATP hydrolysis observed in the presence of GroEL and ATP but in the absence of divalent cation. Data represent the mean ± SEM of at least three independent experiments

observed in SDS-PAGE if it is encapsulated in the GroEL-GroES *cis*-ternary complex with ATP (Fig. 3A). In the control experiment, the denatured METF bound to GroEL was completely digested by proteinase K, 30 min after the addition of the protease in the absence of GroES and divalent cations. GroEL-GroES-METF complexes were found to form in the presence of magnesium, manganese, cobalt, nickel and zinc ions, but not in the presence of calcium, strontium, barium and EDTA due to a complete digestion of METF by proteinase K. Furthermore, the proteinase K digestion of METF during the refolding reaction was also examined in the absence of BeFx (Fig. 3B). In the presence of magnesium ion, denatured METF was gradually digested by proteinase K, whereas in the presence of manganese, cobalt, nickel and zinc ions, denatured METF was barely digested by proteinase K even after 30 min incubation.

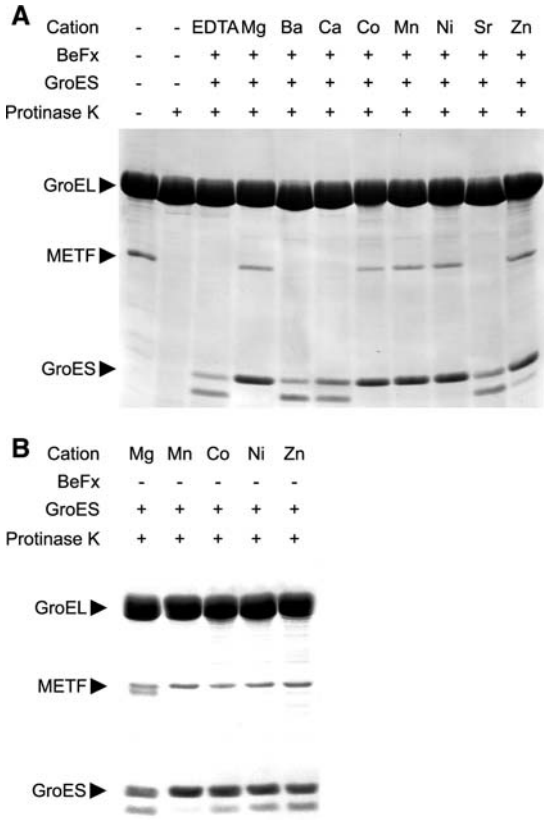

Fig. 3 Protection of encapsulated METF from proteinase K in the presence of various cations. METF-saturated GroEL (1 μM), GroES (2 μM), ATP (1 mM) and indicated components were added to the refolding buffer and incubated for 30 min at 25°C. After incubation, the solutions were analyzed by 13% SDS-PAGE. Formation of GroEL-GroES-METF *cis*-ternary complexes with ATP and various cations in the presence (**A**) and absence (**B**) of BeFx

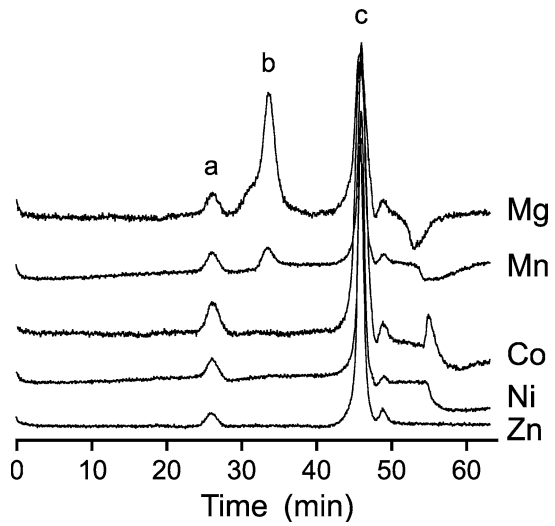

Fig. 4 Detection by gel filtration of METF, which is released from the GroEL/ES complex in the presence of various cations. Refolding mixtures in the presence of various cations were applied to the gel filtration column (see Materials and methods for details) and detected by absorbance at 450 nm for the absorption maximum of FAD. GroEL containing METF with FAD bound (**a**), refolded METF with FAD bound (**b**) and free FAD (**c**) were eluted at 27, 35 and 47 min, respectively

Release of METF from the GroEL-GroES-METF *cis*-ternary complexes

Finally, we investigated by gel filtration whether METF is released from the GroEL-GroES-METF *cis*-ternary complexes (Fig. 4). METF-saturated GroEL containing the enzyme co-factor FAD was eluted at 27 min (Fig. 4, peak a). METF-free GroEL was also eluted at the same time as monitoring at 280 nm (data not shown). FAD-bound METF, which was released from GroEL, was eluted at 35 min (Fig. 4, peak b), whereas free FAD was eluted at 47 min (Fig. 4, peak c). The released FAD-bound METF was detected in the presence of magnesium and manganese ions in refolding reaction mixtures, but not in the presence of other cations.

Discussion

Of the divalent cations, zinc ion has been shown to enhance the stability of GroEL as revealed by an increase of cross-linking species by glutardialdehyde (Azem et al. 1994) and the affinity for hydrophobic probes 4,4′-bis(1-anilino-8-naphthalenesulfonic acid) (Brazil et al. 1998). In contrast, manganese ion has been found to enhance not only the stability of GroEL (Azem et al. 1994), but also the binding of GroES to GroEL (Diamant et al. 1995). Also, effects of divalent cations on the ATP hydrolysis by GroEL have been studied by Melkani et al. (2003). However, to our knowledge, there have been no reports on the effects of divalent cations on the GroEL-assisted protein encapsulation and release. Excluding magnesium, only manganese ion permitted GroEL-assisted refolding of METF (Fig. 1), suggesting that it was available for the

GroEL-assisted folding reaction cycles. However, manganese ion forms more stable METF-GroEL-GroES *cis*-ternary complex than magnesium ion so that encapsulated METF was protected from protease digestion in the absence of BeFx (Fig. 3B). These results seem to reflect an enhancement of the GroEL stability and the GroES affinity for GroEL as reported (Azem et al. 1994; Diamant et al. 1995).

Zinc ion inhibited the ATP hydrolysis by GroEL, whereas cobalt and nickel ions permitted it (Fig. 2). The addition of BeFx markedly suppressed the cobalt and nickel ion-induced ATP hydrolysis, but GroES failed to stop it (Fig. 2), as observed for the magnesium and manganese-induced ATP hydrolysis. Thus, it is conceivable that the ATP hydrolysis in the presence of cobalt and nickel ions occurs in a similar manner to that in the presence of magnesium and manganese ions.

METF was protected from protease digestion in the GroEL-GroES complex in the presence of cobalt, nickel and zinc ions (Fig. 3), but no FAD-bound METF was detected in the gel filtration assay (Fig. 4). These results suggest that cobalt, nickel and zinc ions permit the formation of a stable METF-GroEL-GroES *cis*-ternary complex, but prevented the release of METF from the complexes. It is of interest that the ATP hydrolysis occurred in the METF-GroEL-GroES *cis*-ternary complex in the presence of cobalt and nickel ions, despite the protein release failure. The mechanism for such observation remains to be studied in future.

In conclusion, we have investigated the effects of divalent cations on the functional refolding of METF, which involves GroEL-GroES complex formation. Alkaline earth metal ions such as calcium, strontium and barium ions did not induce the GroEL-assisted refolding. Of the divalent cations other than magnesium ion, only manganese ion permitted, but to a lower extent than magnesium, GroEL-assisted refolding and release of METF, which is accompanied by ATP hydrolysis. By contrast, cobalt, nickel and zinc ions permitted the encapsulation of METF in the GroEL-GroES complex, but prevented the release of the protein. Although cobalt and nickel ions induced the ATP hydrolysis, addition of BeFx suppressed it, as observed for magnesium ion. These findings provided a new insight into the role of divalent cations in the chaperonin-assisted protein folding reaction.

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References

Azem A, Diamant S, Goloubinoff P (1994) Effect of divalent cations on the molecular structure of the GroEL oligomer. Biochemistry 33:6671–6675

Badcoe IG, Smith CJ, Wood S, Halsall DJ, Holbrook JJ, Lund P, Clarke AR (1991) Binding of a chaperonin to the folding intermediates of lactate dehydrogenase. Biochemistry 30:9195–9200

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1474

Braig K (1998) Chaperonins. Curr Opin Struct Biol 8:159–165

Brazil BT, Ybarra J, Horowitz PM (1998) Divalent cations can induce the exposure of GroEL hydrophobic surfaces and strengthen GroEL hydrophobic binding interactions. Novel effects of Zn^{2+} GroEL interactions. J Biol Chem 273:3257–3263

Chaudhuri TK, Farr GW, Fenton WA, Rospert S, Horwich AL (2001) GroEL/GroES-mediated folding of a protein too large to be encapsulated. Cell 107:235–246

Diamant S, Azem A, Weiss C, Goloubinoff P (1995) Increased efficiency of GroE-assisted protein folding by manganese ions. J Biol Chem 270:28387–28391

Ellis RJ (1996) The chaperonins. Academic Press, San Diego

Farr GW, Fenton WA, Chaudhuri TK, Clare DK, Saibil HR, Horwich AL (2003) Folding with and without encapsulation by *cis*- and *trans*-only GroEL-GroES complexes. Embo J 22:3220–3230

Fisher MT (1992) Promotion of the in vitro renaturation of dodecameric glutamine synthetase from *Escherichia coli* in the presence of GroEL (chaperonin-60) and ATP. Biochemistry 31:3955–3963

Goloubinoff P, Christeller JT, Gatenby AA, Lorimer GH (1989) Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfoleded state depends on two chaperonin proteins and Mg-ATP. Nature 342:884–889

Hartman DJ, Surin BP, Dixon NE, Hoogenraad NJ, Hoj PB (1993) Substoichiometric amounts of the molecular chaperones GroEL and GroES prevent thermal

denaturation and aggregation of mammalian mitochondrial malate dehydrogenase *in vitro*. Proc Natl Acad Sci USA 90:2276–2280

Holl-Neugebauer B, Rudolph R, Schmidt M, Buchner J (1991) Reconstitution of a heat shock effect *in vitro*: influence of GroE on the thermal aggregation of alpha-glucosidase from yeast. Biochemistry 30:11609–11614

Kerner MJ, Naylor DJ, Ishihama Y, Maier T, Chang HC, Stines AP, Georgopoulos C, Frishman D, Hayer-Hartl M, Mann M, Hartl FU (2005) Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. Cell 122:209–220

Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 100:95–97

Makino Y, Amada K, Taguchi H, Yoshida M (1997) Chaperonin-mediated folding of green fluorescent protein. J Biol Chem 272:12468–12474

Martin J, Mayhew M, Langer T, Hartl FU (1993) The reaction cycle of GroEL and GroES in chaperonin-assisted protein folding. Nature 366:228–233

Melkani GC, Zardeneta G, Mendoza JA (2003) The ATPase activity of GroEL is supported at high temperatures by divalent cations that stabilize its structure. Biometals 16:479–484

Mizobata T, Akiyama Y, Ito K, Yumoto N, Kawata Y (1992) Effects of the chaperonin GroE on the refolding of tryptophanase from *Escherichia coli*. Refolding is enhanced in the presence of ADP. J Biol Chem 267:17773–17779

Motojima F, Yoshida M (2003) Discrimination of ATP, ADP, and AMPPNP by chaperonin GroEL: hexokinase treatment revealed the exclusive role of ATP. J Biol Chem 278:26648–26654

Rye HS, Burston SG, Fenton WA, Beechem JM, Xu Z, Sigler PB, Horwich AL (1997) Distinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL. Nature 388:792–798

Sheppard CA, Trimmer EE, Matthews RG (1999) Purification and properties of NADH-dependent 5, 10-methylenetetrahydrofolate reductase (MetF) from *Escherichia coli*. J Bacteriol 181:718–725

Taguchi H, Tsukuda K, Motojima F, Koike-Takeshita A, Yoshida M (2004) BeF(x) stops the chaperonin cycle of GroEL-GroES and generates a complex with double folding chambers. J Biol Chem 279:45737–45743

Todd MJ, Viitanen PV, Lorimer GH (1993) Hydrolysis of adenosine 5′-triphosphate by *Escherichia coli* GroEL: effects of GroES and potassium ion. Biochemistry 32:8560–8567

Xu Z, Horwich AL, Sigler PB (1997) The crystal structure of the asymmetric GroEL-GroES-$(ADP)_7$ chaperonin complex. Nature 388:741–750