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DnaK and DnaJ facilitated the folding process and reduced inclusion body formation of magnesium transporter CorA overexpressed in *Escherichia coli*

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Abstract

Overexpression of CorA, the major magnesium transporter from bacterial inner membrane, in *Escherichia coli* resulted in the synthesis of 60 mg of protein per liter of culture, most of which however was in the form of inclusion bodies. The levels of inclusion body formation were reduced by lowering the cell culture temperature. To dissect CorA inclusion body formation and the folding process involved, we co-expressed the protein with various chaperones and other folding modulators. Expression of DnaK/DnaJ (Hsp70) prevented inclusion bodies from forming and resulted in the integration of more CorA into the membrane. GroEL/GroES (Hsp60/Hsp10) were less effective at reducing CorA inclusion body formation. Co-expression with either Ffh/4.5S-RNA, the signal recognition particle, or SecA, the ATPase that drives protein insertion into the membrane, had little effect on CorA folding. These results indicate: (1) that CorA inclusion bodies form immediately after synthesis at 37 °C, (2) that CorA solubility in the cytosol can be increased by co-expressing a chaperone system, (3) membrane targeting is probably not a rate-limiting factor, and (4) that membrane insertion becomes a limitation only when large amounts of soluble CorA are present in the cytosol. These co-expression systems can be used for producing other membrane proteins in large quantities.

Keywords: Magnesium transporter; Membrane protein folding; Inclusion bodies; Chaperones; Co-expression

Membrane proteins overexpressed in *Escherichia coli* (*E. coli*) often accumulate in the cytosol in the form of inclusion bodies, which are insoluble aggregates of misfolded proteins [18,20,46]. Although these inclusion bodies can be easily purified, the constituent protein must be refolded in vitro for biochemical and structural studies. Such refolding procedures are complicated and costly, and the yields are usually low [7,26,37]. Ideally, one wants to prevent inclusion body formation in vivo which, in turn, requires a full understanding of the folding process of membrane proteins in the cell and the causes for inclusion body formations.

The folding process of inner membrane proteins in *E. coli* can be either co- or post-translational, probably depending on the protein [15,19]. For many of these proteins, the post-translational folding process can be divided into four steps: keeping the newly synthesized polypeptide soluble in the cytosol, targeting it to the membrane, inserting it into the membrane, and folding in the membrane [15,21]. The efficiencies of these four steps have to be well balanced to maximize the amount of correctly folded proteins. Except in the last step, newly synthesized membrane proteins require the assistance of chaperones or other folding modulators at each of the first three folding stages. A homologue of the eukaryotic Hsp70, DnaK/DnaJ binds to hydrophobic segments of the unfolded polypeptide in order to maintain solubility and prevent aggregation. Disassociation of DnaK and DnaJ from the polypeptide is facilitated by protein GrpE, which triggers nucleotide exchange [9,12,23]. The GroEL/GroES chaperonin system, in contrast, binds to misfolded polypeptide and allows it to refold when released. Next, the signal

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recognition particle (SRP), Ffh/4.5S-RNA, together with its receptor FtsY, guides the newly synthesized polypeptide to the inner membrane [29]. There the polypeptide inserts into the membrane at SecYEG-translocase, driven by secA ATPase [19,35]. Recent experiments show that another component, YidC, can also assist inner membrane protein integration [39].

Folding modulators play such critical roles in membrane protein biogenesis that efforts have been made to increase the folding efficiency of membrane proteins with chaperones. In vitro translation studies indicate that DnaK/DnaJ facilitates import and assembly of the α- and β-polypeptides of the light-harvesting complex I of *Rhodobacter capsulatus* [36]. While the presence of GroEL/GroES increases the post-translational insertion of the *E. coli* lactose permease into reconstituted liposome [5], their overexpression accelerates folding of the tetracycline/H^+ antiporter protein, thereby reducing its susceptibility to proteolytic digestion [40]. Interestingly, co-expression of the serotonin transporter with the endoplasmic reticulum chaperone calnexin in insect cells results in a threefold increase of the active transporter [43]. However, no comparison has been made on the importance of various folding modulators for membrane protein overexpression and their role in preventing inclusion body formation remains unclear.

In the present work, we dissected the folding process of CorA, the major magnesium transporter from *E. coli* inner membrane [24]. The protein consists of 316 amino acids and its topology was determined by BlaM and LacZ fusion [42]. The N-terminal 235 amino acid residues of the CorA protein are located within the periplasmic space. The C-terminal region of CorA is composed of three membrane-spanning segments, thus depositing the C-terminus within the cytoplasm. SecA and SecY were found necessary for CorA membrane integration [42]. We overexpressed CorA in *E. coli* at high levels. However, most of the expressed protein accumulated as inclusion bodies in the cytosol. When the cell culture temperature was reduced, inclusion body formation was minimized and consequently, more CorA was integrated into the membrane. Reasons for inclusion body formation were investigated by co-expressing CorA with chaperones and other folding modulators, followed by monitoring its cellular distribution by biochemical techniques and electron microscopy.

Materials and methods

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA), cloning kits from QIAGEN (Chatsworth, CA, USA), T4 ligase and DNA markers from Promega (Madison, WI, USA), and detergents from Anatrace (Maumee, OH, USA). All other chemicals were from Sigma (St. Louis, MO, USA) and were of analytical grade or higher.

Plasmid construction

The *corA* gene was amplified by conventional PCR, using the chromosomal DNA purified from *E. coli* LMG194 strain as a template. The PCR product was cloned into *Nco*I/*Hin*dIII sites of the pBAD vector which carried C-terminal *myc*-His-tags (Invitrogen, Carlsbad, CA, USA). A thrombin cleavage site was introduced between residue Trp315 and the *myc*-epitope. The combined length of the thrombin site and the *myc*-His-tags was 31 residues. The forward and reverse primers used for the cloning were: CATGCCAT GGGAAGCGCATTTCAACTGGAAAA and GGGC CCAAGCTTGGATCCACGCGGAACCAGCCAGTT CTTCCGCTTAAA, respectively. The Leu2 → Gly2 mutation was introduced to facilitate cloning [2]. The plasmid produced was assigned as pBAD-*corA*.

Plasmids encoding various folding modulators used for co-expression are listed in Table 1. Plasmids pOFX-JK2, pOFX-JKE2, and pOFX-LS2, encoding for the *dnaK*/*dnaJ*, *dnaK*/*dnaJ*/*grpE*, and *groEL*/*groES* genes, respectively, were gifts from Dr. O. Fayet. Their expression was controlled by the P*tac* promoter and

Table 1
Plasmids used for co-expression with CorA in *E. coli*

Plasmid	Coded protein and RNA	Promoter	Inducer	Resistance gene	Source
pBAD-*corA*	CorA	pBAD	Arabinose	Amp	This work
pOFX-JK2	DnaK/DnaJ	P*tac*	IPTG	Cam	[11]
pOFX-JKE2	DnaK/DnaJ/GrpE	P*tac*	IPTG	Cam	[11]
pOFX-LS2	GroEL/GroES	P*tac*	IPTG	Cam	[11]
pACYC-LS	GroEL/GroES	Native	N/A	Tet	[51]
pHQ3	Ffh/4.5S-RNA	Native	N/A	Cam	[29]
pHQ4	Ffh/4.5S-RNA/FtsY	Native	N/A	Cam	[29]
pOFX-*SecA*	SecA	P*tac*	IPTG	Cam	This work
pOFX-*SecB*	SecB	P*tac*	IPTG	Cam	This work
pOFX-*SecAB*	SecA/SecB	P*tac*	IPTG	Cam	This work

Notes. Amp, ampicillin and Cam, chloramphenicol.

was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) [11]. The pACYC-LS plasmid, which carried the *groEL* and *groES* genes, was a gift from Dr. A.L. Horwich and Dr. W. Fenton. Their expression was controlled by the native promotor and therefore no induction was required [51]. Plasmids pHQ3 and pHQ4, which encoded *ffh/4.5S-RNA* and *ffh/4.5S-RNA/ftsY* genes, were controlled by their native promotor [29], and were gifts from Dr. H.D. Bernstein. To express SecA and SecB, we constructed three separated plasmids. We amplified the *secA* and *secB* genes by PCR, using *E. coli* genomic DNA from the JB1655F′ strain as a template. The two genes were cloned into the pOFX-tac2 vector between *Xba*I and *Bam*HI sites, and *Bam*HI and *Sac*I sites, respectively, resulting in plasmids pOFX-*secA* and pOFX-*secB*. We then cloned the *secB* gene into pOFX-*secA*, again using the *Bam*HI and *Sac*I sites, and thus obtained the pOFX-*secAB* plasmid which contained both the *secA* and *secB* genes.

The folding modulator-encoding plasmids are compatible with the pBAD vector used for CorA expression, since they were all derived from pACYC184. In addition, they conferred resistance to tetracycline or chloramphenicol. This differs from the ampicillin resistance encoded by pBAD-*corA*. This allowed for selection of colonies that contained both CorA- and modulator-encoding plasmids.

Cell culture and CorA overexpression

Escherichia coli LMG194 strain was transformed with the pBAD-*corA* plasmid for CorA overexpression. In a 4-liter flask, 1 liter LB medium containing 0.01% ampicillin was inoculated with overnight culture and grown at 37 °C. The temperature was reduced to the intended post-induction temperature 15 min prior to induction. Following induction at an OD_{600} of 0.5–0.6 with 0.01% arabinose, the cells were grown to an OD_{600} of 2.5 at 15, 20, 25, 30, or 37 °C separately and later harvested by centrifugation. The saturation OD_{600} for cell growth was 2.9. The number of *E. coli* cells per liter culture was calculated from the dry weight of the cell pellet, assuming an intracellular volume of 10^{-12} cm^3 and a density of 1 g/cm^3 [14].

CorA co-expression with folding modulators

Escherichia coli LMG194 strain was co-transformed with the pBAD-*corA* and a plasmid encoding a folding modulator (Table 1). Cells were grown to an OD_{600} of 0.5–0.6 in LB medium containing 0.01% ampicillin and 0.0035% chloramphenicol (or 0.0015% tetracycline), followed by induction with 0.01% arabinose and 1 mM IPTG. After induction, cells continued to grow at 37 °C to an OD_{600} of 1.8 and were harvested by centrifugation.

Cell fractionation and SDS–PAGE analysis

To study the cellular distribution of expressed CorA, harvested *E. coli* cells were fractionated into four parts: inclusion body, cytosol, unsolubilized membrane, and solubilized membrane. Cell pellet was resuspended to 5 ml/g cell in TBS buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), DNase I, and protease inhibitor cocktail (Sigma). Cells were broken by three cycles of French Press at 18,000 psi. Approximately 85% of the cells were successfully broken. The whole cell lysate was centrifuged at 8000*g* for 20 min to isolate inclusion bodies from cytosol and membranes [38]. An additional centrifugation step (100,000*g*, 35,000 rpm, 2.5 h Ti45 rotor, Beckman Instruments, Palo Alto, CA, USA) further separated the membrane from the cytosol. Membrane was homogenized and solubilized in solubilization buffer A (50 mM Tris–HCl, pH 8.0, 400 mM NaCl, 10 mM imidazole, 1 mM fresh PMSF, 20% glycerol, protease inhibitor cocktail, and 1.0% dodecylmaltoside (DDM)) for 30 min, at a ratio of 10 ml/g. The insoluble membrane fraction was removed by another centrifugation step at 100,000*g* for 30 min. CorA was later purified from the solubilized membrane fraction. All cell fractionation and protein purification steps were carried out at 4 °C. The protein concentration in each fraction was measured by the Micro-BCA assay (Pierce, Rockford, IL, USA).

Whole cell lysate as well as the four cellular fractions were analyzed by 12% SDS–PAGE. The inclusion body and the insoluble membrane fractions were first solubilized in 6 M urea then added to the sample buffer. Quantitative analysis of the Coomassie blue-stained gel using Imagequant (Molecular Dynamics, Sunnyvale, CA, USA), in combination with BCA protein assay, was used to determine the amounts of CorA in various cellular fractions. Other image analysis software gave similar results. The expressed modulator proteins were detected either by Coomassie blue-stained SDS–PAGE, or by Western blot analysis. Antibodies against Ffh and FtsY were presents from Dr. H. Bernstein, and antibodies for *E. coli* SecB were purchased from Minotech (Crete, Greece).

Determination of CorA synthesis rate

Since *E. coli* cells were induced and harvested in the log phase, where the cells grew exponentially, the number of cells per liter culture at time t after induction was $N(t) = N_{\text{ind}} \times 2^{t/\tau}$, where N_{ind} was the number of cells per liter at induction and τ the doubling time for cell growth. Then we arrived at:

$$\tau = \frac{T_{\text{harv}}}{\log_2(N_{\text{harv}}/N_{\text{ind}})}, \quad (1)$$

where N_{harv} was the cell number per liter at the time of harvesting T_{harv}. N_{ind} and N_{harv} were determined from the dry cell weights, using 1.0×10^{-12} g as the weight of a single *E. coli* cell. Assuming that CorA synthesis rate per cell, V, was a constant at a given temperature, we obtained the total number of CorA molecules per liter of culture at time t:

$$M(t) = \int_0^t N(t) \times V \, dt = \int_0^t N_{ind} \times 2^{t/\tau} \times V \, dt = \frac{N_{ind} \times V \times \tau}{\ln 2} \times (2^{t/\tau} - 1). \quad (2)$$

From the number of total CorA molecules synthesized per liter of culture at the time of harvesting, M_{harv}, as determined by BCA measurements, we obtained the rate of CorA synthesis rate per cell:

$$V = \frac{\ln 2 \times M_{harv}}{N_{ind} \times \tau \times (2^{T_{harv}/\tau} - 1)} = \frac{\ln 2 \times M_{harv}}{\tau \times (N_{harv} - N_{ind})}. \quad (3)$$

CorA purification and characterization

Membrane prepared from *E. coli* cells grown at 15 °C was solubilized in solubilization buffer containing 1% DDM and incubated with Ni^{2+}–NTA resin (QIAGEN, Chatsworth, CA, USA) at 1.5 ml/g membrane. The resin was washed twice with buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20% glycerol, and 0.1% DDM) containing 20 and 40 mM imidazole, respectively. CorA was eluted in three steps using the washing buffer containing 150, 300, and 500 mM imidazole, respectively, and subsequently digested 22 °C with 4 NIH U/mg of thrombin (ICN, Casta Mesa, CA, USA). The identification of CorA was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in the laboratory of Dr. T. Neubert in the Skirball Institute.

The Stokes radius of CorA in DDM detergent was measured by analytical size-exclusion chromatography [6,50]. Purified CorA samples were loaded onto a Shodex KW804 size-exclusion column on HPLC (Waters, Milford, MA, USA) in buffer (50 mM Tris, pH 8.0, 200 mM Na_2SO_4, 3 mM NaN_3, and 0.05% DDM). The Stokes radius of the CorA–detergent complex was determined by using the following soluble proteins as references: thyroglobulin (86 Å), apoferritin (63 Å), aldolase (46 Å), and albumin (35 Å) [22,27].

Transmission electron microscopy

Specimens for electron microscopy were prepared according to [1]. Briefly, *E. coli* cells were fixed first in 2.5% (w/v) glutaraldehyde at 4 °C overnight and then in 1% OsO_4 (w/v) at 22 °C for 2 h. After dehydration in acetone, cells were embedded in Epon 812. Specimens were sectioned and subsequently stained with uranyl acetate and lead citrate. Stained sections were examined in a Philips CM120 electron microscope.

Results

Overexpression of CorA in E. Coli

Expression of CorA in *E. coli* LMG194 strain at 37 °C using the pBAD vector (Table 1) produced the membrane protein at high levels. SDS–PAGE analysis and BCA measurements of the whole cell lysate showed that approximately 60 mg of CorA (23% of the total cellular protein) was produced per liter of culture (Fig. 1A and Table 2). Following the cell breakage and the low-speed centrifugation step [38], however, most of the expressed CorA was found in the pellet in the form of inclusion bodies. The existence of these inclusion

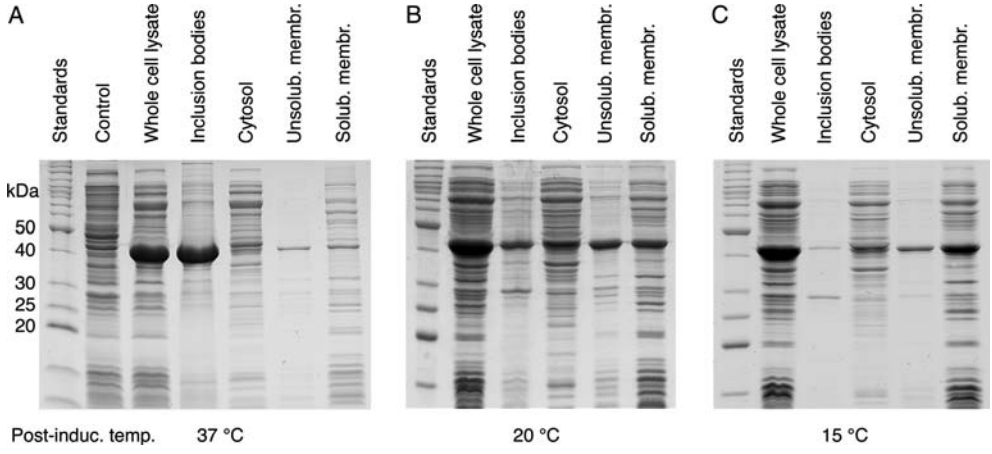

Fig. 1. SDS–PAGE analysis (12%, Coomassie blue-stained) of CorA overexpressed in *E. coli* at (A) 37 °C, (B) 20 °C, and (C) 15 °C. Control lane at 37 °C: uninduced whole cell lysate. The expressed CorA protein migrated as a 40 kDa polypeptide. Inclusion bodies were solubilized in 6 M urea before the sample was loaded onto the gel.

Table 2
Expression of CorA at different temperatures

Post-induction temperature	Time to reach OD_{600} 2.5 (h)	Total cellular protein (mg)	Total CorA in cell (mg)	Soluble CorA in membrane (mg)	Percentage of CorA in membrane
37	2.5	278	60	4	7
30	3	297	57	13	23
25	4	340	58	13	22
20	8	363	51	15	29
15	14	332	36	15	42

Notes. All experiments were repeated at least three times. The errors of measurements were less than 15%.

bodies was confirmed by electron microscopy of sectioned *E. coli* cells (Fig. 2A). As a result, little soluble CorA was present in the cytosol and only 3 mg was detected in the solubilized membrane fraction (Fig. 1A). Similar levels of CorA expression and inclusion body formation were observed in the *E. coli* Top10F′ strain (data not shown).

Temperature effect on CorA expression

Various cell culture and induction conditions were tested in an effort to reduce inclusion body formation and increase CorA's integration into the membrane. The most drastic effect on CorA cellular distribution was due to the post-induction temperature, although inducing cells with less arabinose led to lower CorA expression and less inclusion body formation. As expected, cells grew slower when the temperature was reduced (Table 2). At 37 °C, cell density doubled in 63 min, but the doubling time was as long as 530 min at 15 °C. The total amount of CorA expressed was unchanged between 25 and 37 °C, but a slight decrease was observed at 15 and 20 °C. The average rates for CorA synthesis were calculated to be 600 and 5500 molecule/cell/min for 15 and 37 °C, respectively, assuming that the protein synthesis rate at a certain temperature was a constant. Some inclusion bodies were still present at 20 °C (Fig. 1B), but very few were detected at 15 °C (Figs. 1C and 2B). The decrease in inclusion body formation correlated with increased amounts of membrane-embedded CorA, from 3 mg/liter at 37 °C to 15 mg/liter at 15–20 °C. A small portion of CorA from the membrane remained insoluble in detergent DDM, indicating the existence of some partially inserted CorA. Reducing the inducer concentration had a similar effect to CorA expression as lowering the cell culture temperature (data not shown).

CorA purification and characterization

CorA expressed in *E. coli* at 15 °C was purified using Ni^{2+}–NTA affinity chromatography (Fig. 3). Various non-ionic detergents were tested for CorA solubilization, including nonyl-glucoside, decyl-maltoside, dodecyl-maltoside, and $C_{12}E_8$. DDM was found to solubilize CorA more completely than the others, although there was still some unsolubilized protein reminded even after stirring for 90 min. From Ni^{2+}–NTA resin, most CorA was eluted in the first two fractions and they were combined for later experiments. From a starting yield of 15 mg per liter of culture in the solubilized membrane fraction, 9 mg of CorA was purified. Overnight incubation with thrombin at 22 °C completely removed the *myc*-His-tags, as shown by SDS–PAGE analysis. In addition to providing a spacer for the thrombin cleavage of the His-tag, the *myc*-tag with 19 amino acids also allowed easy detection of the cleavage of the tags by SDS–PAGE. MALDI-TOF mass spectroscopy of the digested protein showed a

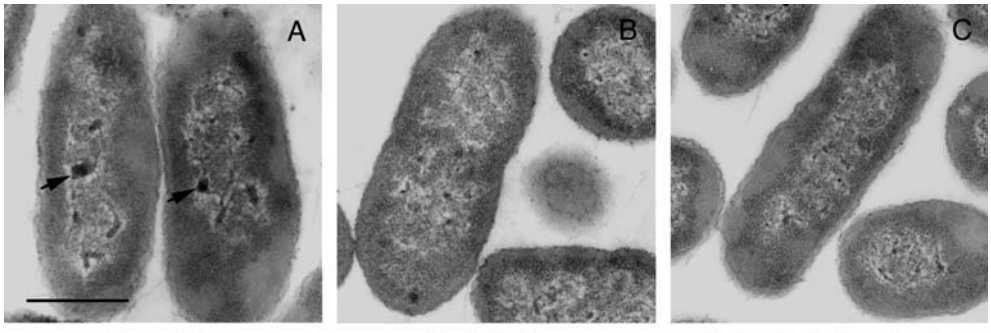

Fig. 2. Electron micrographs of *E. coli* cells overexpressing CorA that were grown at (A) 37 °C and (B) 15 °C. (C) *E. coli* cells co-expressing CorA with DnaK/DnaJ at 37 °C. Inclusion bodies are indicated by arrowheads. The scale bar represents 0.5 μm.

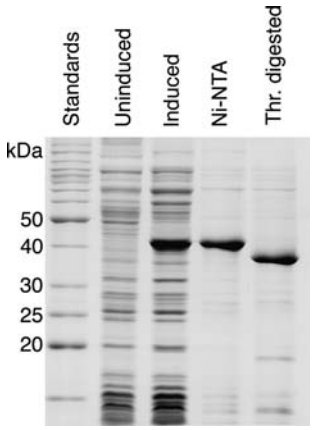

Fig. 3. SDS–PAGE analysis of CorA purification. CorA expressed at 15 °C was purified in DDM detergent using Ni^{2+}–NTA affinity chromatography. Overnight treatment with thrombin at 22 °C removed the His- and *myc*-tags.

single, sharp peak, with a molecular mass of 36,761.4 Da. This agrees with the molecular weight of 36,754.8 Da calculated from the CorA sequence of Gly2–Arg319, which suggests a post-translationally cleaved N-terminal methionine, as is often observed for proteins expressed in *E. coli* [2,33,48]. Therefore, the expressed CorA was free of proteolytic degradation and the preparation did not contain significant amounts of endogenous CorA.

The Stokes radius and oligomeric state of CorA were characterized by analytical size-exclusion HPLC. CorA in DDM solution eluted at 19.415 min from a KW804 size-exclusion chromatography column. This corresponds to a Stokes radius of 70 Å. Comparing this value with the Stokes radii of 66 Å for the dimeric band three membrane domain (2×53 kDa) in DDM [10,32], and 50 Å for the monomeric erythrocyte glucose transporter (54 kDa) in decylmaltoside [6], we concluded that in solution CorA is an oligomer with probably 3–5 subunits. This indicates that the protein oligomerized following membrane insertion and agrees with the suggestion that CorA functions as an oligomer in the membrane [25].

CorA co-expression with DnaK/DnaJ

To understand CorA inclusion body formation and identify the most critical folding steps for overexpression, we dissected its folding process by co-expressing CorA with various chaperones and modulator proteins in *E. coli*. In the following co-expression experiments, *E. coli* cells were always grown at 37 °C.

CorA was co-expressed in *E. coli* with DnaK/DnaJ (Table 1). At respective levels of 41 and 12 mg per liter of culture, the expression of DnaK and DnaJ was readily detected by SDS–PAGE (Fig. 4). The DnaK level was therefore eightfold above its concentration of 50 μM in wild type *E. coli* [23]. The expression of DnaK/DnaJ only changed the *E. coli* growth slightly (doubling time 57 min) (Table 3) and affected little CorA expression. The CorA synthesis rate was calculated to be 6000 molecule/cell/min, similar to that when it was overexpressed alone. However, the expression of the chaperones almost completely prevented CorA inclusion body formation at 37 °C, and resulted in significant amounts of soluble CorA in cytosol, presumably protected by the chaperones (Fig. 4A). Furthermore, about 12 mg of CorA per liter of culture was found in the solubilized membrane fraction (Table 3), a fourfold

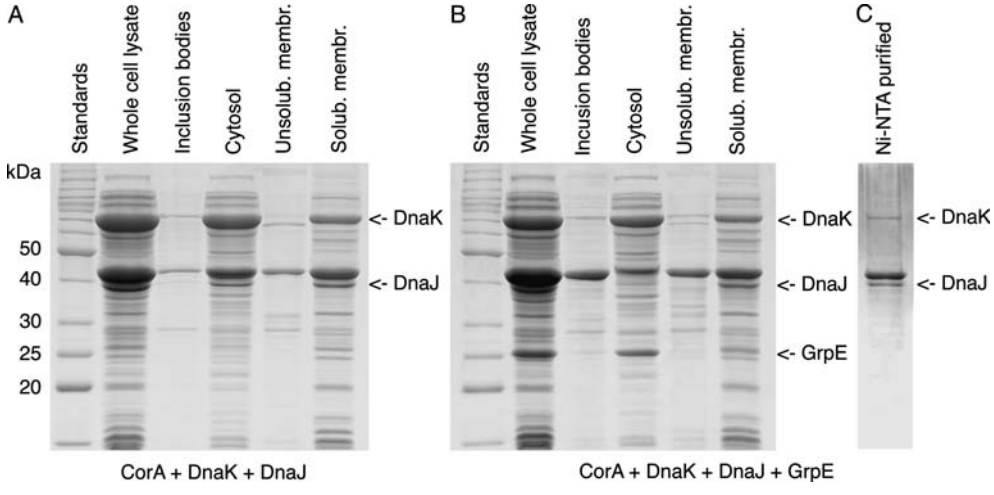

Fig. 4. SDS–PAGE showing the effects of expression of DnaK/DnaJ and DnaK/DnaJ/GrpE on the cellular distribution of CorA at 37 °C. (A) Co-expression of CorA with DnaK/DnaJ. (B) Co-expression of CorA with DnaK/DnaJ/GrpE. (C) Purification of CorA from cells expressing both CorA and DnaK/DnaJ/GrpE, using Ni^{2+}–NTA affinity chromatography. Only CorA was His-tagged, therefore DnaK and DnaJ co-purified with the CorA. DnaK, DnaJ, and GrpE proteins are clearly visible on the gels as bands with expected molecular weights of 72, 40, and 20 kDa, respectively. The positions of DnaK, DnaJ, and GrpE are indicated by arrows.

Table 3
Co-expression of CorA at 37 °C with various chaperones and targeting factors

Chaperone or targeting factors	Cell growth after induction (h)	OD_{600} at harvesting	Total cellular protein (mg)	Total CorA in cell (mg)	Soluble CorA in membrane
None	1.5	1.8	253	51	3
DnaK/DnaJ	1.5	1.6	227	33	12
DnaK/DnaJ/GrpE	4	1.5[a]	225	35	13
GroEL/GroES	2	1.8	265	45	5
Ffh/4.5S-RNA	3.5	1.8	152	30	1
Ffh/4.5S-RNA/FtsY	5.5	1.7[a]	125	19	2
SecA	4	1.8	133	8	1
SecB	2	1.9	183	34	3
SecA/SecB	3.5	1.4[a]	164	25	2

Note. All experiments were repeated at least three times. The errors of measurements were less than 15%.
[a] OD_{600} when cells reached saturation.

increase from the 3 mg obtained in the absence of chaperone co-expression.

CorA was also co-expressed with DnaK/DnaJ/GrpE (Table 1). The introduction of GrpE actually led to more inclusion bodies and less soluble CorA in the cytosol than in the CorA/DnaK/DnaJ co-expression system (Fig. 4B), probably by reducing the level of stable chaperone–CorA complexes, as GrpE is known to catalyze the disassociation of DnaK/DnaJ/polypeptide [23].

In the above two co-expression experiments, small amounts of DnaK and DnaJ proteins co-precipitated with the membrane (Figs. 4A and B), probably via interactions with CorA. Isolating CorA from the membrane using Ni^{2+}–NTA affinity chromatography resulted in co-purification of DnaK and DnaJ (Fig. 4C). This indicated that DnaK and DnaJ were bound to CorA, and probably also implied that a small fraction of CorA polypeptide was only partially inserted into the membrane.

CorA co-expression with GroEL/GroES

CorA was then co-expressed with the GroEL/GroES chaperonins (Table 1). Expression of GroEL/GroES was readily detected in the whole cell lysate by SDS–PAGE (Fig. 5). Although not changing the total amounts of CorA synthesized, their expression reduced the level of CorA inclusion bodies by approximately 20% (Table 3). At the same time, the CorA in the membrane was observed to increase from 3 to 5 mg per liter of cell culture. CorA co-expression with plasmid pACYC-LS (Table 1), which produced GroEL/GroES constitutively, produced similar results (data not shown).

Large amounts of GroEL and GroES were also present in the membrane fraction prepared by 2.5 h centrifugation at 35,000 rpm in a Ti45 rotor (Fig. 5). Control experiments with cells overexpressing GroEL/GroES only but not CorA showed that chaperonin complexes indeed sedimentated under the above centrifugation conditions, as was observed previously [49].

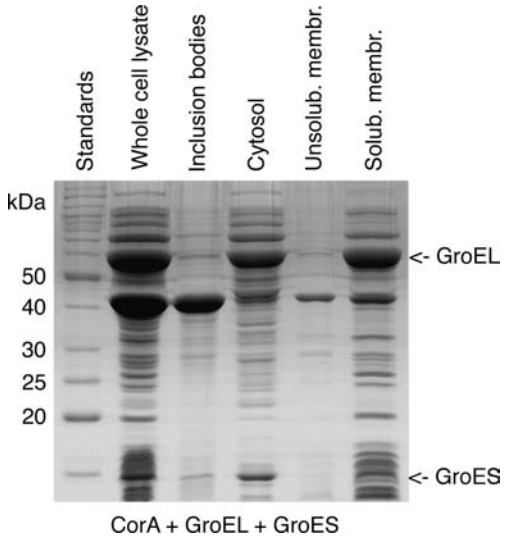

Fig. 5. SDS–PAGE showing the effects of expression of GroEL/GroES on the cellular distribution of CorA at 37 °C. *E. coli* cells carrying both pBAD-CorA and pOFX-LS2 were induced with both arabinose and IPTG. Positions of GroEL and GroES are indicated by arrows.

Therefore, the presence of large amounts of GroEL and GroES in the membrane fraction was chiefly a result of co-sedimentation and not membrane association.

CorA co-expression with Ffh/4.5S-RNA

Next, we co-expressed CorA with the *E. coli* signal recognition particle. Two types of co-expression experiments were performed, one with Ffh/4.5S-RNA, and the other with Ffh/4.5S-RNA and their receptor, FtsY. In both experiments, all the SRP-related genes were cloned into a single plasmid (Table 1). Expression levels of Ffh and FtsY protein were too low to be detected by Coomassie blue-stained SDS–PAGE of the whole cell lysate or cytosol (Figs. 6A and B). However, Western blot analysis showed that they expressed at approximately nine- and fivefold higher than their respective endogenous levels (Fig. 6C). Expression of Ffh/4.5S-RNA and particularly, FtsY, reduced CorA expression

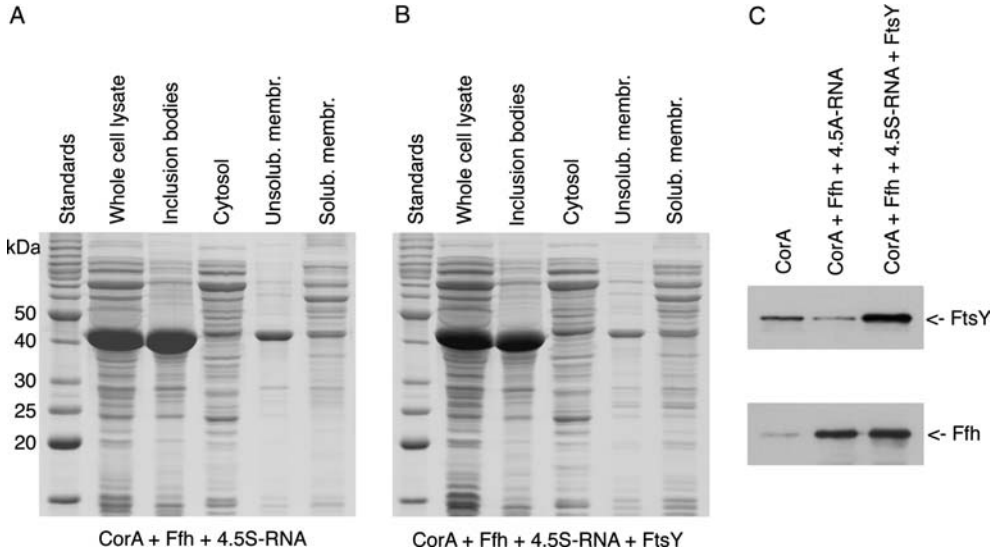

Fig. 6. SDS–PAGE and Western blot showing the effects of expression of Ffh/4.5S-RNA and Ffh/4.5S-RNA/FtsY on CorA cellular distribution at 37 °C. (A) Co-expression of CorA with Ffh/4.5S-RNA. (B) Co-expression of CorA with Ffh/4.5S-RNA/FtsY. (C) Anti-Ffh and anti-FtsY Western blots of whole cell lysate from three different expression experiments: CorA, CorA/Ffh/4.5S-RNA, and CorA/Ffh/ 4.5S-RNA/FtsY.

as well as the total cellular protein significantly (Table 3). Importantly, both the expression of Ffh/4.5S-RNA and Ffh/4.5S-RNA/FtsY failed to prevent CorA inclusion body formation or change its cellular distribution.

CorA co-expression with SecA and SecB

Finally, we co-expressed CorA with the SecA which provides the energy required for protein membrane insertion (Table 1). Expression of SecA reduced the CorA protein biogenesis by about 75% (Table 3), probably due to the large amounts of SecA synthesized (Fig. 7A). On the other hand, expression of SecB, at a level 2–3 times above the endogenous amounts as determined by Western blot, slowed down cell growth minimally but hardly affected CorA synthesis (Fig. 7B). Simultaneous expression of SecA and SecB resulted in less SecA and largely restored CorA expression to previous levels (Fig. 7C). Approximately 90% of the expressed CorA aggregated in the form of inclusion bodies in all the three co-expression systems.

Discussion

CorA overexpressed in *E. coli* at 37 °C mostly formed inclusion bodies, probably because the cell's protein folding machinery was unable to keep pace with the high CorA synthesis rate. We set out to determine which stage of CorA folding—maintaining solubility in the

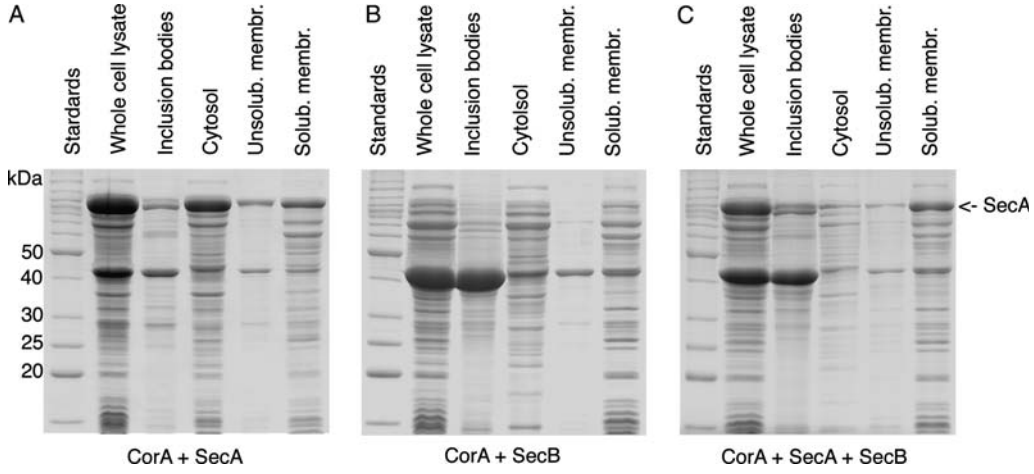

Fig. 7. SDS–PAGE showing the effects of expression of SecA, SecB, and SecB/SecA on CorA cellular distribution at 37 °C. (A) Co-expression of CorA with SecA. (B) Co-expression of CorA with SecB. (C) Co-expression of CorA with SecA/SecB. The position of SecA is indicated by arrows.

cytosol, targeting it to the membrane, or inserting into the membrane—was most limiting the production of large amounts of membrane-embedded CorA. Each of the above three folding steps is assisted by one or more folding modulator [8,19], we have therefore investigated the folding process by co-expressing CorA with various modulators. The high expression levels of CorA at 37 °C allowed us to monitor its cellular distribution using SDS–PAGE and Western blot analysis.

CorA inclusion bodies form immediately after polypeptide synthesis

Our experiments suggest that the most critical step for preventing CorA inclusion body formation is keeping newly synthesized polypeptides soluble. Based on a translation elongation rate of 15 amino acids/ribosome/second under normal growth conditions [13], CorA synthesis in *E. coli* takes about 20 s. A synthesis rate of 5500 molecule/cell/min observed at 37 °C means that, at any moment, 1800 newly synthesized CorA polypeptides per cell need protection from aggregating. DnaK and DnaJ are the major chaperones for newly synthesized, unfolded polypeptides in the cell [23]. Each DnaK molecule binds to a peptide segment of 5–7 amino acids that is typically hydrophobic in its central region. In soluble proteins, such regions occur statistically in every 40 amino acids [9], but are more frequently occurring in membrane proteins. Thus, 8–10 DnaK molecules are required to protect a completely unfolded CorA polypeptide and a total of 10,000 DnaK molecules per cell are needed for the levels of CorA expression that we have achieved. Given that the CorA polypeptides constitute only 23% of all proteins being synthesized, the expected level of 30,000 copies of DnaK molecules per wild type *E. coli* cell (50 μM) is insufficient to protect the newly expressed CorA effectively. As a result, most unfolded CorA polypeptides aggregate in the cytosol before they ever reach the inner membrane.

The above analysis predicts that by reducing the protein synthesis rate, or by increasing the cytosolic concentrations of DnaK/DnaJ and GroEL/GroES chaperones, one can decrease CorA aggregation and thus the formation of inclusion bodies. Indeed, both mechanisms have been observed to work in our experiments. Decreasing expression rates by lowering either inducer concentration or cell culture temperature reduced incorporation of CorA into inclusion bodies (Fig. 1 and Table 2). Alternatively, overexpression of DnaK/DnaJ chaperones completely prevented the inclusion body formation at 37 °C and substantially increased the amounts of soluble CorA in the cytosol and of folded CorA in the membrane (Fig. 4 and Table 3). In contrast, GroEL/GroES, the chaperone system downstream of DnaK/DnaJ [23], was less effective, presumably because CorA already had aggregated into inclusion bodies (Fig. 5). Here, we assume that DnaK/DnaJ directly protected newly synthesized CorA. It is possible that their effect on CorA expression is indirect, e.g., by increasing the cellular concentration of other chaperones or translocation components, although we think that such an effect, if exists, is less important than the direct protection.

Membrane targeting is probably not a limiting factor for CorA overexpression

Our results of CorA co-expression with the *E. coli* signal recognition particle and the Sec system suggest that membrane targeting is probably not the rate-limiting step in CorA folding. Substantial evidence indicates that the Ffh/4.5S-RNA complex is the major targeting pathway for inner membrane proteins in bacteria [19,29]. However, overexpression of Ffh/4.5S-RNA at levels of ninefold above the endogenous expression levels, with or without its membrane receptor FtsY, did not reduce inclusion body formation or increase CorA integration into the membrane (Fig. 6 and Table 3). This is consistent with the suggestion that *E. coli* cells normally contain excess levels of SRP to target effectively a single type of overproduced protein to the membrane [4]. Similarly, overexpression of SecB, the major targeting factor for outer membrane proteins and secretory proteins [19,35], did not affect CorA membrane targeting,

Membrane insertion becomes a limiting factor when large amounts of soluble CorA are present in the cytosol

CorA inclusion body formation is not caused by any limitation imposed by the endogenous levels of membrane insertion in *E. coli*. However, membrane insertion indeed becomes a rate-limiting factor for folding when large amounts of CorA polypeptides are available in the cytosol in a soluble form. This is supported by our observations that only 30–40% of total synthesized CorA was found in the membrane fraction when CorA was overexpressed either alone at 15–20 °C or co-expressed at 37 °C with DnaK/DnaJ—even though no significant amounts of inclusion bodies were formed in either case (Figs. 1, 2, and 4). Indeed, DnaK/DnaJ-bound CorA was found to co-precipitate with the membrane (Fig. 4C), presumably due to interaction with the translocase, indicating that the cell's membrane insertion machinery was overwhelmed with large amounts of soluble CorA polypeptides.

The above observations can be explained by comparing CorA's synthesis and membrane insertion rates in *E. coli*. Keyzer et al. [16] recently determined the translocation rate of outer membrane protein proOmpA in *E. coli* to be 4.5 molecule/translocase/min at 37 °C, or 450–900 molecules/cell/min, assuming 100–200 functional translocase units per cell [17,45]. CorA is similar

to proOmpA both in its numbers of amino acids and in the requirement for the entire polypeptide to insert into (or translocate through) the inner membrane. It then follows that CorA's insertion rate is close to that of proOmpA, namely, on the order of several hundred molecules per cell per minute. Such a rate is much lower than the CorA synthesis rate of 6000 molecule/cell/min at 37 °C, thus imposing a limit to the membrane insertion of CorA when co-expressed with DnaK and DnaJ (Fig. 4). At 15 °C, the rates of expression and membrane insertion are probably much lower than that at 37 °C [30], leading to a similar situation (Fig. 1).

Previous genetic experiments showed that SecA and SecY are both required for CorA's membrane integration [42]. Overexpression of SecA in the cell, however, failed to increase the amounts of CorA in the membrane fraction (Fig. 7). We therefore propose that overexpression of both SecA- and SecYEG-translocase would be necessary for increasing the efficiency of CorA's membrane insertion. It is unknown whether higher levels of additional components, such as accessory proteins SecD and SecF [35], are also required. Co-expression of CorA with the SecYEG-translocase represents a technical challenge, because SecY, SecE, and SecG are all membrane proteins and simultaneous expression of four membrane proteins in *E. coli* is likely to be difficult. This will be a possible future experiment.

Although it is yet to be determined experimentally whether CorA membrane integration is co- or post-translational, we suspect it adopts the post-translational mode. SPR only binds to hydrophobic sequence with high affinity [29] and it then guides the transient polypeptide–ribosome complex to the translocon for co-translational insertion into the membrane. The N-terminal domain of CorA (first 235 out of the total 316 amino acids) lacks such hydrophobic stretches and co-translation is therefore less likely.

Co-expression with chaperones as a way of producing membrane proteins in E. coli

Inclusion body formation is also a problem for certain soluble proteins when overexpressed in *E. coli* [44,47]. In a few cases, decreasing the cell culture temperature helped produce these proteins in a soluble form [3,41]. Co-expression with GroEL/GroES or DnaK/DnaJ also increased the solubility of several overexpressed cytosolic proteins [28,31,52]. Here, we have successfully applied these approaches to a bacterial inner membrane protein, for which the in vivo folding process is more complex. Unlike soluble proteins or bacterial outer membrane proteins, which can both be refolded at a reasonable yield [7,34], proteins from both prokaryotic and eukaryotic cytoplasmic membrane are more hydrophobic and thus more difficult to refold [26]. Co-expression with chaperones and other folding modulators may provide an attractive alternative for producing these membrane proteins in large quantities in vivo.

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