Protein Cell 2012, 3(1): 44–50
DOI 10.1007/s13238-011-1133-z

Protein & Cell

RESEARCH ARTICLE

Study on the chaperone properties of conserved GTPases

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Received November 12, 2011 Accepted November 29, 2011

ABSTRACT

As a large family of hydrolases, GTPases are widespread in cells and play the very important biological function of hydrolyzing GTP into GDP and inorganic phosphate through binding with it. GTPases are involved in cell cycle regulation, protein synthesis, and protein transportation. Chaperones can facilitate the folding or refolding of nascent peptides and denatured proteins to their native states. However, chaperones do not occur in the native structures in which they can perform their normal biological functions. In the current study, the chaperone activity of the conserved GTPases of *Escherichia coli* is tested by the chemical denaturation and chaperone-assisted renaturation of citrate synthase and α-glucosidase. The effects of ribosomes and nucleotides on the chaperone activity are also examined. Our data indicate that these conserved GTPases have chaperone properties, and may be ancestral protein folding factors that have appeared before dedicated chaperones.

KEYWORDS protein folding, chaperone, conserved GTPase

INTRODUCTION

GTPases are a large family of hydrolases that can bind and hydrolyze GTP into GDP and inorganic phosphate. GTPases are often referred to as molecular switches, and execute their functions in a cell cycle, where a GTPase has three states. First, the GTPase is in its original state of binding no nucleotide. After binding a GTP and maybe other ligands, its conformation is changed. The GTPase then hydrolyzes GTP into GDP and releases inorganic phosphate, which results in a GDP-binding state. After the release of GDP, the GTPase returns to its original state. GTP binding and hydrolysis takes place in the highly conserved G domain common to all GTPases, although the GTPase family can be divided into numerous different subfamilies based on peptide sequencing analysis. Sequence alignment indicates there are eleven highly conserved GTPases in all bacteria (Caldon et al., 2001). Four take part in the ribosome translation process as translation factors. The others are known as Ffh, FtsY, Era, TrmE/ThdF, EngA, YchF, ObgE, and SelB.

These conserved GTPases have different biological functions. As an important part of a signal recognition particle (SRP), Ffh constitutes an SRP with a 4.5S RNA. The SRP interacts with the N-terminus signal peptide of a nascent peptide, binds to the SRP receptor FtsY, transfers the target protein to the membrane, and finally transports it out of the membrane. Era plays an important role in the regulation of the cell cycle as well as C and N metabolism. An Era gene-deleted *E. coli* is able to normally replicate its DNA but cannot reproduce by fission. EngA and ObgE can interact with ribosomes. ObgE may be related to the maturation of the ribosome. TrmE takes part in the modification of U34 in tRNAs. The major function of SelB is to assist the accommodation of selenocysteinyl-tRNA into ribosomes. YchF is rarely researched and its functions have not yet been clarified (Leipe et al., 2002).

Biological functions in cells are executed by proteins. The biological activities of proteins guarantee the physiological functions in cells and organisms. The biological activity of a protein is determined by its three-dimensional structure. Even a tiny change in the structure may lead to a complete loss of biological activity. Although the thermodynamically stable structure of a protein has been determined by its amino acid sequence, the actual situation is much more complicated. Some small proteins with a single domain can spontaneously fold into their native structures. However, most proteins with

multiple domains cannot, and their folding processes require the assistance of molecular chaperones (Anfinsen et al., 1961; Ellis and Hemmingsen, 1989; Nissen et al., 2000; Kramer et al., 2009). Molecular chaperones are proteins that can take part in the folding process of nascent peptides into their native structures, or in the refolding of misfolded proteins into correct structures. However, chaperones do not occur in the final and biologically active state of proteins. Many proteins are molecular chaperones. They are unrelated in sequence but have similar functions (Ellis, 1990, 1993; Hendrick and Hartl, 1993; Hartl and Hayer-Hartl, 2009). Generally, chaperones can recognize and bind with the exposed hydrophobic residues of non-native proteins, protect them from aggregations, and assist them to fold into their native structures (Anfinsen et al., 1961; Ellis and Hemmingsen, 1989).

Among the 11 highly conserved GTPases, only EF-G, EF-Tu, and IF2 have been indicated to have chaperone properties (Kudlicki et al., 1997; Caldas et al., 1998, 2000; Suzuki et al., 2007). There are no such reports on other GTPases yet. Determining the possible chaperone properties of these GTPases will benefit the understanding of the chaperone network in prokaryotic cells and the evolutionary development of chaperones. Citrate synthase and α- glucosidase are appropriate proteins for testing chaperone activity, considering that their assays are both convenient and the results are easily distinguishable (Jakob et al., 1993). We chose them as denaturation and renaturation substrates in the current study. Trigger factor (TF), a confirmed chaperone, was used as the positive control (Deuerling et al., 1999; Teter et al., 1999; Maier et al., 2001; Genevaux et al., 2004). We determined the chaperone activities of the conserved GTPases by the chemical denaturation and chaperone-assisted renaturation of citrate synthase and α-glucosidase. We also examined the effects of ribosomes and nucleotides on the chaperone properties of these GTPases.

RESULTS

Molecular cloning, protein expression, and purification

We successfully cloned *E. coli* proteins SelB, FtsY, Ffh, TrmE, ObgE, YchF, and Era in pET-28a plasmid. Isopropyl-ß-D-thiogalactoside-induced proteins were first purified by Ni-column affinity chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The crude purified proteins were further purified by gel filtration chromatography using an AKTA purifier system with a prepacked Superdex 200 10/300 GL column from GE Company. Protein concentration was determined by UV absorption using extinction coefficients at 280 nm. The purified proteins were stored at −80°C after liquid nitrogen freezing (Supplemental Fig. 1).

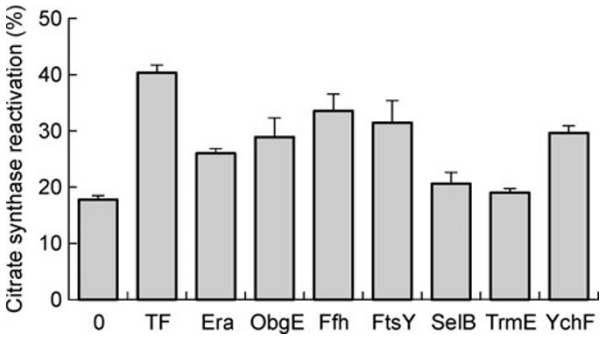

Figure 1. Influence of different proteins on the refolding of denatured citrate synthase. Denatured citrate synthase (0.1 μmol/L) was incubated at 25°C for 40 min with or without different proteins (5 μmol/L) before its enzyme activity was measured.

Effect of conserved GTPases on the renaturation of denatured citrate synthase

Denatured citrate synthase (0.1 μmol/L) was added to a renaturation buffer with 5 μmol/L GTPases, and incubated at 25°C for 40 min for renaturation. Most tested proteins could significantly assist citrate synthase refolding (Fig. 1). Column 0 was the self-reactivation level of citrate synthase alone, which was about 18%. TF, as a positive control, showed the greatest chaperone property among all GTPases and increased the reactivation level to 40%. Ffh and FtsY increased the reactivation to 34%. ObgE and YchF could also improve the reactivation level to about 30%. Although the impact was relatively weak, Era could increase the reactivation level to 26%. SelB and TrmE produced slight improvements in citrate synthase reactivation.

Effect of conserved GTPases on the renaturation of denatured α-glucosidase

In the α-glucosidase assay, 2 μmol/L of GTPases was used to assist 0.1 μmol/L denatured α-glucosidase. Enzyme activity was determined after 1 h of renaturation at 25°C. Most GTPases could increase α-glucosidase reactivation (Fig. 2). The self-reactivation level seemed higher (36%) because in the enzyme activity determination, about 30% denatured α-glucosidase refolded when it was incubated with its substrates. TF could increase the reactivation level of denatured α-glucosidase 12% more than that of its self-renaturation. This reactivation level could also be increased by ObgE, FtsY, and TrmE to a higher degree than TF, as well as by Era, SelB, and YchF to a lower degree than TF. Surprisingly, Ffh completely inhibited α-glucosidase renaturation (1%) even when it was incubated with its substrate. Ffh did not affect the enzyme activity of native α-glucosidase either.

Among all proteins whose chaperone properties were

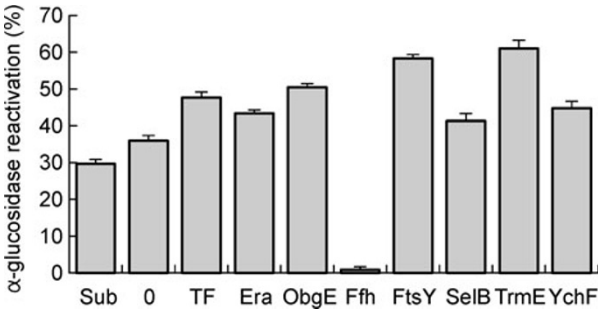

Figure 2. Influence of different proteins on the refolding of denatured α-glucosidase. Denatured α-glucosidase (0.1 μmol/L) was incubated at 25°C for 1 h with or without different proteins (2 μmol/L) before its enzyme activity was measured. Sub, renaturation yield of denatured α-glucosidase in the enzyme activity determination.

tested, only Ffh could inhibit the renaturation of denatured α-glucosidase, which meant that Ffh may have some unique characteristics. We calculated different physical and chemical parameters of these proteins with the ProtParam tool on the ExPASy website, and found that Ffh is the only one whose theoretical isoelectric point (pI), 9.47, is higher than that of the refolding buffer, 7.6 (Table 1). Therefore, in our buffer condition, Ffh was the only positively charged protein, and all other proteins including α-glucosidase were negatively charged. Given that denatured α-glucosidase stretches out its peptide and loses its protective three-dimensional structure, it may bind to the oppositely charged Ffh and not be able to refold. The theoretical pI of citrate synthase is 7.01. The charge of citrate synthase in our condition should be much less than the pI of α-glucosidase. Hence, no similar inhibitory effect was observed in the citrate synthase assays.

Effect of ribosomes on the assisted renaturation of denatured α-glucosidase

Ribosomes are peptide factories, and a nascent peptide begins its folding process as early as when its first few residues come out from the peptide tunnel of a ribosome. EngA, ObgE, Ffh, and SelB could interact with ribosomes. Thus, determining the effects of ribosomes on the chaperone properties of the conserved GTPases is prudent. Reassociated ribosomes (0.2 μmol/L) from *E. coli* were added to the assisted-refolding assays. $MgAc_2$ (6 mmol/L) was also added to stabilize the ribosomes, which showed no effect on the renaturation of both citrate synthase and α-glucosidase. All other conditions were the same as in the preceding section. The ribosomes alone only slightly affected the renaturation yield of denatured citrate synthase. However, when the ribosomes were combined with other protein factors, the reactivation level of denatured citrate synthase decreased in different levels, except for FtsY, which increased by merely 1% (Fig. 3A). This result was probably due to the binding of these proteins to the ribosomes, which decreased the available protein concentration for assisted refolding. The reactivation yield of citrate synthase dramatically decreased when Ffh and ribosomes were both present because there a large amount of precipitation occurred.

In the α-glucosidase assays, the ribosomes showed some chaperone properties. The renaturation yield of denatured α-glucosidase was increased by 11% by 0.2 μmol/L ribosomes, and was totally improved with GTPases (Fig. 3B). Interestingly, when ribosomes and Ffh were both present, no inhibitory effect from Ffh was observed. The reactivation level was even higher than that with ribosomes alone. This result could be explained by the interaction of ribosomes with Ffh as well as the decreased affinity between Ffh and denatured α-glucosidase such that denatured α-glucosidase was able to refold again.

Effect of nucleotides on the assisted renaturation of denatured α-glucosidase

To determine whether the GTPase activity affects the chaperone properties of conserved GTPases, 2 mmol/L each of GTP, GDP, or guanosine 5′-(β,γ-imido)triphosphate (GDPNP) was added to the assisted-refolding assays. The other conditions were the same as in the above assays, and the reactivation yields were compared with those without nucleotides. GDPNP, an analog of GTP, can bind to a

Table 1 Relationship between the pI values of proteins and their ability to assist α-glucosidase refolding

Protein	pI	pH − pI[a]	α-glucosidase reactivation (%)
α-glucosidase	5.53	2.07	7.6
TF	4.97	2.63	17.6
SelB	6.19	1.41	11.6
Era	6.71	0.89	13.5
ObgE	4.91	2.69	20.7
YchF	5.05	2.55	15.0
Ffh	9.47	−1.87	−28.9
FtsY	4.56	3.04	28.3
TrmE	5.08	2.52	31.1

[a] pH in the refolding buffer was 7.6. pI, isoelectric point.

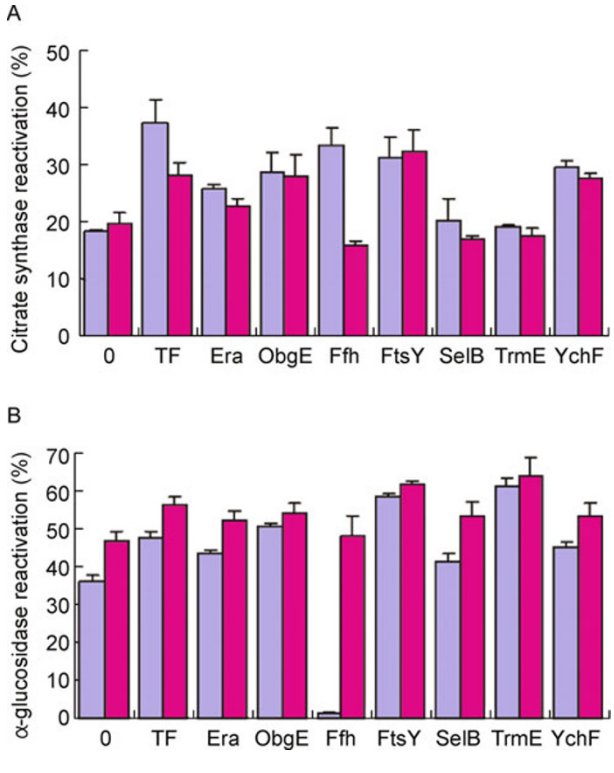

Figure 3. Influence of ribosomes on the refolding of denatured citrate synthase and α-glucosidase. (A) Denatured citrate synthase (0.1 μmol/L) was incubated at 25°C for 40 min with or without chaperone protein (5 μmol/L), and with (magenta) or without (blue) ribosome (0.2 μmol/L). (B) Denatured α-glucosidase (0.1 μmol/L) was incubated at 25°C for 1 h with or without chaperone protein (2 μmol/L), and with (magenta) or without (blue) ribosome (0.2 μmol/L).

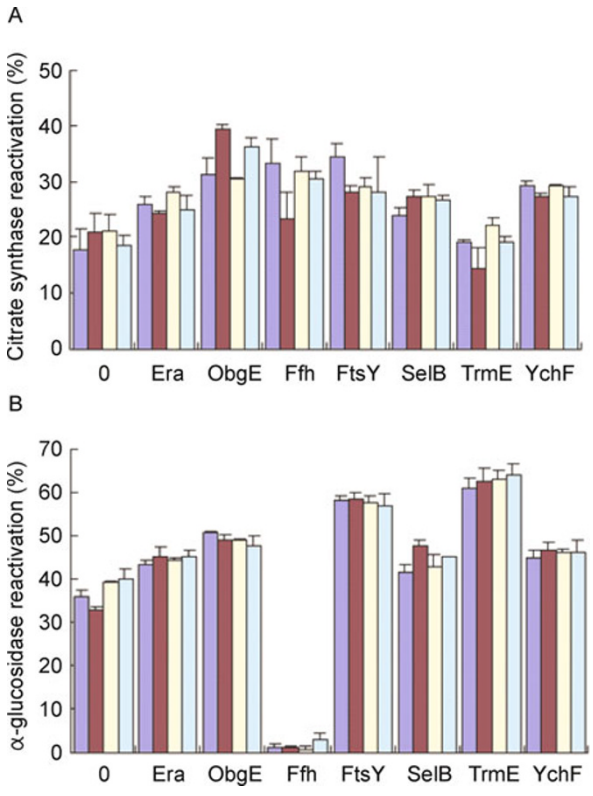

Figure 4. Influence of nucleotides on the refolding of denatured citrate synthase and α-glucosidase. (A) Denatured citrate synthase (0.1 μmol/L) was incubated at 25°C for 40 min with or without chaperone protein (5 μmol/L), and without (blue) or with 2 mmol/L GTP (wine), 2 mmol/L GDP (yellow) or 2 mmol/L GDPNP (cyan). (B) Denatured α-glucosidase (0.1 μmol/L) was incubated at 25°C for 1 h with or without 2 μmol/L chaperone protein, without (blue) or with 2 mmol/L GTP (wine), 2 mmol/L GDP (yellow), or 2 mmol/L GDPNP (cyan).

GTPase similarly as GTP. However, GDPNP cannot be hydrolyzed, keeping the GTPase as its GTP-binding conformation at all times. In the assisted-refolding assays of either denatured citrate synthase or α-glucosidase, the three nucleotides showed no significant effect on most GTPases (Fig. 4). Although a few GTPases seemed to be affected by one or two nucleotides, there was no universal rule. Therefore, we suggest that the conformational change of GTPases with different nucleotides does not affect their chaperone properties. Our results are also consistent with the research on the conformation of EF-Tu and its chaperone properties by Suzuki et al. (2007).

DISCUSSION

In most cases, the greatest obstacles in protein folding are hydrophobic groups exposed to a solvent. In the folding process, these hydrophobic groups may interact with each other and result in protein aggregation as well as deposition. The function of chaperones is often to recognize and bind to these non-natural surfaces to prevent false folding (Wang, 2004). TF has been confirmed as the first chaperone that nascent peptides encounter after their synthesis. The results of the current study show that TF could significantly raise the reactivation yield of denatured citrate synthase and α-glucosidase, and that most of the conserved GTPases could also increase the refolding level of the two enzymes. Although TF showed the greatest chaperone properties in citrate synthase refolding, ObgE, FtsY, and TrmE could improve the refolding of denatured α-glucosidase to a greater extent than TF. The reactivation of denatured α-glucosidase was apparently affected by the charges of the chaperones because Ffh inhibited its refolding.

The effects of ribosomes on protein refolding were also determined. The refolding level of denatured citrate synthase with either TF or Ffh significantly decreased after ribosome addition. Both proteins have a binding site on ribosomes, and they could interact with L23, a ribosomal protein on the peptide exit tunnel of ribosomes (Gu et al., 2003). Era, ObgE,

SelB, and YchF could also interact with ribosomes (Sayed et al., 1999; Leipe et al., 2002; Sato et al., 2005). The binding of these GTPases to ribosomes reduced the amount of dissociated proteins, which could improve denatured citrate synthase refolding, thereby decreasing the reactivation level. Unlike that of citrate synthase, the refolding yield of denatured α-glucosidase was positively affected by ribosomes alone; the assisted refolding levels increased when ribosomes were added to all the assisting proteins. No inhibition effect from Ffh was observed with ribosomes. Binding with ribosomes may have decreased the affinity of Ffh to denatured α-glucosidase, such that the binding process of Ffh and denatured α-glucosidase became reversible and allowed its refolding. FtsY can bind to Ffh, but not to ribosomes (Buskiewicz et al., 2004), and TrmE is involved in tRNA modification (Leipe et al., 2002). These two GTPases seem to be unable to interact directly with ribosomes. The effects of ribosomes on the renaturation assay with FtsY and TrmE were also relatively minor compared with the assays with the other GTPases (Fig. 3).

Generally, although the solvent-accessible surface of a protein is hydrophilic as a whole, it also contains some hydrophobic areas. These areas could possibly be able to bind temporarily with the hydrophobic area of an unfolded peptide. This binding process may protect against false folding or aggregation, and is thereby beneficial to peptide renaturation. Therefore, although not dedicated chaperones, conserved GTPases could also assist the refolding of denatured proteins. These GTPases improved the refolding of denatured citrate synthase and α-glucosidase by different degrees. Some GTPases assisted one enzyme much more than the other, and some exhibited similar effects on both enzymes. This effect may be due to the differences in the hydrophobic areas of the GTPases. The area, shape, and hydropathicity may all affect the binding to an unfolded peptide and the resulting reactivation level. The chaperone properties of the conserved GTPases did not seem to be affected by their conformation with different nucleotides. The difference induced by the nucleotides may not have been significant in the hydrophobic areas.

In conclusion, the conserved GTPases in *E. coli* have chaperone properties. Although they are not dedicated, they are able to interact with some denatured proteins and assist their reactivation. Given that they are ancient and very much conserved, they may have been responsible for maintaining the stability of proteins *in vivo* before dedicated chaperones evolved.

MATERIALS AND METHODS

Materials

The plasmid with TF gene was provided by Dr. Junmei Zhou and Dr. Jiangbi Xie of the Institute of Biophysics, Chinese Academy of Sciences. Citrate synthase (from porcine heart), α-glucosidase (from *Saccharomyces cerevisiae*), and all other chemicals were from Sigma and were of reagent grade.

Molecular cloning, protein expression, and purification

Genes of SelB, FtsY, Ffh, TrmE, ObgE, YchF, and Era were inserted into pET-28a plasmids. Proteins were expressed in *E. coli* BL21 (DE3), and purified with a Ni-NTA column as well as a molecular sieve. The purified proteins were frozen in liquid nitrogen and stored at −80°C in 20 mmol/L Tris-HCl (pH 8.0), 500 mmol/L NaCl, and 1 mmol/L dithiothreitol (DTT). The purities of all proteins were confirmed by SDS-PAGE. The concentrations of all proteins were determined by UV absorption using the following extinction coefficients ($\times 10^4$ $(mol/L)^{-1} \cdot cm^{-1}$) at 280 nm: TF (1.59), Sel B (8.09), FtsY (5.47), Ffh (4.98), TrmE (2.65), ObgE (4.33), YchF (3.97), and Era (3.38).

Purification of EF-G, LepA, their truncations, other translation factors, and GTPases

Proteins were constructed in pET22b and pET28a plasmids, expressed in *E. coli* BL21 (DE3), and purified with a Ni-NTA column and gel filtration with column buffer [for LepA and its truncations: 20 mmol/L Tris-HCl (pH 8.0), 500 mmol/L NaCl, and 1 mmol/L DTT; for other proteins: 20 mmol/L Tris-HCl (pH 8.0), 500 mmol/L NaCl, and 1 mmol/L DTT].

Refolding of citrate synthase

The denaturation and renaturation of citrate synthase were performed as described by Caldas et al. (2000) with some modifications. Citrate synthase (10 μmol/L) was denatured in 6 mol/L GdnHCl, 50 mmol/L Tris-HCl (pH 8.0), and 2 mmol/L DTT at 25°C for over 2 h. Renaturation was initiated by a 100-fold dilution in 40 mmol/L 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)-KOH (pH 8.0), 50 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, and 2 mmol/L KAc at 25°C for an indicated time with or without chaperone and other additives. The activity of renatured citrate synthase was determined in 50 mmol/L Tris-HCl (pH 8.0), 0.1 mmol/L acetyl coenzyme A, 0.2 mmol/L oxaloacetate, and 0.2 mmol/L 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm absorbance.

Calculation of the citrate synthase renaturation efficiency

The slope ΔA412/s was calculated based on the curve of absorbance at 412 nm *versus* time. The slope represented the activity of citrate synthase. Renaturation efficiency was the ratio of the activity of renatured citrate synthase to that of native citrate synthase in the same amount with the same manipulation except denaturation. All assays were repeated at least three times.

Refolding of α-glucosidase

The denaturation and renaturation of α-glucosidase were performed as described by Caldas et al. (2000) with some modifications. A test amount of 3 μmol/L α-glucosidase was denatured in 8 mol/L urea, 0.1 mol/L $KHPO_4$ (pH 7.0), 1 mmol/L EDTA, and 20 mmol/L DTT at 25°C for 1.5 h. Renaturation was initiated by a 30-fold dilution in

40 mmol/L HEPES-KOH (pH 7.6), at 25°C for an indicated time with or without chaperone and other additives. Renatured α-glucosidase (2 μL) was mixed with 57 μL of a solution containing 60 mmol/L $KHPO_4$ (pH 7.0), 0.1 mmol/L reduced glutathione, and 0.9 mmol/L p-nitrophenyl α-D-glucoside. The mixture was incubated at 37°C for 20 min. Then, 20 μL of the mixture was drawn and added to 80 μL of 0.1 mol/L Na_2CO_3. The absorbance at 400 nm was recorded.

Calculation of α-glucosidase renaturation efficiency

The absorbance at 400 nm represented the activity of α-glucosidase. The renaturation efficiency is the ratio of the activity of renatured α-glucosidase to that of native α-glucosidase in the same amount with the same manipulation except denaturation. All assays were repeated at least three times.

Purification of reassociated ribosomes

Highly purified reassociated ribosomes were prepared as described by Márquez et al. (2004). *E. coli* cells in the exponential phase were poured into ice and collected by centrifugation at 4°C. All subsequent steps were performed at 4°C or in ice if not indicated. The cells were disrupted under high pressure, and the cell debris was removed. Crude ribosomes were separated by ultracentrifugation and applied to three zonal centrifugations with sucrose gradients: crude separation, dissociation, and reassociation. The purified ribosomes were incubated at 40°C for 20 min for stabilization.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 31170756) and CAS-NN Research Foundation to Y. Qin.

Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s13238-011-1133-z and is accessible for authorized users.

ABBREVIATIONS

DTNB, 5,5'-dithiobis (2-nitro-benzoic acid); DTT, dithiothreitol; EF-G, elongation factor G; EF-Tu, elongation factor Tu; Era, *E. coli* Ras-like protein; EngA, a GTP binding protein; Era, *E. coli* Ras-like protein; Ffh, signal sequence recognition protein; FtsY, signal recognition particle receptor; GdnHCl, guanidine hydrochloride; GDPNP, 5'-(β,γ-imido) triphosphate; IF2, initiation factor 2; ObgE, Obg in *E. coli*; SelB, selenocysteinyl-tRNA-specific translation factor; SRP, signal recognition particle; TrmE/ThdF, a tRNA modification GTPase; YchF, another member of the Obg family

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