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Comparing the functional properties of the Hsp70 chaperones, DnaK and BiP

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ABSTRACT

The Hsp70 family of molecular chaperones is an essential class of chaperones that is present in many different cell types and cellular compartments. We have compared the bioactivities of the prokaryotic cytosolic Hsp70, DnaK, to that of the eukaryotic Hsp70, BiP, located in the endoplasmic reticulum (ER). Both chaperones helped to prevent protein aggregation. However, only DnaK provided enhanced refolding of denatured proteins. We also tested chaperone folding assistance during translation in the context of cell-free protein synthesis reactions for several protein targets and show that both DnaK and BiP can provide folding assistance under these conditions. Our results support previous reports suggesting that DnaK provides both post-translational and co-translational folding assistance while BiP predominately provides folding assistance that is contemporaneous with translation.

1. Introduction

The Hsp70 family of heat shock proteins represents a ubiquitous class of molecular chaperones whose structure and function are largely conserved across both prokaryotic and eukaryotic hosts. Hsp70s serve multiple roles in their hosts but generally function by transiently binding to exposed hydrophobic sequences of nascent proteins [1–5]. The well characterized *Escherichia coli* Hsp70, DnaK, is located in the cytoplasm and serves to help fold and assemble native proteins. In mammalian cells, Hsp70s are located in the cytoplasm, nucleus, mitochondria, and endoplasmic reticulum [2,6], but the molecular chaperone BiP is the sole Hsp70 homolog identified in the lumen of the endoplasmic reticulum (ER). BiP participates in protein folding and assembly, translocation of proteins across the ER membrane, targeting misfolded proteins for degradation, and in controlling ER calcium stores [7]. Unlike DnaK and other cytoplasmic Hsp70s that diffuse relatively freely in their cellular environments, BiP is typically localized to the Sec translocon in the ER lumen, effectively forming a permeation barrier to this organelle. BiP associates with polypeptides as they are secreted through the translocon and detaches from the translocon to follow the nascent protein after about 70 amino acids have been translated, thereby providing co-translational folding and translocation assistance to nascent proteins [8,9].

The function of all Hsp70 chaperones is facilitated by ATP. ATP is accepted by a highly conserved N-terminal domain that allows the chaperone to rapidly associate with low affinity to potential substrates. Hydrolysis of ATP to ADP causes a conformational change in the chaperone to increase this affinity. Finally, exchange of ADP for fresh ATP allows the substrate to be released and initiates a new cycle of chaperone–substrate interactions [10,11]. Several co-chaperones also contribute to the functioning of Hsp70s. For DnaK, the co-chaperone DnaJ helps accelerate the hydrolysis rate of ATP while GrpE assists in the exchange of ADP for ATP [12]. Several DnaJ homologs exist in the mammalian ER including the ERdj class of proteins (ERdj1–5) [13,14]. A GrpE homolog, BAP, is also present [15].

Several groups have studied the roles that the Hsp70 chaperone family plays in the prevention of protein aggregation [16–20], *in vitro* refolding [20–24], and co-translational protein folding of model proteins [25–28] such as firefly luciferase and β-galactosidase. Many Hsp70 systems improve the refolding of heat or chemically denatured firefly luciferase [21,22,29,30], while refolding proceeds inefficiently in the absence of Hsp70 chaperones [31,32]. Additionally, both prokaryotic and eukaryotic Hsp70 systems improve refolding of chemically denatured β-galactosidase [6,33,34].

The cytoplasmic Hsp70 chaperones also improve co-translational folding during the cell-free protein synthesis of firefly luciferase. For example, Frydman et al. [35] demonstrated that the eukaryotic cytoplasmic Hsp70, Hsc70, and its DnaJ homolog, Hsp40, were necessary for productive co-translational folding of firefly luciferase in a rabbit reticulocyte translation system. In contrast, the cell-free protein synthesis of active firefly luciferase using an *E. coli* translation system occurs without augmenting chaperone concentrations even

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though DnaK, DnaJ, and GrpE have been diluted about 20-fold relative to cytoplasmic concentrations [26,36]. Agashe et al. [28] showed that additional DnaK + DnaJ + GrpE increased the yield of active firefly luciferase.

However, the refolding and co-translational folding efficacy of the ER Hsp70 chaperone (BiP) and its co-chaperones (for example, ERdj3 and BAP) has not been studied as extensively. BiP is known to interact with the common β-barrel immunoglobulin fold that is present in the domains of many secreted eukaryotic proteins (e.g., antibodies and T-cell receptors) [37,38] and has been shown to help refold antibodies *in vitro* [39]. In this study, we compare the chaperone functions of the mammalian ER Hsp70 (BiP) and its co-chaperones ERdj3 and BAP to those of the prokaryotic cytosolic Hsp70 (DnaK) and its co-chaperones DnaJ and GrpE. ERdj3 is one of several Hsp40 (J-domain) chaperones found in the ER and was chosen for these experiments because it is not a membrane bound chaperone [14]. We show that although it discourages aggregation, the BiP system (BiP + ERdj3 + BAP) is deficient in its ability to refold both denatured prokaryotic and denatured eukaryotic cytoplasmic targets as compared to the DnaK system (DnaK + DnaJ + GrpE). Despite this, the BiP system effectively assists in the folding of several different extracellular eukaryotic polypeptide targets during cell-free protein synthesis. The use of exogenously added chaperones such as BiP and its co-chaperones offers the potential for improved production of important eukaryotic protein targets using cell-free protein synthesis reactions in which other important influences such as redox potential and disulfide isomerase activity can also be modulated.

2. Materials and methods

2.1. Gene constructs, protein production and purification

Coding DNA segments for the *E. coli* chaperones DnaK, DnaJ, and GrpE were obtained through PCR from *E. coli* genomic DNA. Expression genes for the mammalian chaperones hamster BiP [40] and human ERdj3 [13] and BAP [15] were generous gifts from Dr. Linda Hendershot, St. Jude Children's Hospital, Memphis, TN. PCR products for these genes without their signal sequences were generated, and each gene was cloned into the pY71 vector using NdeI and SalI restriction sites. pY71 is a reduced size plasmid (2.45 kb) with a pUC19 origin of replication and a kanamycin resistance element under the control of the T7 promoter [41]. Additionally, in order to obtain the human BiP sequence, primers to change 6 amino acids in the hamster sequence were used to amplify the gene by PCR in 2 pieces followed by amplification of the full length, human sequence [42].

Plasmids were transformed into *E. coli* BL21(DE3) pLysS cells and were grown in 1 L LB medium supplemented with 40 μg/ml kanamycin and 20 μg/ml chloramphenicol at 37 °C. At an OD of 0.6, cells were induced with 1 mM IPTG and incubated for an additional 3 h. (For the production of BAP, cells were incubated for 3 h at 17 °C after induction to increase product solubility and reduce degradation.) Cells were harvested by centrifugation and resuspended in 15 mL of load buffer (10 mM imidazole, 50 mM sodium phosphate, 300 mM sodium chloride, pH 8). A single pass through an Avestin EmulsiFlex C-50 high-pressure homogenizer at 20,000 psig lysed cells. Protease inhibitor (PMSF) was added to all samples before lysis to a concentration of 1.33 mM. After centrifugation at 30,000 ×*g*, 2200 U of DNaseI (Invitrogen) was added to the supernatant and incubated at room temperature for approximately 45 min to reduce viscosity. The clarified lysates were loaded on 1 mL nickel-chelating columns (His GraviTrap, GE Healthcare) equilibrated with load buffer and eluted with load buffer with 250 mM imidazole. Pooled fractions were dialyzed into 10 mM potassium phosphate, pH 7.2. Sucrose was added to 10%, and proteins were stored at −80 °C. A nominal amount of sucrose was carried over into subsequent experiments, but the fraction was almost always less than 3% w/v.

Cell-free production from plasmid templates was completed (see below) for the following genes: single chain T-cell receptor (scTCR) (kind gift from the Ronald Levy Lab, Stanford University), which is composed of the variable alpha and variable beta regions linked with 3 gly-gly-gly-gly-ser domains ($(G_4S)_3$) and followed by the constant beta region of the T-cell receptor for C6VL, a T-cell tumor of C57BL/Ka origin [43]; anti-digoxin single chain antibody fragment (scFv) [44] which is composed of the variable light chain linked to the variable heavy chain with a $(G_4S)_3$ linker (kind gift from the George Georgiou lab, University of Texas at Austin); the CD-19 [45] extracellular domain (amino acids 19–287) without the N-terminal leader sequence and the C-terminal transmembrane and cytoplasmic domains; β-galactosidase from *E. coli* [46]; and *Photinus pyralis* (firefly) luciferase (see Table 1) [47,48]. In each case, the gene was cloned behind the T7 promoter in plasmid pY71 [41].

2.2. ATPase activity assay

The ATP hydrolysis rate was monitored over 30 min using a luciferase assay to measure ATP concentration [49]. In a 50 μl reaction, the purified chaperones were incubated in 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 100 μM ATP, and 20 mM HEPES, pH 7.2 at 37 °C. Samples were taken immediately after addition of ATP and every 10 min thereafter. To stop ATP hydrolysis, the samples were diluted 100-fold in ice cold luminescence buffer (20 mM Tris, 5 mM MgAc, 1 mM DTT, and 0.1 mM EDTA) and were stored on ice until analysis. A mix containing 50 μM luciferin and 0.2 μg/mL firefly luciferase in 0.2% m/v BSA plus luminescence buffer was mixed into the diluted samples. Immediately after mixing, the luminescence was measured for 10 s (Mithras LB 940, Berthold Technologies). The ATP concentration of the time course samples was determined by comparing the luminescence readings to those from ATP standard curves, and the ATPase activities were determined from the slope of the decreasing ATP concentrations.

2.3. Luciferase and β-galactosidase aggregation assay

Firefly luciferase (Promega, Madison, WI) (20 μM) and β-galactosidase (Sigma, St. Louis, MO) (20 μM) were denatured with 6 M guanidine-HCl in 5 mM $MgCl_2$, 50 mM KCl, 5 mM β-mercaptoethanol, and 25 mM HEPES, pH 7.4 for 15 min at 22 °C. Denatured proteins were diluted 100-fold (0.2 μM final concentration) into a quartz cuvette containing 26 mM HEPES, 5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, and 1 mM DTT (dilution buffer) with or without chaperones present. Immediately after addition of the denatured protein, aggregation was monitored continuously at 320 nm [16–18] for 45 min. BiP + ERdj3 and DnaK + DnaJ were supplemented at concentrations of 2 μM and 0.5 μM for each protein tested here.

Table 1
Protein domains used for model proteins to test Hsp70 chaperone effectiveness.

Abbreviation	Gene/description	Protein domains	References
Luciferase	Firefly luciferase	Native sequence	de Wet et al. [47]
β-gal	*E. coli lacZ*	Native sequence	Kesseler et al. [46]
scTCR	Single chain T-cell receptor	V_α-V_β-$(G_4S)_3$-C_β	Okada et al. [43]
Dig scFv	Anti-digoxin single chain variable region	V_L-$(G_4S)_3$-V_H	Levy et al. [44]
CD-19 ECD	CD-19 extra cellular domain	Extra cellular domain only (aa 19–287)	Tedder et al. [45]

2.4. Single chain T-cell receptor aggregation assays

ScTCR was produced under standard cell-free protein synthesis conditions with the incorporation of ^{14}C-leucine using the PANOx SP system [50] (see below). After cell-free protein synthesis of the scTCR, the insoluble pellet was resuspended in 1 mL load buffer with 6 M urea and 5 mM DTT and was purified under denaturing conditions using a nickel-chelating column (see protocol above for purification of his-tagged proteins). All buffers contained 6 M urea and 5 mM DTT to ensure that denaturing conditions were maintained throughout the entire purification protocol. After purification, buffer exchange into 10 mM potassium phosphate, 6 M urea, and 5 mM DTT was completed using an Amicon Ultra-10 (10 kDa cutoff) ultrafiltration device. Aliquots were stored at −80 °C. Aggregation monitored at 320 nm in a quartz cuvette was performed as described above except that denatured scTCR was only diluted 50-fold in dilution buffer before the absorbance was measured.

To further quantify aggregation prevention using scintillation counting, denatured purified scTCR was diluted 50-fold (0.28 μM final concentration) into 100 μl dilution buffer (without DTT but with the addition of 50 μg/ml pyruvate kinase, 0.27 mM Coenzyme A, 30 mM phosphoenolpyruvate, 0.5 mM GSSG, and 5 mM GSH to encourage disulfide bond formation) containing either chaperones or 3.5 μM BSA. After incubating at 22 °C for 15 min, the samples were centrifuged at 14,000 rpm for 15 min to separate the soluble fraction from insoluble aggregates. Soluble fractions were spotted onto Whatman 3MM filter paper (50 μl each) and insoluble fractions were resuspended in 100 μl of water and spotted onto Whatman 3MM filter papers (50 μl each). The spotted fractions were dried for 1 h under a heat lamp and radioactivity from both filter papers was determined with a liquid scintillation counter (Beckman LS3801, Beckman Coulter, Fullerton, CA).

2.5. Heat denatured luciferase refolding assay

Hsp70 chaperones (BiP or DnaK; 4 μM) and co-chaperones (ERdj3 or DnaJ; 1 μM) were pre-incubated with excess ATP (1 mM) for 15 min at room temperature in Buffer A (20 mM HEPES, 5 mM $MgCl_2$, 50 mM KCl, and 2 mM DTT). Next, luciferase (80 nM) was added to the chaperones and incubated at room temperature for 10 min. After addition of the nucleotide exchange factors (BAP or GrpE; 1 μM) the mix was heat shocked at 42 °C for 10 min. Immediately after the heat shock, the reaction mixtures were incubated at room temperature. To determine resultant luciferase activity, 2.5 μl samples were diluted 20-fold with Buffer A (including 1 mM ATP and 0.2 mM luciferin), and the luciferase activity (light emission) was determined using a luminometer (Mithras LB 940, Berthold Technologies) by integrating luminescence over 10 s. The luminescence readings were compared to luminescence readings of each sample taken before heat shock.

2.6. Urea-denatured luciferase refolding assay

Luciferase at 8 μM was denatured in 26 mM HEPES, 14 mM $MgCl_2$, 27 mM KCl, 1.7 mM DTT, and 8 M urea for 30 min at room temperature. Next, the denatured luciferase was diluted 100-fold into refolding mix containing 26 mM HEPES, 5 mM $MgCl_2$, 50 mM KCl, and 1 mM ATP with either chaperones (3.5 μM BiP or DnaK, 0.7 μM ERdj3 or DnaJ, and 0.35 μM BAP or GrpE) or BSA (3.5 μM). Luciferase activity was determined with the addition of luciferin as described above.

2.7. Cell-free protein synthesis in PANOx SP

The PANOx SP [50] cell-free protein synthesis system reaction mixture contained 20 mM magnesium glutamate, 10 mM ammonium glutamate, 170 mM potassium glutamate, 1.2 mM ATP, 0.86 mM each of GTP, UTP, and CTP, 34 μg/ml folinic acid, 170 μg/ml *E. coli* tRNAs, 2 mM each of the 20 amino acids, 2 mM additional cysteine, 30 mM phosphoenolpyruvate, 0.33 mM NAD, 0.27 mM Coenzyme A, 2.7 mM oxalic acid, 1 mM putrescine, 1.5 mM spermidine, 8.4 μM L-[U–^{14}C] Leucine (306 mCi/mmol), 0.1 mg/ml T7 RNA polymerse, 13.3 μg/ml plasmid DNA, and 24% v/v *E. coli* KC6 [51] cell extract. For proteins containing disulfide bonds, the KC6 cell-free extract was pretreated with 1 mM iodoacetamide and the reaction mixture was supplemented with an oxidizing glutathione buffer consisting of 4 mM GSSG and 1 mM GSH as well as *E. coli* DsbC at 75 μg/ml as a disulfide isomerase except for the scTCR for which better solubility and folding was obtained by using more reducing conditions with 1 mM GSSG and 4 mM GSH. The concentrations of the Hsp70 chaperones added to the reactions were 8 μM and the co-chaperone concentrations were each kept at 0.5 μM in all reactions containing chaperones. These transcription/translation reactions were incubated at 37 °C for 3.5 h. After incubation, insoluble cell debris was removed by centrifugation at 14,000 rpm for 15 min at 4 °C. Protein yields were determined by scintillation counting of the incorporated ^{14}C-leucine after TCA washes of the cell-free supernatant sample [50].

2.8. Cell-free protein synthesis in the PURE SYSTEM

Cell-free protein synthesis also was conducted in the PURESYSTEM classic II *in vitro* translation system (Cosmo Bio USA Co., Ltd.) with the addition of 8.4 μM ^{14}C-leucine. To allow for correct disulfide bond formation, the glutathione buffer and DsbC were added as described above. Reactions were conducted for 1 h at 37 °C. Scintillation counting was used to determine protein concentrations as described above.

3. Results

3.1. Confirmation of ATPase activity

To demonstrate that BiP and DnaK display characteristic activities, the ATP hydrolysis activity of the purified recombinant chaperones was determined. BiP and DnaK were incubated either alone or with their appropriate co-chaperones and hydrolysis of ATP to ADP was measured over time using a luminescence assay [49]. BiP, ERdj3, and BAP concentrations of 0.5 μM, 1 μM, and 1 μM were used in accordance with previously published studies [15]. DnaK, DnaJ, and GrpE concentrations of 0.5 μM, 0.6 μM, and 1 μM were used as they provided maximal specific ATP hydrolysis rates. As indicated in Fig. 1, addition of BAP to BiP increased ATP hydrolysis rate almost 2-fold (Fig. 1a), as expected [14,15]. On the other hand, the addition of ERdj3 to either BiP or BiP + BAP did not increase the ATP hydrolysis rate, which contradicts previous findings that suggest a 1.8-fold stimulation [14,15]. While it is possible that our *E. coli* produced ERdj3 was inactive, it accumulated as a soluble product and was readily purified, so we continued to use it in subsequent experiments. The lack of ATP turnover stimulation may relate to the biochemical environment used in our assay. The hydrolysis rates of DnaK were increased by addition of DnaJ and GrpE (Fig. 1b), and the rates compare well with previously published results [12].

3.2. Aggregation prevention

Several studies show that Hsp70 chaperones suppress aggregation of denatured proteins [16–18]. In the absence of chaperones, 6 M guanidine-denatured luciferase immediately aggregates after a 100-fold dilution as noted by an increase in light scattering (optical density measured at 320 nm as in previous studies) [16–18]. A lower rate of increase in light scattering would indicate aggregation suppression. Fig. 2 shows such results for three different model proteins: firefly luciferase, β-galactosidase, and a single chain T-cell receptor (scTCR).

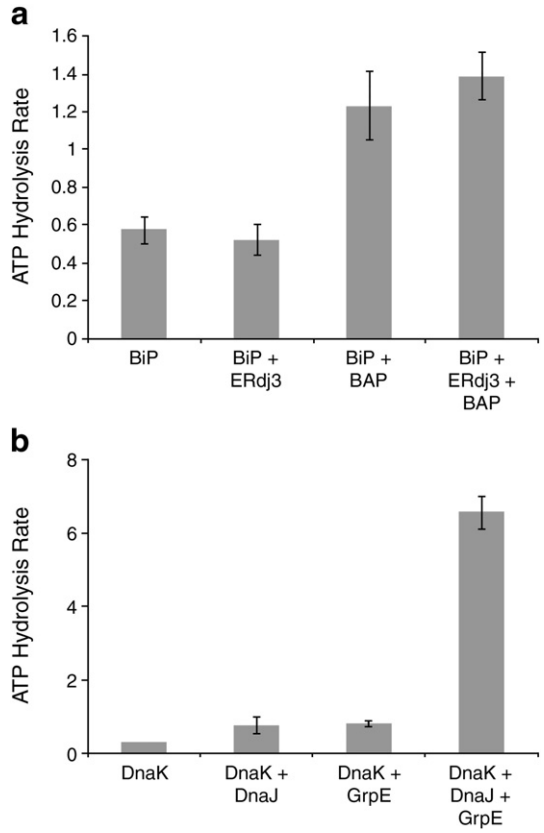

Fig. 1. ATP hydrolysis rate (μmol ATP/μmol Hsp70/min) of the Hsp70s with co-chaperones. Reactions were in 20 mM HEPES (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, and 100 μM ATP at 37 °C [15]. (a) The concentrations used were BiP at 0.5 μM, ERdj3 at 1 μM, and BAP at 1 μM; error bars show standard deviation of two separate experiments. (b) The concentrations used were DnaK at 0.5 μM, DnaJ at 0.6 μM, and GrpE at 1.2 μM; error bars show standard deviation of three separate experiments.

Without chaperones, the light scattering initially increases rapidly. However, after ~1.5 min, the signal decreases as the larger aggregates settle out of the light path (Fig. 2). 2 μM BiP + 0.5 μM ERdj3 prevents guanidine-denatured luciferase from forming large aggregates (optical density increases only very slowly); however, some luciferase aggregation was observed as noted by a gradual increase in optical density readings. DnaK + DnaJ has been shown to prevent luciferase aggregation [21] and was more effective than BiP + ERdj3 for all three of the model proteins. However, BiP + ERdj3 still provided significant benefit. As with previous studies [16–18], the nucleotide exchange factors BAP and GrpE were not included in these experiments as it was anticipated that nucleotide exchange would not contribute to aggregation prevention.

The light scattering results indicate that both the DnaK and BiP systems retard aggregation of the scTCR. To confirm this result, a centrifugation based aggregation assay [19] was performed with radio-labeled scTCR produced using a cell-free protein synthesis system [50]. The scTCR was denatured with 6 M urea and 5 mM DTT and diluted into solutions containing either chaperones (2 μM Hsp70 and 0.5 μM for each co-chaperone) or bovine serum albumin (BSA, 20 μM). (In this case, BSA was added to prevent adsorption of scTCR unto the polypropylene tube walls). After incubation at room temperature for 15 min, the mixture was centrifuged at 22,000 ×g for 15 min to remove insoluble fractions. Both soluble and insoluble fractions were quantified by scintillation counting of radio-labeled scTCR (Fig. 3). This assessment confirmed the light scattering results showing that both chaperone systems suppress aggregation of denatured scTCR with the DnaK system again being more effective.

3.3. Refolding of denatured proteins

Many studies have previously characterized the ability of Hsp70 chaperones to improve refolding of either heat or chemically denatured substrates. We compared the refolding activities of the ER Hsp70 chaperone, BiP, with the prokaryotic Hsp70 chaperone, DnaK. The BiP and DnaK chaperone systems (4 μM DnaK or BiP, 1 μM DnaJ or ERdj3, and 1 μM GrpE or BAP) were incubated with 80 nM luciferase and the mixture was heat shocked at 42 °C for 10 min. Post heat shock luciferase activities were compared to initial readings

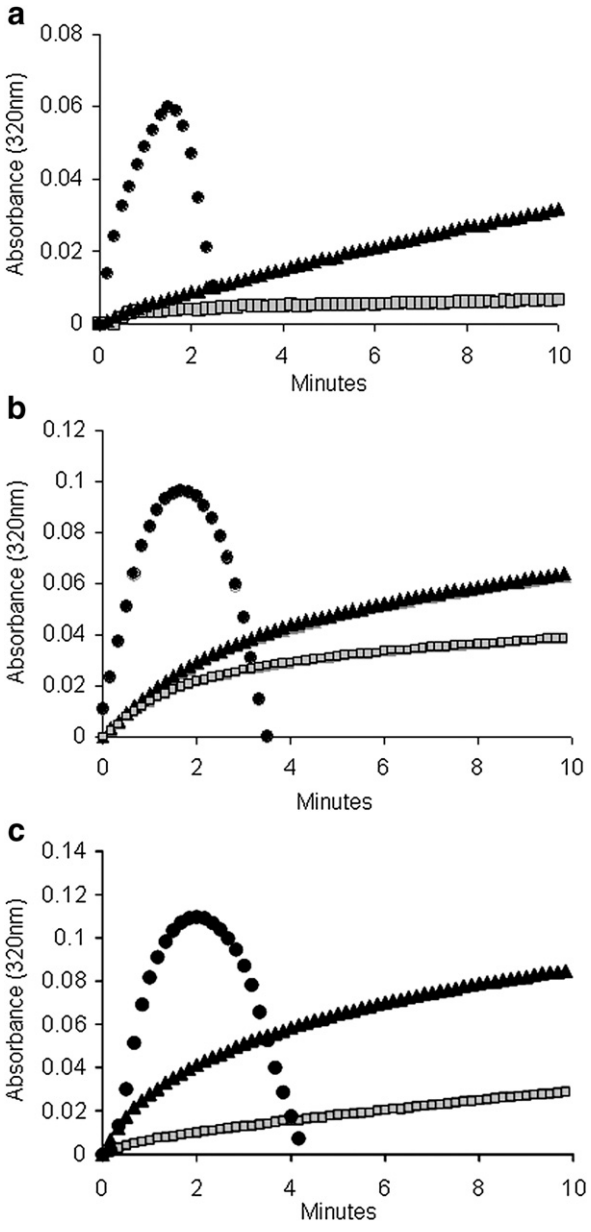

Fig. 2. Suppression of aggregation of guanidine-denatured (a) luciferase (0.2 μM) and (b) β-galactosidase (0.2 μM) and of (c) urea-denatured scTCR (0.28 μM). Luciferase and β-galactosidase were denatured in 6 M guanidine-HCl and diluted 100-fold into solutions containing 5 mM $MgCl_2$, 50 mM KCl, 1 mM ATP, 1 mM DTT, 25 mM HEPES, pH 7.4 and the indicated chaperones. ScTCR was denatured in 6 M urea and diluted 50-fold in the same buffer. Absorbances were measured at 320 nm with or without chaperone additions. Gray squares: DnaK + DnaJ (2 μM; 0.5 μM), black triangles: BiP + ERdj3 (2 μM; 0.5 μM), black circles: control (no chaperones). Representative figures are from triplicates. To normalize values, initial OD values were subtracted from each data point. The secondary decrease in absorbances for the controls resulted from large aggregates settling out of the solution.

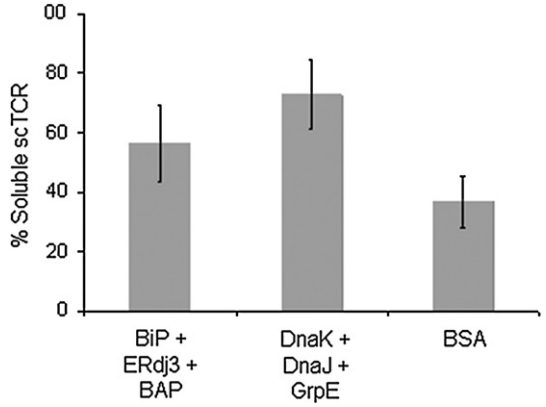

Fig. 3. Using chaperones to inhibit aggregation of urea-denatured scTCR. Error bars show standard deviation $n = 4$. Denatured scTCR was added at 0.28 μM into mixtures containing chaperones (2 μM + 0.5 μM + 0.5 μM) and incubated at room temperature for 15 min. The insoluble fraction was then removed by centrifugation. Scintillation counts of soluble and insoluble fractions were used to calculate the percentage of soluble scTCR. The BSA interacts non-specifically with the scTCR to prevent aggregation, although to a lesser extent than the chaperone systems.

(Fig. 4a), and percent luciferase activity recovery was determined over time.

The DnaK + DnaJ + GrpE system improved the reactivation of heat shocked luciferase as expected [21,22,30]. In contrast, BiP + ERdj3 + BAP was ineffective. It is interesting to note that the luciferase activity immediately after heat denaturation was significantly higher with the DnaK chaperone system. Thus, with this procedure it is difficult to separate the effects of chaperone protection during heat shock from assistance of folding. However, BiP + ERdj3 + BAP also failed to refold 8 M urea-denatured luciferase even though the DnaK + DnaJ + GrpE chaperone system improved the folding of urea-denatured luciferase producing about 10% active luciferase after 90 min (Fig. 4b). Similar results were obtained for β-galactosidase denatured with 6 M guanidine-HCl and diluted 125-fold to assess refolding (results not shown).

3.4. Folding assistance during cell-free protein expression

We then used cell-free protein synthesis to determine the folding benefits from the Hsp70 chaperone systems for several proteins in the context of translation and a more relevant chemical environment designed to mimic the cytoplasmic intracellular solution. The following two *E. coli*-based cell-free systems were chosen for these studies: the PANOx SP system [50] and the PURE system [52]. The PURE system is reconstituted from purified *E. coli* proteins, ribosomes, etc., and is devoid of chaperones intrinsic in the *E. coli* cytoplasmic extracts used in the PANOx SP system. Thus, the PURE system was chosen for comparison to the PANOx SP system in order to reveal the potential benefits of native *E. coli* chaperones on co-translational protein folding. The PURE system is also buffered with HEPES, which could affect protein folding, whereas the PANOx SP system does not contain synthetic buffers. Plasmids containing the gene of interest following a T7 promoter were mixed with either the PANOx SP or the PURE cell-free system and incubated at 37 °C for either 4 h or 1 h, respectively. Protein production was determined from scintillation counts of incorporated ^{14}C-leucine [50]. For all cases, Hsp70 chaperones (BiP or DnaK) were added at 8 μM with co-chaperones added at 0.5 μM each. The higher concentration of the Hsp70 chaperones was used to ensure that the effects seen were from exogenously added chaperones and not from DnaK present in the cell extracts (2–3 μM) [53]. The ratios used here also more accurately approximate *in vivo* ratios for Hsp70s and co-chaperones [48,53]. Fig. 5 shows that addition of chaperones to the cell-free protein synthesis reactions for either luciferase or β-galactosidase did not significantly increase soluble yields, percent solubility, or activity of the two model proteins compared to the no chaperone control (see Table 2 for control soluble yields).

Fig. 6 shows the results of cell-free expression of immunoglobulin domain proteins in the PURE system. Proteins derived from three secreted or membrane eukaryotic proteins: CD-19 extracellular domain (ECD), an anti-digoxin single chain antibody (scFv), and a single chain T-cell receptor (scTCR) were tested for soluble expression with and without Hsp70 chaperones (see Table 2 for control soluble yields). Each of these proteins contains immunoglobulin domains, which have been shown to interact with BiP *in vivo* [37,38]. Adding the BiP family (BiP + ERdj3 + BAP) of chaperones to the PURE system resulted in statistically significant increases in soluble protein yields. For example, an almost 2-fold increase in soluble scTCR was produced compared to the control. The DnaK chaperone system also increased soluble yields in the PURE system for all proteins tested except for the scTCR.

Fig. 7 shows the results of cell-free expression of immunoglobulin domain proteins in the PANOx SP cell-free protein synthesis system. Although results suggest improvements in soluble yields for each protein, statistically significant increases were observed only for scTCR. In this case, both the BiP family and the DnaK family were effective. However, the percent soluble protein (soluble protein/total protein) increased for each immunoglobulin domain protein,

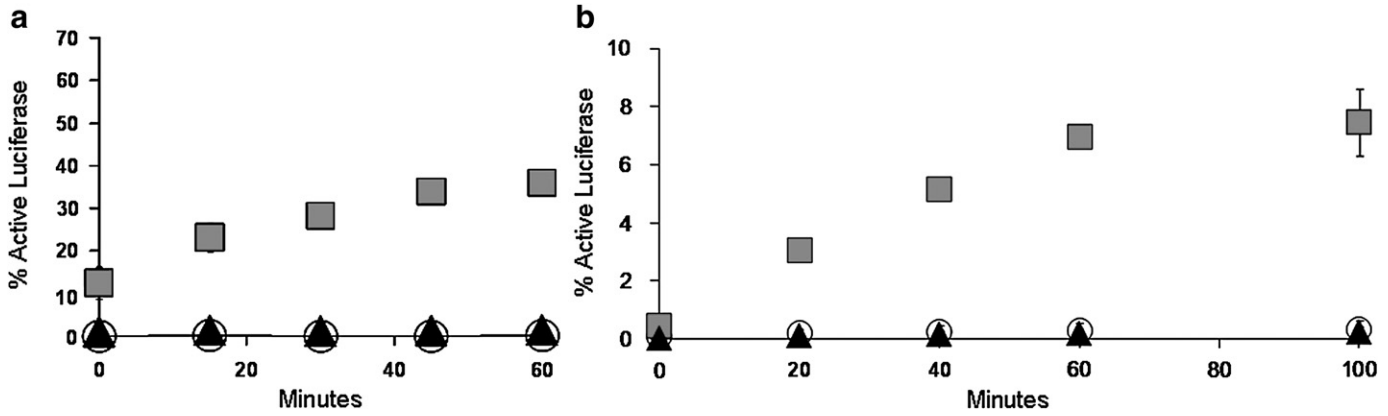

Fig. 4. Refolding of luciferase. (a) Refolding of heat denatured luciferase. Squares: 4 μM DnaK + 1 μM DnaJ + 1 μM GrpE, triangles: 4 μM BiP + 1 μM ERdj3 + 1 μM BAP, circles: 0.5 μM BSA control. (b) Refolding of urea-denatured luciferase. Squares: 3.5 μM DnaK + 0.7 μM DnaJ + 0.35 μM GrpE, triangles: 3.5 μM BiP + 0.7 μM ERdj3 + 0.35 μM BAP, circles: 3.5 μM BSA control. The % activity was calculated compared to that of the non-denatured samples at time 0. Error bars show standard deviation of $n = 3$.

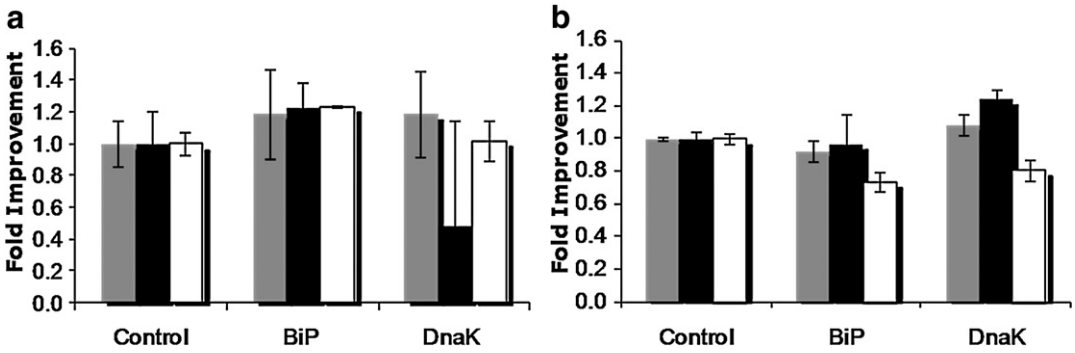

Fig. 5. Cell-free protein synthesis of model proteins. (a) Luciferase, $n=3$ and (b) β-galactosidase, $n=2$. Fold improvement is for PANOx SP cell-free yields compared to control (no chaperones present). In each case 8 μM of the Hsp70 was added (BiP or DnaK) along with 0.5 μM of each of the appropriate co-chaperones (ERdj3 + BAP or DnaJ + GrpE). Gray bars: improvement in soluble yield, black bars: improvement in % soluble, white bars: improvement in activity.

likely because the chaperones interacted with newly synthesized polypeptides to avoid protein aggregation and encourage proper folding.

4. Discussion

The prokaryotic cytoplasmic Hsp70 chaperone system (DnaK + DnaJ + GrpE) has been well characterized, but this is not the case for the eukaryotic ER Hsp70 chaperone system (BiP + ERdj1–5 + BAP). Only the ATPase activities of the ERs Hsp70 chaperone system have been measured (BiP, ERdj1–5, and BAP) [13–15,54–57], and little is known about its ability to prevent denatured protein aggregation or its ability to assist in refolding or *in situ* protein folding. In this report we characterize the BiP + ERdj3 + BAP ER Hsp70 chaperone system's ability to prevent protein aggregation, improve refolding of denatured proteins, and assist in the contemporaneous folding of several model proteins during protein synthesis.

Both BiP and DnaK bind transiently to exposed hydrophobic regions approximately seven amino acids in length. BiP, in particular, binds to regions where large hydrophobic amino acids occur in alternating positions [58]. Blond-Elguindi et al. [58] developed a computer algorithm to predict the interaction between BiP and any seven amino acid peptide. In order to increase the likelihood of BiP interaction with model substrates, we used this algorithm to choose only substrates that contained several hydrophobic regions with a high probability for BiP interaction. Eukaryotic cytoplasmic (firefly luciferase) and secreted (immunoglobulin domain proteins) substrates as well as a prokaryotic cytoplasmic protein (β-galactosidase) were selected based on this criteria.

Because some chaperones may only prevent aggregation rather than actively assisting with the refolding of aggregated proteins (see Gething and Sambrook [5] for review), the efficacy of BiP in preventing luciferase, β-galactosidase, and scTCR aggregation was determined. As shown in the aggregation prevention studies (Figs. 2 and 3), the BiP system does reduce the formation of large aggregates for β-galactosidase, luciferase, and scTCR, albeit less efficiently than the DnaK system. This aggregation prevention provided by BiP suggests that BiP can indeed interact with model substrates as predicted by the previously developed computer algorithm.

In contrast to the aggregation prevention result, we show that the BiP system does not improve refolding of heat or chemically denatured firefly luciferase or β-galactosidase. This is surprising considering the number of Hsp70 systems that have been shown to assist firefly luciferase [20–24] and β-galactosidase [33,34,59] folding. The DnaK system, on the other hand, improved refolding of both substrates after either heat or chemical denaturation. Most likely because DnaK + DnaJ could remain active during heat denaturation and therefore could interact with luciferase during unfolding, a larger percentage of the heat denatured luciferase was refolded (shown here and elsewhere [30]) compared to chemically denatured substrates. Taken together, these results suggest that the DnaK system can both mitigate denaturation and actively refold denatured proteins, consistent with its role in post-translational folding [28]. Alternatively, the BiP family may only be able to prevent off-pathway interactions and, therefore, be more effective during co-translational folding rather than post-translational folding.

We examined the effect of Hsp70 chaperones on nascent protein folding by utilizing cell-free protein synthesis. While the cell-free protein synthesis system provided a folding environment that is similar to the native *E. coli* cytoplasmic environment of DnaK, the native conditions in the ER where BiP resides are poorly understood. Nevertheless, after adjusting the oxidizing environment to encourage disulfide bond formation, we observed BiP folding assistance for several model proteins.

In the case of the co-translational folding of luciferase, previous reports conclude that chaperones are not necessary for luciferase production and folding in an *E. coli* system [26], and our results confirm this observation (Fig. 5). The yields of active β-galactosidase were also not increased with the addition of chaperones, possibly because the protein was expressed and folded at high yields without chaperones (Table 2), suggesting highly efficient folding mechanisms that render the addition of chaperones unnecessary. Because BiP is known to interact co-translationally with immunoglobulin domains *in vivo*, cell-free protein synthesis of several proteins that contain immunoglobulin domains (scTCR, scFv, CD-19 ECD) was also performed. The efficacy of both the BiP family and the DnaK family for folding assistance of proteins *in situ* was confirmed by an increased soluble production of these model proteins. These results suggest the ability of both the BiP and DnaK Hsp70 families to facilitate co-translational protein folding of these protein domains that are naturally co-translationally translocated through the ER membrane. Other work from our lab using BiP and DnaK fusions that have been localized to the ribosome exit tunnel (manuscript in preparation) also show yield increases for model substrates, further suggesting a co-translational folding mechanism.

Table 2
Cell-free protein synthesis soluble yields (μg/ml) for proteins produced without any chaperones present (controls).

Protein	CFPS system	Soluble yields (μg/mL)	±	% Soluble	±
Dig scFv	PURE	22	4	33	8
	PANOx SP	80	22	34	9
CD-19 ECD	PURE	23	0.4	41	4
	PANOx SP	61	17	14	7
scTCR	PURE	22	1.3	33	1.2
	PANOx SP	34	1.2	6	0.2
Luciferase	PANOx SP	140	20	15	3
β-gal	PANOx SP	470	7	56	2.6

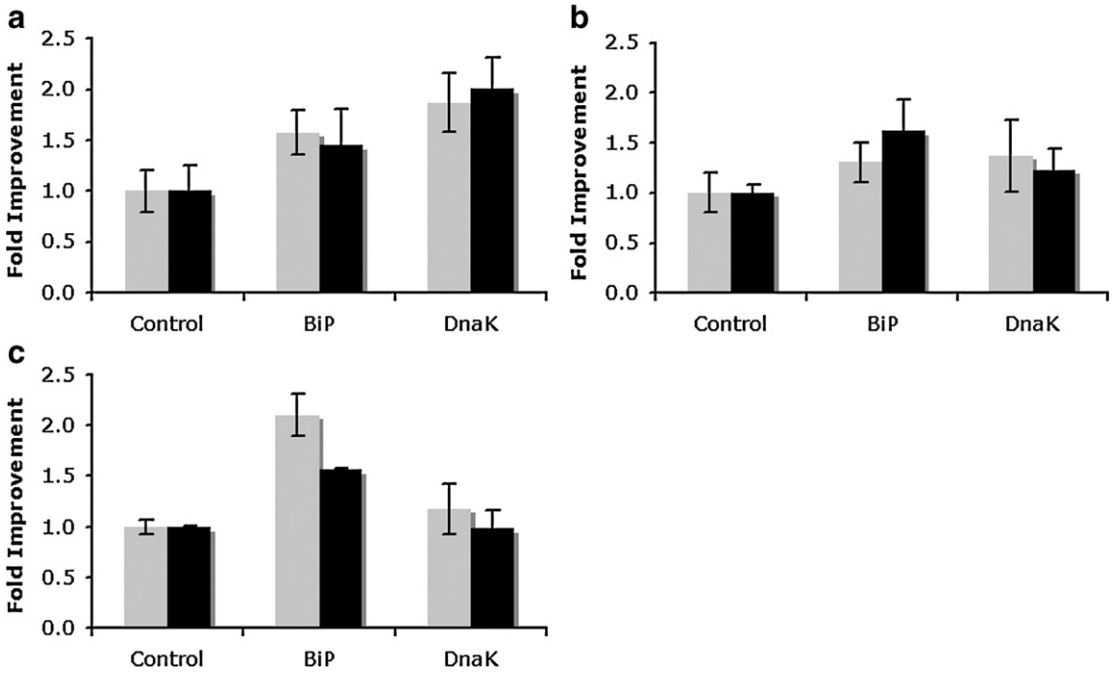

Fig. 6. Cell-free protein synthesis of immunoglobulin domain containing proteins in the PURE classic II system. Gray bars indicate fold improvement for soluble yields and black bars for percent of total that is soluble. (a) scFv, $n=2$, for the soluble yields compared to the control $p=0.04$ for BiP and 0.05 for DnaK. (b) CD-19 ECD, $n=4$, for the soluble yields compared to the control $p=0.02$ for BiP and 0.06 for DnaK. (c) scTCR, $n=2$, for the soluble yields compared to the control $p=0.02$ for BiP and 0.3 for DnaK. In each case 8 μM of the Hsp70 was present (BiP or DnaK) along with 0.5 μM of each of their appropriate co-chaperones (ERdj3 + BAP or DnaJ + GrpE). See Table 2 for control (no chaperone present) cell-free protein synthesis yields. *P*-values were determined using a one tailed Student's *t*-test based on fold improvement of protein yields with chaperones compared to the control (no chaperones).

The exogenous addition of these chaperones to cell-free protein synthesis reactions could help increase the production of complex eukaryotic targets. *E. coli*-based cell-free protein synthesis technology has advanced in recent years to offer a scalable protein production technology [60,61] for many targets, including non-native proteins containing disulfide bonds [62,63]. However, producing multi-domain, secreted eukaryotic proteins at high yields has remained a challenge. Addition of the BiP family of chaperones to cell-free

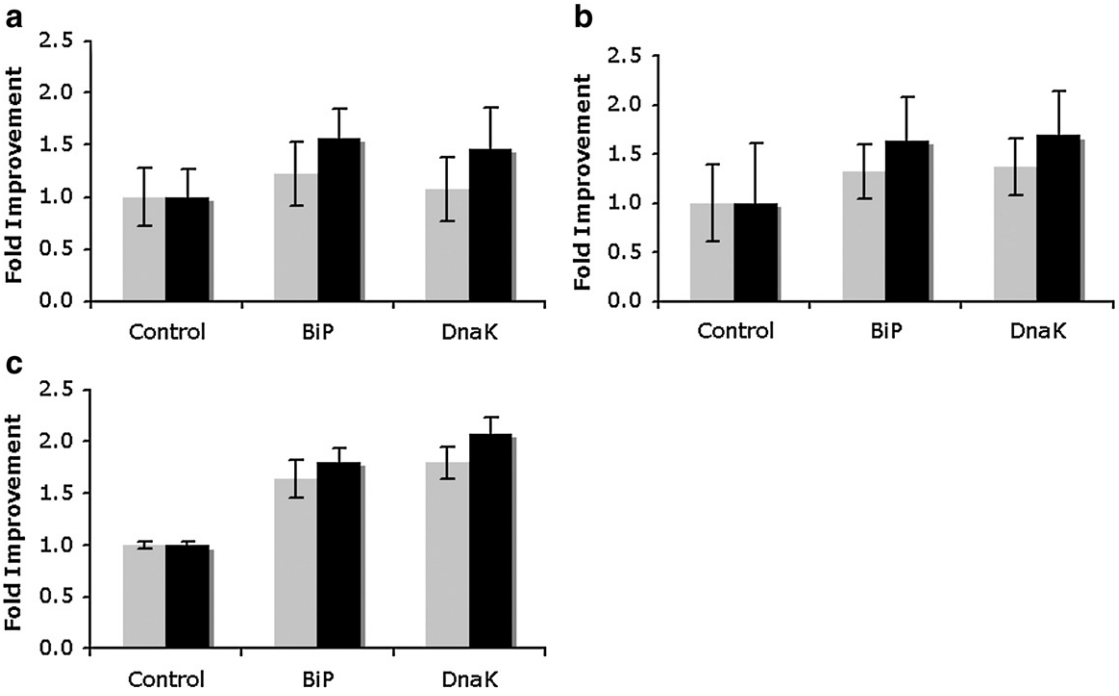

Fig. 7. Cell-free protein synthesis of immunoglobulin domain containing proteins in PANOx SP reactions. Gray bars indicate fold improvement for soluble yields and black bars for percent of total that is soluble. (a) scFv, $n=3$, for the soluble yields compared to the control $p=0.15$ for BiP and 0.34 for DnaK. (b) CD-19 ECD, $n=2$, for the soluble yields compared to the control $p=0.12$ for BiP and 0.11 for DnaK. (c) scTCR, $n=2$, for the soluble yields compared to the control $p=0.04$ for BiP and 0.03 for DnaK. In each case, 8 μM of the Hsp70 was added (BiP or DnaK) along with 0.5 μM of each of their appropriate co-chaperones (ERdj3 + BAP or DnaJ + GrpE). See Table 2 for control (no chaperone present) cell-free protein synthesis yields. *P*-values were determined using a one tailed Student's *t*-test based on fold improvement of protein yields with chaperones compared to the control (no chaperones).

reactions helps with the production of these complex targets, and the open nature of the cell-free system allows for facile addition of these chaperones to the folding environment. This open system provides a convenient means for optimizing chaperone assistance to improve the production of these valuable eukaryotic proteins.

Supporting previous reports [28], our results suggest that the prokaryotic cytoplasmic DnaK Hsp70 chaperone system can participate in both post-translational and co-translational protein folding mechanisms. In agreement with a model for predominately co-translational folding in the ER of eukaryotes [2,25,35], however, the BiP Hsp70 chaperone system was only observed to be effective for protein folding that was contemporaneous with translation.

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