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Accelerated Article

Molecular Chaperones, Folding Catalysts, and the Recovery of Active Recombinant Proteins from *E. coli*

To Fold or to Refold

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ABSTRACT

The high-level expression of recombinant gene products in the gram-negative bacterium *Escherichia coli* often results in the misfolding of the protein of interest and its subsequent degradation by cellular proteases or its deposition into biologically inactive aggregates known as inclusion bodies. It has recently become clear that in vivo protein folding is an energy-dependent process mediated by two classes of folding modulators. Molecular chaperones, such as the DnaK-DnaJ-GrpE and GroEL-GroES systems, suppress off-pathway aggregation reactions and facilitate proper folding through ATP-coordinated cycles of binding and release of folding intermediates. On the other hand, folding catalysts (foldases) accelerate rate-limiting steps along the protein folding pathway such as the *cis/trans* isomerization of peptidyl-prolyl bonds and the formation and reshuffling of disulfide bridges. Manipulating the cytoplasmic folding environment by increasing the intracellular concentration of all or specific folding modulators, or by inactivating genes encoding these proteins, holds great promise in facilitating the production and purification of heterologous proteins. Purified folding modulators and artificial systems that mimic their mode of action have also proven useful in improving the in vitro refolding yields of chemically denatured polypeptides. This review examines the usefulness and limitations of molecular chaperones and folding catalysts in both in vivo and in vitro folding processes.

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Index Entries: Protein folding; protein refolding; protein aggregation; inclusion body; heat-shock protein; DnaK; GroEL; foldase; protein disulfide isomerase; peptidyl-prolyl *cis/trans* isomerase.

INTRODUCTION

The gram-negative bacterium *Escherichia coli (E. Coli)* is a popular choice for the production of recombinant proteins of commercial or therapeutic value for a number of reasons: it grows to high cell densities on inexpensive substrates, its genetics have been heavily researched and are generally well understood, and a large number of cloning vectors engineered to achieve the high-level synthesis and facilitate the purification of recombinant gene products are commercially available. In 1993 alone, almost $5 billion of therapeutic protein products were produced in *E. coli*, including insulin, human growth hormone, α- β-, and γ-interferons, interleukin-2, and granulocyte colony-stimulating factor *(1)*. In general, however, the production of recombinant proteins is not well tolerated by bacteria and it is common for overexpressed polypeptides to be proteolytically degraded or to accumulate within the cell as biologically inactive aggregates known as inclusion bodies. Not surprisingly, extensive research has been directed at improving the production of full-length and soluble heterologous proteins in vivo, and at devising new approaches to optimize the refolding of aggregated polypetides in vitro *(2–6)*. Although *E. coli* is unable to perform certain kinds of post-translational modifications (e.g., glycosylation), which may be required for the full activation of some eukaryotic proteins of interest, it may synthesize complex proteins more efficiently than eukaryotic cells in some cases. For example, while the production of the 160 kDa rat neuronal nitric oxide synthase met with limited success in human kidney cells or in the baculovirus system, large amounts of soluble and active enzyme containing proper protoporphyrin IX heme, FAD, FMN, and BH_4 prosthetic groups were recovered from genetically engineered *E. coli* cells grown in optimized medium *(7)*.

Although the formation of inclusion bodies was first observed in *E. coli (8)*, it has since been documented in a variety of host organisms, including yeast, insect, and mammalian cells *(9,10)*. To date, the precise mechanisms responsible for protein misfolding and aggregation in the cellular environment remain obscure. Statistical attempts to correlate the tendency of a protein to aggregate with its intrinsic characteristics, including size, proline fraction, and hydrophobicity have been largely unsuccessful *(11)*. It was however reported that the charge and fraction of turn-forming residues correlate more strongly with the propensity of a protein to aggregate *(11)*. Several lines of evidence suggest that inclusion bodies arise from the interaction of exposed hydrophobic domains and elements of secondary structure present in partially folded intermediates. This hypothesis

is supported by both theoretical arguments *(12,13)*, and the observations that interleukin-1β *(14)*, and β-lactamase *(15)* inclusion bodies contain a significant amount of secondary structure.

Because inclusion bodies are composed mainly of the desired product and can be separated from other cellular components by differential centrifugation, the production of heterologous proteins in an aggregated form may be desirable from a purification standpoint *(6,9)*. The formation of inclusion bodies is also advantageous in the case of proteolytically unstable gene products or when the protein of interest is toxic to the cell. However, the recovery of biologically active protein requires that the insoluble material be solubilized by chemical treatment and refolded in vitro. Unfortunately, refolding is often a low-yield and expensive operation that may not be readily amenable to scale-up. Thus, while targeted inclusion body formation may be valuable, being able to produce overexpressed proteins in a correctly folded form in vivo remains a critical concern.

It is now clear that, although denatured proteins can reach biologically active conformation by relying solely on the information contained in their amino acid sequence, in vivo folding is an energy-dependent process that is assisted by molecular chaperones and folding catalysts (foldases) (reviewed in *16–23*). An important difference between these two types of folding modulators is that while folding catalysts accelerate rate-limiting steps along the folding pathway (e.g., the isomerization of peptidyl-prolyl bonds and the formation and reshuffling of disulfide bridges), molecular chaperones facilitate proper folding by binding partially folded proteins and maintaining them in a soluble or translocation-competent conformation. Through this process, molecular chaperones reduce the likelihood that two or more folding intermediates associate with each other to give rise to unproductive aggregation reactions. A number of reports have demonstrated that the co-overexpression of folding modulators can improve the soluble recovery yields of many, but not all, aggregation-prone recombinant polypeptides. In addition, both molecular chaperones and folding catalysts show great promise in improving the in vitro refolding of proteins recovered in an inclusion body form. This review examines the usefulness of folding modulators in enhancing recombinant protein folding in vivo and in vitro. A description of "artificial chaperone" systems and of the relevant patent literature is also included.

IMPROVING RECOMBINANT PROTEIN FOLDING IN VIVO

Because the refolding of recombinant proteins from the aggregated state is a difficult operation that must be optimized on a case-by-case basis, a significant amount of effort has been directed at improving the production of aggregation-prone polypeptides in a soluble form in the cellular environment. Three approaches that have been employed to

achieve this goal, including: fermentation engineering; engineering of the amino acid sequence (and thus of the folding pathway) of the overexpressed polypeptide; and manipulation of the intracellular concentration of folding modulators.

Fermentation Engineering

While the proper folding of small, monomeric proteins that have been denatured by chemical treatment is a first-order isomerization process, aggregation reactions between two or more partially folded species follow second or higher order kinetics. As a result, in vitro refolding is typically performed at low protein concentrations to favor correct folding over aggregation. Since the cell cytoplasm is a highly concentrated environment that contains a variety of folding modulators able to interact with nascent and partially folded proteins (*see* below), the series of events leading to proper folding in vivo and in vitro are likely to differ considerably. Nevertheless, extensive experimental evidence indicates that approaches resulting in a net reduction in the concentration of newly synthesized proteins can significantly increase the solubility of recombinant gene products. Two simple and highly effective techniques leading to a decrease in the transcription and/or translation of overproduced proteins consists in using suboptimal levels of gratuitous inducers for those gene products placed under the control of chemically regulated promoters, or in decreasing the fermentation temperature (reviewed in *2,3,9*). The main drawbacks associated with the use of these methods are longer fermentation times and lower accumulation levels of the desired gene product. More importantly, they do not always guarantee an increase in solubility. Other fermentation parameters including the pH and composition of the growth medium, the level of aeration and choice of host strain have all been shown to influence the process of inclusion body formation *(2,3,9)*. However, the increase in solubility obtained by manipulating these variables is generally modest.

Genetic Engineering

Since inclusion body formation is thought to arise from the association of folding intermediates that are kinetically trapped off-pathway *(12,13)*, mutations affecting the stability of these species would also be expected to alter the tendency of a protein to aggregate. In agreement with this hypothesis, certain genetically engineered mutants of the bacteriophage P22 tailspike protein *(24)*, human interferon-γ *(25)*, human interleukin-1β *(26,27)*, and S1 dihydrofolate reductase *(28)* have been shown to exhibit dramatically improved solubility in vivo and in vitro. Unfortunately, the judicious use of protein engineering approaches often requires an *a priori* knowledge of the crystal structure and/or folding pathway of the target protein. Even under these conditions, the effect of specific mutations on folding is very

difficult to predict and amino acid substitutions in the primary sequence are more likely to promote aggregation rather than suppress it. Finally, it is important to bear in mind that genetic engineering inherently results in the production of a mutated gene product.

An alternate protein engineering approach consists in fusing the target gene to a so-called "solubilizing" partner (reviewed in *29*). Several fusion partners that can be cleaved from the desired product by sequence-specific peptidases are available commercially. They include thioredoxin (Invitrogen), glutathione *S*-transferase (Pharmacia), *S. aureus* protein A (Pharmacia), and maltose-binding protein (New England Biolabs). The usefulness of the fusion approach was recently highlighted by the demonstration that 11 different mammalian cytokines that aggregate in the cytoplasm of *E. coli* could be produced at high level in an active and soluble form when fused to thioredoxin *(30)*. While the precise mechanisms leading to a solubility increase remain unclear, fusion partners do not always suppress inclusion body formation and the hybrid proteins may be more difficult to express than the unfused polypeptides. With the discovery that in vivo protein folding can be facilitated by co-overexpressing molecular chaperones and folding catalysts, the manipulation of the intracellular concentration of folding modulators has become an increasingly popular method to enhance the solubility of overexpressed proteins *(2,4,31,32)*.

Molecular Chaperones and the Heat-Shock Response

The best characterized folding modulators in the cytoplasm of *E. coli* are the DnaK-DnaJ-GrpE and GroEL-GroES molecular chaperone systems. Both sets of chaperones belong to the σ^{32} heat-shock regulon, a group of 20–30 molecular chaperones, proteases, and other heat-shock proteins (hsps) that are present at a relatively abundant levels under normal growth conditions. The transcription of hsps becomes transiently upregulated when the cells are subjected to temperature increase or other forms of stress *(33)*, including the overproduction of recombinant proteins *(34–36)*. *Escherichia coli* maintains a precise control on hsp synthesis through an intricate series of positive and negative control loops affecting the heat-shock transcription factor σ^{32} (reviewed in *37*). The intracellular concentration of this alternative σ factor that directs the RNA polymerase core enzyme to heat-shock promoters is tightly regulated at the transcriptional level by four different promoters, and at the translational level by temperature-dependent changes in the σ^{32} mRNA secondary structure. In addition, the DnaK-DnaJ-GrpE hsps themselves negatively regulate the heat-shock response by sequestering native σ^{32} and presenting it to the heat-shock protease HflB (a.k.a. FtsH) *(38)*. It has been argued that when confronted by an insult that increases the concentration of misfolded and

otherwise damaged proteins in the cell, DnaK-DnaJ-GrpE preferentially bind to the non-native polypeptides, thereby allowing σ^{32} to direct the high-level transcription of hsps *(39)*. As the effect of stress is abated through the repair of misfolded polypeptides by molecular chaperones and the degradation of irreversibly damaged proteins by heat-shock proteases, the DnaK-DnaJ-GrpE system is once again able to bind and sequester σ^{32}, thus ending the sustained transcription of hsps.

Extensive research in a number of laboratories indicates that DnaK-DnaJ-GrpE and GroEL-GroES form a cooperative network that mediates the folding of many proteins, as illustrated in Fig. 1. Evidence for a general role of these molecular chaperones in cellular protein folding comes from the observation that the "wholesale" aggregation of host proteins in a σ^{32} mutant at 42°C is suppressed upon overexpression of either the *dnaKJ* or *groE* operon *(40)*. The same study highlighted the cooperative nature of the two chaperone machines by demonstrating that host protein aggregation in the mutant strain was also suppressed when DnaK-DnaJ-GrpE and GroEL-GroES were expressed together at physiological levels *(40)*. In vitro studies have established that while certain proteins must interact first with the DnaK-DnaJ-GrpE chaperones, and subsequently with the GroEL-GroES system to reach an active conformation *(41,42)*, others can shuttle between the two chaperone machines until properly folded *(43)*. The existence of a cascade-like folding pathway in vivo is supported by the observation that mutations in any one of the *dnaK, dnaJ, grpE, groEL,* or *groES* genes block the reactivation of certain newly synthesized and heat-denatured proteins in vivo *(44,45)*. However, it is also clear that other proteins require only DnaK-DnaJ-GrpE, but not GroEL-GroES, to reach a native conformation both in vivo *(46)* and in vitro *(47)*. Although several eukaryotic hsps (e.g., Hsp90, Hsp100s, and small Hsps) are known to act as molecular chaperones in vitro and in vivo *(22)*, little is known about the function of their respective *E. coli* homologs in cellular and recombinant protein folding.

Despite the fact that the heat-shock promoters of the *dnaKJ* and *groE* operons are among the strongest of all known *E. coli* promoters, it is not difficult to imagine that the high-level production of recombinant proteins could titrate the cellular supply of chromosomally encoded molecular chaperones and lead to unproductive aggregation reactions between improperly chaperoned folding intermediates *(46,48)*. Under these conditions, an increase in the intracellular concentration of molecular chaperones that are limiting for folding would be expected to lessen inclusion body formation.

Molecular Chaperones and Recombinant Protein Folding in the Cytoplasm

Goloubinoff et al. *(49)* first demonstrated that chaperone co-overexpression was a powerful tool to improve the folding of heterologous

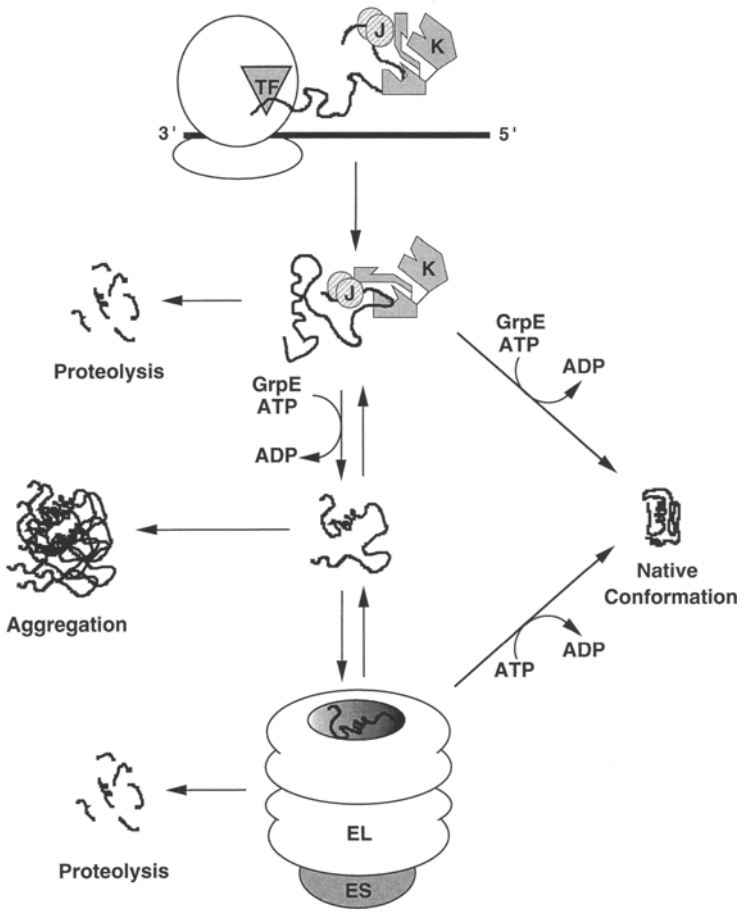

Fig. 1. Possible model for chaperone-assisted protein folding in *E. coli.* Both DnaK and GroEL bind and release protein substrates in an ATP-dependent manner that is coordinated by interactions with the DnaJ-GrpE or GroES cofactors, respectively. DnaJ (J) binds to nascent polypeptides emerging from the ribosome, and targets newly synthesized protein chains for recognition by DnaK (K). DnaK-DnaJ shield exposed hydrophobic stretches in the polypeptide chain, thus preventing unproductive interactions with other folding intermediates and cellular components. Binding of the GrpE nucleotide exchange factor to DnaK catalyzes the release of the bound protein substrate which may: fold into the native conformation; undergo additional cycles of DnaK-DnaJ-GrpE binding and release; be transferred to the GroEL-GroES chaperonin system (EL and ES, respectively); or misfold and aggregate. The GroE chaperonins are believed to facilitate the productive isomerization of proteins that have already reached a compact intermediate conformation. If the protein discharged from the GroE complex is unable to fold into its native conformation, it may undergo another cycle of interaction with either chaperone system or aggregate. If proper folding cannot be achieved after several cycles of chaperone interaction, DnaK-DnaJ-GrpE or GroEL-GroES may present their substrates to the cellular protease machinery. Recently it was shown that trigger factor (TF), a protein that binds to ribosomes and nascent polypeptide chains possesses a peptidyl-prolyl-*cis/trans* isomerase activity (*107*). The possibility that trigger factor acts as a general cotranslational folding catalyst and stabilizes nascent proteins before their transfer to chaperones remains to be examined.

Table 1
Effects of Molecular Chaperone Co-Overexpression on the Production of Recombinant Proteins in the Cytoplasm of *Escherichia coli*

Recombinant protein	Chaperone(s) co-overexpressed and result	Reference
Ribulose bisphosphate carboxylase (Rubisco)	GroEL-GroES increase the yields of active *A. nidulans* L_8S_8 Rubisco 10-fold, although GroEL alone has no effect; GroEL-GroES from *C. vinosum* also facilitate folding of phototrophic bacterial Rubisco in *E. coli*	*(49,162)*
Human growth hormone (hGH)	DnaK improves solubility 1.5–5 fold depending on hGH synthesis level; average number of inclusion bodies increases from 1 to 5 per cell, but inclusion body size is reduced; GroEL-GroES have no effect on hGH solubility	*(10)*
Plant ferredoxin-$NADE^+$ oxidoreductase	GroEL-GroES from *C. vinosum* partially restores activity in *E. coli groEL* and *groES* mutants	*(163)*
Bacterial luciferases	GroEL-GroES improve the accumulation and yield of active *V. harveyi* MAV heterodimers and monomeric fusions, but GroEL alone is detrimental to activity recovery; GroEL-GroES or GroEL have little effect on the yields of active heterodimers and monomeric fusions derived from *V. harveyi* CTP5	*(164)*
Human procollagenase	Both DnaK and GroEL-GroES improve solubility and proteolytic stability, resulting in a 10-fold increase in intracellular accummulation levels	*(50)*
Mitochondrial branched-chain α-keto acid decarboxylase (E1)	GroEL-GroES lead to a 500-fold increase in E1α subunit activity for a maltose-binding protein-E1α fusion produced in a *groES* mutant; GroEL alone is ineffective; GroEL-GroES also improve solubility of E1β subunits and facilitate the folding of certain His-tagged E1α mutants	*(165,166)*
Xenopus laevis and human Cu, Zn superoxide dismutases	GroEL-GroES increase the yield of active enzyme 2–3 fold over a wide range of temperatures (30–42°C) by preventing degradation of metal-deficient superoxide dismutase molecules	*(51)*

Medium-chain acyl-CoA dehydrogenase (MCAD)	GroEL-GroES have little effect on wild-type MCAD, which is produced in a soluble form, but greatly improve the yields of active K304Q, R28C, and other MCAD mutants; GroEL-GroES improve solubility, but not oligomerization or activity of a K304E mutant	*(167,168)*
Bovine pyruvate dehydrogenase phosphatase (PDPc)	GroEL-GroES improve production level and solubility of wild-type enzyme, but have little effect on the aggregation of PDPc fusions to glutathione *S*-transferase or maltose-binding protein	*(169)*
Human non-receptor tyrosine protein kinase	DnaK-DnaJ-GrpE suppress the aggregation of Csk, Fyn and Lck kinases, but decrease overall accumulation; GroEL-GroES improve the solubility of Csk, but have only a slight effect on solubility of Fyn and Lck	*(53,119,170)*
S. aureus type 1 dihydrofolate reductase (DHFR)	3–4 fold increase in yield of active enzyme with GroEL-GroES or GroEL. Improvement in solubility is accompanied by a decrease in intracellular accumulation	*(52)*
Single-chain Fv antibody fragments	Two fold increase in the intracellular accumulation of soluble—but inactive—scFv fragments with GroEL-GroES	*(171)*
Phage 22 coat and tailspike structural protein mutants	GroEL-GroES, but not GroEL or GroES alone, facilitate the folding of 17 coat protein mutants having thermolabile folding intermediates; GroEL-GroES do not suppress the misfolding of mutant tailspike proteins (all experiments were performed in *Salmonella typhimurium*)	*(172)*
E. coli glutamate racemase	GroEL-GroES lead to a 2–4 fold increase in the yields of active enzyme by reducing aggregation	*(173)*
Branched-chain α-keto acid dehydrogenase kinase	GroEL-GroES result in a large solubility increase of a fusion to maltose-binding protein	*(63)*
Rat neuronal nitric oxide synthase (nNOS)	GroEL-GroES increase the yield of active enzyme from undetectable levels to 20–24 mg/L of culture	*(7)*

(continued)

Table 1 *(Continued)*

Recombinant Protein	Chaperone(s) co-overexpressed and result	Reference
Xenopus mos proto-oncogene product (Ser/Thr kinase)	GroEL-GroES lead to a significant increase in solubility	*(119)*
Human SPARC	DnaK-DnaJ significantly suppress aggregation but result in lower accumulation levels; GroEL-GroES have little effect on solubility	*(54)*
PreS2-S′-β-galactosidase	DnaK-DnaJ increase the yields of active enzyme 3–6-fold over a wide temperature range (30–42°C); 1.5 fold increase in activity with GroEL-GroES at 30°C only	*(48)*
Human cAMP response element binding protein 1	Slight increase in solubility with GroEL-GroES	*(119)*
Human p53 tumor suppressor gene product	Slight increase in solubility with GroEL-GroES	*(119)*
Mouse *myb* proto-oncogene product (c-Myb)	Protein is completely insoluble in control cells, but about 10% is soluble with GroEL-GroES	*(119)*
β-galactosidase-bovine somatotropin fusion protein	Neither DnaK nor GroEL-GroES have a significant effect on solubility or accumulation	*(50)*
Chloramphenicol acetyltransferase	Neither DnaK nor GroEL-GroES have a significant effect on solubility or accumulation	*(50)*
Yeast *N*-myristoyl transferase	Neither DnaK nor GroEL-GroES have a significant effect on solubility or accumulation	*(50)*
Adenovirus oncogene product E1A	GroEL-GroES have no effect on solubility	*(119)*
myc proto oncogene product (Myc)	GroEL-GroES have no effect on solubility	*(119)*
ski-related gene product SnoN	GroEL-GroES have no effect on solubility	*(119)*

proteins by showing that the yield of active *A. nidulans* Rubisco heteroligomers was increased 10-fold in cells harboring a plasmid encoding the *groE* operon. Subsequently, a number of laboratories have investigated the influence of co-overproducing the GroE chaperonins or components of the DnaK-DnaJ-GrpE system on the recovery yields of other aggregation-prone proteins expressed at high level in the cytoplasm of *E. coli*. Table 1 shows that while this approach has been very successful for a large number of unrelated substrates, there is no guarantee that chaperone co-overproduction will improve the folding of a recombinant protein. The beneficial effect of higher intracellular concentrations of molecular chaperones can generally be explained by facilitated folding of newly synthesized protein chains *(48)*, which leads to an increase in the ratio of soluble to aggregated material without affecting steady-state accumulation levels. However, in the case of human procollagenase *(50)* and superoxide dismutase *(51)*, the improvement in yield was traced to a reduction in proteolysis. Thus, a net increase in accumulation levels was observed in the chaperone-overproducing cells. For reasons that remain unclear, chaperone-mediated improvements in the yield of soluble and active recombinant proteins may also occur at the expense of their overall production. For instance, in the case of S1 dihydrofolate reductase (DHFR), co-overexpression of the GroE chaperonins led to a threefold decline in the steady-state levels of the protein. Nevertheless, the net amount of soluble and active DHFR was 3–4-fold higher because of a 10-fold increase in solubility *(52)*. Lower accumulation levels of overexpressed proteins have also been observed in cells overexpressing the *dnaKJ* operon *(53,54)*.

While a large number of studies have examined the effect of GroEL-GroES overproduction on the solubility of aggregation-prone recombinant proteins, much less attention has been paid to the DnaK-DnaJ-GrpE system (Table 1). Yet, the latter set of proteins may be more useful than the GroE chaperonins in suppressing aggregation based on the following observations. First, both DnaK and DnaJ have been shown to interact with nascent polypeptides *(55,56)* and are suspected to play an important role in early folding events. Thus, if DnaK and DnaJ become limiting, newly synthesized proteins may aggregate before they are able to reach a conformation recognized by GroEL. Second, the GroEL-GroES system is not required for the proper folding of all host and recombinant proteins in *E. coli (46,57,58)* and it has been argued that proteins larger than ~60 kDa may not be able to completely fit within the central cavity of GroEL and undergo productive GroES-dependent folding *(59,60)*. In contrast, DnaK and DnaJ could potentially recognize all newly synthesized proteins. Indeed, whereas overproduction of the GroE chaperonins had little effect on the folding of preS2-S'-β-galactosidase *(48)*, human SPARC *(54)*, and three human tyrosine kinases *(53)*, co-overexpression of the *dnaKJ* operon efficiently suppressed the aggregation of these polypeptides. The overpro-

duction of either DnaK or DnaJ alone may lead to little or no improvement in the folding and secretion of host and recombinant proteins (*38,44,59*, unpublished data). Furthermore, overexpression of DnaK in the absence of DnaJ may lead to plasmid instability, defective filamentation, and ultimately result in cell death *(61)*. It is therefore recommended that DnaK and DnaJ be overproduced simultaneously. Increasing the intracellular levels of GrpE may be less essential because of the catalytic manner in which this protein resolves DnaK·DnaJ·polypeptide complexes *(62)*. It is finally important to bear in mind that co-overexpression of the *dnaKJ* operon downregulates the production of other hsps *(48)*. Although this phenotype may be advantageous in the case of proteolytically-sensitive gene products because of reduction in the synthesis rates of heat-shock proteases (e.g., Lon and ClpP), it may also be detrimental to the correct folding of recombinant proteins that requires not only DnaK-DnaJ-GrpE, but also GroEL-GroES or other hsps, to reach a native conformation.

Although it is reasonable to assume that overproduced chaperones exert their beneficial effect by directly binding to aggregation-prone folding intermediates and mediating their proper folding, their effect may also be indirect. For instance, overproduction of the *groE* operon could reduce the amount of DnaK and DnaJ necessary for the folding of host proteins, and thus free some of these molecules to support the correct folding of recombinant gene products. The observation that overexpression of the *groE* operon improves the solubility of proteins that should be too large to completely fit within the central binding site of GroEL *(7,63,64)* lends some support to this hypothesis. However, it is not yet clear whether the GroE system has an upper size limit in vivo.

Since overproduction of either the DnaK-DnaJ-GrpE or the GroEL-GroES chaperone machines does not enhance the correct folding of all recombinant proteins, there is a pressing need to determine whether the overexpression of other hsps suspected (or known) to exhibit a chaperone function will improve the recovery of recombinant gene products in a soluble form. For example, high levels of the *E. coli* Hsp90 homolog HtpG suppress certain defects in the translocation process *(65,66)*, suggesting that HtpG may facilitate the secretion of aggregation-prone precursor proteins. The *E. coli* Hsp100 family members ClpB and ClpX, and their non-hsp homolog ClpA, are known to play a role in ATP-dependent proteolytic events in the cell *(67,68)*. However, recent evidence suggest that the Clp ATPases can also function as molecular chaperones in a manner similar to DnaK-DnaJ in vivo and in vitro *(68–70)*. Thus, co-overexpression of the Clp proteins may facilitate the folding of overexpressed proteins that interact strongly with DnaK-DnaJ, without leading to the downregulation of GroEL-GroES and other hsps *(48)*. Finally, the *E. coli* small hsps IbpA and IbpB have been found in association with aggregated host and recombinant proteins *(71,72)*. To date, their influence on inclusion body formation

has not been reported. The availability of an arsenal of plasmid-encoded molecular chaperones will undoubtedly be of great help in fighting inclusion body formation in the cytoplasm of *E. coli*.

Molecular Chaperones and Secretion

The production of recombinant gene products in the periplasm of *E. coli* has several advantages over cytoplasmic production *(3,31,73)*. First, the periplasmic space is a more oxidizing compartment than the cytoplasm and is conducive to the formation of disulfide bonds due to the presence of the Dsb family of oxidoreductases and disulfide isomerases *(17,73)*. Second, if the signal sequence is correctly processed, a protein with a correct amino-terminal sequence will be recovered. Third, secretion into the periplasm allows the production of proteins that would be toxic if synthesized in the cytoplasm. Finally, since the periplasm only contains 4% of the total cellular protein *(74)*, the purification of the target polypeptide may be greatly facilitated.

Since secreted proteins must be maintained in a soluble and partially folded conformation to be efficiently engaged by the secretion machinery *(75)*, the finding that the molecular chaperones SecB, DnaK-DnaJ, and GroEL-GroES were involved in the translocation process was not entirely surprising. SecB, which is not a hsp, appears to be a specialized secretory chaperone that binds to a small but significant number of precursor proteins *(76)*. The DnaK-DnaJ-GrpE and GroEL-GroES systems have also been found to associate with precursor proteins and may play an important role in the secretion of SecB-independent proteins *(77–79)*. As illustrated in Table 2, co-overexpression of the above chaperones has a positive influence on the secretion of several recombinant proteins in *E. coli*. However, the usefulness of this approach is highly dependent on the nature of both the secreted protein and the signal sequence, as clearly demonstrated by Bergès et al. *(80)*. Furthermore, an increase in the intracellular concentration of molecular chaperones may actually be detrimental to the secretion process of some target proteins *(50,80)*.

The aggregation of precursor proteins within the cell cytoplasm is not an indication that higher concentrations of cytoplasmic molecular chaperones will improve the yield of mature product. For example, although both DnaK-DnaJ-GrpE *(79)* and GroEL-GroES *(77)* are involved in maintaining pre-β-lactamase in a translocation competent form, their overproduction had little effect on the aggregation of the overexpressed precursor *(81)*. Since substitution of the native β-lactamase signal sequence for that of OmpA alleviates precursor aggregation *(82)*, rate-limiting steps in the secretion process are likely to be responsible for the formation of pre-β-lactamase inclusion bodies. In agreement with this view, overproduction of SecE and SecY improves the translocation and processing of human interleukin 6 fused to the OmpA signal sequence 10-fold *(83)*, and overexpres-

Table 2
Effects of Molecular Chaperone Overexpression on the Secretion of Recombinant Proteins in *Escherichia coli*

Recombinant protein	SS[a]	Chaperone(s) overexpressed and result	Reference
LamB-LacZ(42-1) fusion protein	LamB	DnaK increases the amount of secreted material 40-fold; GroEL-GroES or GroEL alone lead to a 2.5-fold increase in secretion	*(174)*
Penicillin-binding protein 3 (PBP3)	OmpA	SecB improves secretion 2.6-fold; GroEL-GroES or DnaK have no effect	*(175)*
Tetracycline/H^+ antiporter	none	GroEL-GroES increase the half life of a truncated form of this membrane bound protein two fold	*(176)*
Human placental glutathione transferase	PelB	GST remains soluble in the cytoplasm but is not translocated in control (GST) cells; GroEL-GroES allows the secretion of 24% of this material	*(177)*
Human granulocyte-colony stimulating	hybrid OmpA	DnaK-DnaJ, and to a lesser extent either DnaK or DnaJ alone, factor (hG-CSF) increase overall accumulation and facilitate secretion; however, the percentage of aggregated material is higher in the DnaK-DnaJ strain than in control cells; SecB or GroEL-GroES have no effect on accumulation or secretion	*(178)*
Human granulocyte-macrophage colony-stimulating factor (hGM-GSF)	SP1 or SP3	SecB or DnaK-DnaJ lead to a threefold improvement in the recovery of soluble and mature SP3-GM-CSF, but have no effect on SP1-GM-CSF; GroEL-GroES have no effect in either case	*(80)*

Human interleukin-13 (hIL-13)	SP1 or SP3	Twofold improvement in yield of soluble and mature SP1-IL-13 using SecB, although GroEL-GroES have no effect and DnaK-DnaJ lead to a 10-fold reduction; no improvements in secretion with SecB, DnaK-DnaJ or GroEL-GroES for SP3-IL-13	*(80)*
Single chain T-cell receptor fragments (scTCR)	OmpA	GroEL-GroES stabilize cell lysis resulting from CR15 scTCR overproduction	*(110)*
LamB-bovine somatotropin fusion protein	LamB	DnaK or GroEL-GroES have no effect on secretion	*(50)*
LamB-human procollegenase fusion protein	LamB	Both DnaK and GroEL-GroES improve the solubility of the precursor; however, GroEL-GroES have no effect on secretion while DnaK suppresses it	*(50)*
Single-chain Fv antibody fragments	OmpA	GroEL-GroES have no effect on production	*(171)*
TEM-β-lactamase	native	Although precursor aggregates in the cytoplasm, DnaK-DnaJ or GroEL-GroES have only a slight effect on the recovery of enzymatic activity	*(81)*

[a]The abbreviations used are: SS, signal sequence.

sion of PrlF alleviates the toxicity and improves the secretion of *lacZ*-hybrid proteins *(84)* and *B. stearothermophilus* α-amylase in *E. coli (85)*. Even when translocation is carried out to completion, recombinant polypeptides may still form inclusion bodies within the periplasm *(35,82)*. To date, however, no general molecular chaperone has been discovered in this cellular compartment. The observation that the σ^E heat-shock regulon responds to periplasmic stresses *(37)* may however aid in the identification of such factors, as was recently shown by Missiakas et al. *(86)*.

Overproducing all Heat-Shock Proteins

Although the recovery of active recombinant proteins can be greatly enhanced upon overexpression of either the *groE* and *dnaKJ* operon (Table 1), a stoichiometric overproduction of all cellular molecular chaperones (including those yet to be discovered) may be required for optimal heterologous protein folding since these proteins function in a cooperative manner (Fig. 1). However, a plasmid encoding all known chaperones and ancillary proteins is bound to be difficult to construct, unstable, and will likely accumulate mutations in the overexpressed genes *(87)*. Two alternative approaches to raise the concentration of cellular hsps—and therefore of most molecular chaperones—consist in overproducing the heat-shock transcription factor σ^{32}, or inducing the heat-shock response by increasing the cell growth temperature or adding ethanol or other agents to the growth medium *(33)*. These techniques are not without drawbacks. Plasmids encoding σ^{32} may be unstable and only lead to a transient overexpression of hsps due to the sequestration of σ^{32} by DnaK and DnaJ *(88, 89)*. Similarly,induction of the heat-shock response by temperature increase, addition of ethanol, or other insults may result in the partial unfolding of host proteins, thus reducing the availability of chaperones necessary to facilitate proper folding of the target protein. Nevertheless, each of these approaches can be advantageous to the folding of certain recombinant proteins (Table 3). Direct and indirect methods to overproduce specific chaperones and all hsps may also be used in concert to provide additive improvements in the folding of recombinant gene products *(81)*.

Finally, strains bearing mutations in *dnaK, dnaJ, grpE, groEL*, or *groES* contain higher intracellular concentrations of hsps under all conditions and do not downregulate their expression at high temperature *(90)*. Such mutants may be helpful for the expression of aggregation-prone recombinant proteins whose in vivo folding does not exhibit an absolute requirement for the mutated chaperone system (US Patent 5,552,301).

The Heat-Shock Response and Proteolysis

Several of the major *E. coli* protease systems belong to the σ^{32} heat-shock regulon, including the ATP-dependent proteins Lon, FtsH, HslVU,

and various Clp family members *(67, 91, 92)*. As a result, overexpressing all heat-shock proteins in an effort to solubilize a recombinant gene product could also stimulate its degradation. In addition, since several molecular chaperones, including DnaK-DnaJ-GrpE, GroEL-GroES, and the Clp ATPases, have been implicated in proteolysis owing to their ability to maintain folding incompetent polypeptides in a form that can be recognized by cellular proteases (reviewed in *[68,93]*), the overproduction of specific chaperones could also increase the rate of degradation of certain recombinant gene products. This latter activity has not been documented to date. In contrast, chaperone co-overexpression can reduce the degradation of some target proteins, presumably by favoring the proper folding of unstable polypeptide molecules *(50, 51)*. Nevertheless, a reduction in the intracellular levels of all heat-shock proteases may be beneficial when producing recombinant gene products that are highly susceptible to proteolytic degradation. Such global decreases in protease synthesis can be achieved in cells bearing mutations in the *rpoH* gene encoding σ^{32} *(94, 95)*, or by co-overproducing antisense mRNA to *rpoH (96)*. However, limiting the cellular synthesis levels of σ^{32}—and thus those of most molecular chaperones—can also lead to the severe aggregation of normally soluble recombinant gene products *(46)*. Thus, the use of hosts bearing mutations in specific protease genes may be more appropriate in these cases *(94, 97)*.

The Effects of Cellular Folding Catalysts and Other Folding Modulators

In addition to molecular chaperones, *E. coli* contains other folding modulators that catalyze rate-limiting steps in protein folding. These include peptidyl-prolyl *cis/trans* isomerases and thiol/disulfide oxidoreductases. Although the *trans* conformation of X-Pro prolyl peptide bonds (in which X is any aa) is energetically favored in nascent protein chains *(98)*, about 5% of all prolyl bonds have been estimated to be in a *cis* conformation in native polypeptides *(99)*. In vivo, the isomerization of peptidyl-prolyl bonds from *trans* to *cis* conformations is catalyzed by peptidyl-prolyl *cis/trans* isomerases (PPIases, for a review *see* ref. *18*). Based on homology considerations, *E. coli* contains at least eight such enzymes *(31)*. PPIase A, the *rotA* gene product, is a periplasmic enzyme that exhibits peptidyl prolyl *cis/trans* isomerase activity in vitro. However, the total yield or folding kinetics of *E. coli* periplasmic and outer membrane proteins is not affected in *rotA null* mutants *(100)*. Furthermore, overproduction of the *rotA* gene did not alter the recovery yields of soluble and secreted Fab fragments or placental alkaline phosphatase, and had only a small effect on the recovery of ScFv fragments *(101, 102)*. SurA is a soluble periplasmic protein sharing homology with the cytoplasmic PPIase parvulin but displaying only 0.1 to

Table 3
Effect of Overexpression of Chromosomally-Encoded Heat-Shock Proteins on the Production of Recombinant Proteins

Recombinant protein	Method	Effect on protein production	Reference
Cytoplasmic proteins			
A. nidulans Rubisco	HS[a]	Growth at 42°C leads to a 6-fold increase in activity relative to growth at 26°C	*(49)*
E. coli RNA polymerase β subunit	HS	Growth at higher temperatures improves activity of plasmid-borne subunits but effect depends on host strain	*(179)*
E. coli RNA polymerase	EtOH	Activity of plasmid-borne subunits increases with ethanol concentration but β subunit effect depends on host strain	*(179)*
Soybean seed lipoxygenase L-1	EtOH	40% increase in yield with 3% ethanol in cultures grown at 15°C	*(180)*
PreS2-S′-β–galactosidase	EtOH	2–3 fold increase in activity at 30 and 42°C with 3% ethanol; lower effect observed at 37°C; ethanol addition and chaperone co-overexpression additively improve activity at 30°C	*(48,81)*
Human SPARC	EtOH	3% ethanol increases in the percentage of aggregated material at 30, 37, and 42°C	*(81)*

E. coli RNA polymerase β-subunit	$KMnO_4$	Activity of plasmid-borne subunits increases with concentration	*(179)*
PreS2-S′-β–galactosidase	σ^{32}	2–3 fold increase in activity at 30 and 42°C; lower effect at 37°C	*(48)*
Human SPARC	σ^{32}	Slight improvement in solubility	*(54)*
Periplasmic proteins			
TEM-β-lactamase	EtOH	Precursor aggregation is completely suppressed with 3% ethanol but accumulation of active enzyme is reduced	*(81)*
Single chain T-cell receptor fragments (scTCR)	σ^{32}	Cell lysis resulting from synthesis of CR15 scTCR is reduced; large improvement in secretion and solubility when both σ^{32} and DsbA are overproduced; σ^{32} and DsbA have no effect on the normally soluble P14 scTCR and do not affect the rapid degradation of the 8/10-2 scTCR	*(110)*
TEM-β-lactamase	σ^{32}	No effect on precursor aggregation or recovery of enzymatic activity	*(81)*

[a]The abbreviations used are: HS, heat-shock; EtOH, addition of ethanol to the growth medium; $KMnO_4$ addition of $KMnO_4$ to the growth medium; σ^{32}, co-overexpression of plasmid encoded σ^{32}.

1% of its isomerase activity in vitro *(86, 103)*. Strains bearing null mutations in *surA* are unable to properly fold several outer membrane proteins and synthesize high levels of the periplasmic protease DegP, as would be expected if misfolded proteins accumulated in the periplasm *(86, 103, 104)*. Although the folding efficiency of several secreted host proteins remained unchanged in *surA* mutants *(103, 104)*, there is evidence that SurA overproduction may improve the folding of certain secreted recombinant proteins *(86)*. Recently, the *fkpA* gene product was identified as a soluble periplasmic PPIase [Horne, 1994 #242; Missiakas, 1996 #236]. Although very little information is available, FkpA also appears to play an important role in mediating the proper isomerization of secreted proteins. Additional work will be needed to delineate the effect of SurA and FkpA overproduction on the misfolding of recombinant proteins in the periplasm.

It was recently reported that trigger factor, a cytoplasmic protein originally identified as important in protein secretion *(106)*, is a PPIase that associates with the ribosome and nascent polypeptides and interacts with GroEL *(107)*. These results suggested that trigger factor may be part of a ribosome-associated folding system that accelerates prolyl bond isomerization and cooperates with molecular chaperones to guarantee efficient in vivo folding. Nevertheless, this hypothesis and the effect of trigger factor overproduction on cytoplasmic inclusion body formation remain to be tested.

The formation and isomerization (reshuffling) of disulfide bonds is critical for the function and stability of many secreted eukaryotic and prokaryotic proteins. These rate-limiting step in protein folding are catalyzed in vivo by thiol/disulfide oxidoreductases and protein disulfide isomerases, respectively. The periplasm of *E. coli* contains at least four Dsb proteins that are involved in the formation of correct disulfide bonds in secreted and membrane proteins (reviewed in *17*). DsbA accelerates thiol oxidation in reduced protein substrates due to the presence of a highly unstable disulfide bond at its active site. The reduced form of DsbA is reoxidized by the inner membrane protein DsbB. The soluble protein DsbC also acts as a strong oxidant but appears to preferentially catalyze disulfide bond isomerization *(108)*. Finally, the essential inner membrane protein DsbD generates a reducing source in the periplasm and may play a key role in maintaining a proper redox environment in this compartment *(109)*.

A number of studies have examined the effect of DsbA overproduction on the folding of secreted recombinant proteins. While an increase in DsbA concentration had no effect on the recovery yields of antibody fragments *(102)* and human alkaline phosphatase *(101)*, the degradation of secreted T-cell receptor fragments was reduced when both DsbA and σ^{32} (but not when DsbA alone) were overproduced *(110)*. Similarly, a 14-fold increase in the amount of a correctly folded trypsin inhibitor was only observed when DsbA was overexpressed and reduced glutathione was added to the medium *(111)*. Interestingly, inactivation of *dsb* genes may also improve yields as was shown

in the case of a metallo-β-lactamase from *B. fragilis* which is rapidly degraded in wild type strains due to the formation of aberrant disulfide bonds *(112)*. Co-overexpression of the disulfide isomerase DsbC has also been found to improve the yields of tissue plasminogen activator in the periplasm *(113)*. Finally, secreted versions of eukaryotic protein disulfide isomerases (PDI) enhanced the recovery yields of bovine pancreatic trypsin inhibitor *(114)*, *E. carotovora* pectate lyase C *(115)*, and antibody fragments *(116)*, although they do not function as efficient isomerases in the periplasm *(4)*.

Since the cytoplasm of *E. coli* is more reducing than the periplasmic space, stable disulfide bonds are not found in cytoplasmic proteins. However, it was recently shown that the production of disulfide-bonded alkaline phosphatase *(117)*, mouse urokinase *(117)*, single chain Fv *(118)*, and human SPARC *(54)* was possible in the cytoplasm of strains lacking a functional thioredoxin reductase *(trxB)* gene. Since proper cytoplasmic folding can be favored by manipulating the concentration of molecular chaperones, *trxB* mutants may be extremely valuable hosts for the production of disulfide-bridged eukaryotic proteins.

In another promising approach to enhance the folding of recombinant proteins in the cytoplasm of *E. coli*, Yasukawa et al. *(119)* co-overexpressed thioredoxin with eight aggregation-prone eukaryotic gene products. The solubility of all proteins was increased under these conditions, whereas overproduction of the *groE* operon was only beneficial in four cases (Table 1). It was postulated that the positive effect of thioredoxin was related to alterations in the intracellular redox environment *(119)*.

IN VITRO REFOLDING OF INCLUSION BODY PROTEINS

Targeted Inclusion Body Formation

Since inclusion bodies are highly enriched in the protein of interest and easy to purify, in vitro protein refolding may be an attractive alternative to producing recombinant proteins in a soluble form in vivo *(6, 9)*. Two of the most effective means to induce aggregation consist of using strong promoter systems (e.g., the T7 and T4 bacteriophage promoters) to direct the transcription of the gene of interest, and cultivating the cells at high temperatures. Since both approaches increase synthesis rates, they have the additional advantage of improving the accumulation of the desired protein. The use of nonphysiological pH and variations in the growth medium composition can also favor aggregation *(9)*. Finally, it is possible to target proteins that are primarily soluble to the insoluble fraction of the cell by fusing amphiphilic extensions to the gene of interest (US Pats. 5,215,896, 5,302,526, and 5,330,902), or by using host strains containing mutations in the σ^{32} heat-shock transcription factor or in specific molecular chaperones genes *(46, 120)*. Thus, there is little doubt that any gene product can be produced in an insoluble form in *E. coli*.

Protein Refolding In Vitro

With recovery yields ranging from 0 to 100%, the refolding of inclusion body protein can be a rewarding or frustrating operation that is highly dependent on the folding pathway of the protein of interest. The major limitation to efficient refolding stems from the aggregation side reactions that kinetically compete with proper folding. However, since the refolding environment affects the extent of aggregation reactions, refolding conditions can be optimized to maximize proper folding. Recovery yields can usually be increased significantly by working at low protein concentrations since second or higher order aggregation reactions dominate over first order folding reaction at high concentrations. The main disadvantage of this approach is that the final concentration of native protein will also be low. This problem can be circumvented by making use of pulse renaturation techniques in which the denatured polypeptide is added to the refolding buffer in a stepwise manner *(121, 122)*. This process reduces the concentration of aggregation-prone folding intermediates while achieving a high final concentration of refolded proteins *(121, 122)*. Low temperatures may also favor correct folding owing to the fact that hydrophobic interactions are reduced under these conditions. Finally, the composition of the refolding buffer and the methods by which the protein is denatured (e.g., urea or guanidium hydrochloride unfolding) and renatured (e.g., dilution or dialysis) may affect recovery yields. A number of specialized techniques designed to achieve high refolding yields have been developed and recently reviewed *(6, 32, 123)*. They include the addition of L-arginine *(124)* and polyethylene glycol *(125, 126)* to the refolding buffer, and the use of micro-emulsions *(127)*.

Use of the GroEL-GroES System In Vitro

Since molecular chaperones play an essential role in cellular protein folding, the finding that these proteins could improve the recovery yields of chemically unfolded polypeptides in vitro was not surprising (Table 4 and 5). However, the fact that the GroE chaperonins could facilitate the productive folding of a large number of structurally and functionally unrelated proteins (Table 4) was more unexpected. In the past few years, an extraordinary amount of attention has been paid to the GrosEL-GroES system and, although controversies are still raging *(128)*, an understanding of its mechanism of action is starting to emerge and has been discussed in more detail elsewhere *(21, 23)*. GroEL is a weak ATPase organized as a double stack of seven identical 57-kDa monomers arranged around a central, solvent exposed cavity. When diluted into refolding buffer supplemented with GroEL, most denatured proteins form a stable binary complex with the chaperonin (Table 4). Compelling evidence indicates that

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Table 4
Effects of GroEL and Cofactors on In Vitro Protein Refolding

Substrate protein	Complex with GroEL	Effect of nucleotides	Effect of GroES	Enhancement in recovery	Reference
Bovine branched-chain α-keto acid dehydrogenase (E2c)	Stable for release	ATP required were used for release	Both ATP and GroEs with complete system in this study	From 0 to 100%	
Bovine liver rhodanese-I	Stable	ATP required for release	Required; some release with ATP only, but aggregation is extensive	From 0 to ~80% with complete system	*(182,183)*
Rat liver ornithine transcarbamylase	Stable	ATP required; non-hydrolyzable analogs release a non-assembly-competent protein in the presence of GroES	Required for release of active protein	From 0 to 75% with complete	*(184)*
R. rubrum Rubisco	Stable	ATP hydrolysis required for release	Required for progression to the native state	From 5 to 80% at 22°C; from 10 to 90°C at 15°C with complete system	*(188–187)*
Avena sativa L. Phytochrome photoreceptor	Stable	ATP required for release	No effect	From 0 to ~65% at 4°C with GroEL and ATP	*(188)*
E. coli tryptophanase	Stable	ATP, nonhydrolyzable analogs, or ADP required for release	No effect	From 13 to 80% at 25°C with complete system	*(189)*

(continued)

Table 4 *(Continued)*

Substrate protein	Complex with GroEL	Effect of nucleotides	Effect of GroES	Enhancement in recovery	Reference
Pig malate dehydrogenase	Stable	ATP hydrolysis required for release	Required for optimal refolding	From 0 to 50% at 36°C, from 30 to 90% at 20°C with complete system	*(190,191)*
E. coli glutamine synthetase	Stable	ATP or ADP required for release	Accelerates release slightly	From 20–40% to 70–80% at 37°C with complete systemPig heart citrate	*(192–194)*
ATP hydrolysis required synthase	Increases yields	From 3 to 28% at 25°C for release	*(195,196)*	with complete system	Stable
S. cerevisiae invertase	Stable	ATP required for release	Increases yields	From 14 to 36% at 20°C with complete system	*(197)*
B. stearo-thermophilus lactate dehydrogenase	Stable, slow but spontaneous dissociation	ATP accelerates release	Accelerates release	From 12 to 30% with GroEL and ATP	*(198,199)*
Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase	Transient below 30°C, but stable above 30°C	ATP accelerates release at low temperatures; required for release above 30°C	N.D.	From 35 to 80% with GroEL only, up to ~90% with GroEL and ATP at 20°C	*(130)*
F(ab) fragment	Transient	ATP binding increases release rate	Increases refolding yields slightly	From 30 to 70% at 25°C with complete system	*(200)*
B3(Fv)-PE38KDEL (recombinant immunotoxin)	Stable	ATP required for release	Required for progression to the native state	Twofold at 22°C with complete system	*(135)*
E. coli pre-β-lactamase	Stable	ATP required for release	Allows more efficient release	Twofold with complete system	*(201)*

Firefly luciferase	Stable	Both ATP and GroES were used for release in this study	Both ATP and GroES were used for release in this study	From ~10 to 20% at 30°C with complete system	*(43)*
Yeast α-glucosidase	Stable	ATP required for release	Accelerates release and increases yields	From ~20 to ~30% at 25°C with complete system	*(202)*
E. coli β-galactosidase	Transient	ATP inhibits the transient interaction with GroEL	GroES and ATP eliminate the transient interaction with GroEL	From 11 to ~20% at 25°C with GroEL only	*(132)*
Phage P22 tailspike protein	Stable above 30°C	Cooling to 25°C or addition of ATP required for release	No effect	Slight increase in yield with GroEL and ATP	*(129)*
Mouse dihydrofolate reductase (DHFR)	Stable except in the presence of DHFR ligands	ATP required for release; partial discharge with nonhydrolyzable ATP analogs	Facilitates ATP-dependent release	N.D.[a]	*(185)*
Barnase	Transient	ATP binding increases release rate, slower release with AMP-PNP and ADP	Accelerates release	100% spontaneous and with complete system	*(200)*
β-actin	Stable	ATP required for release	No effect	No change*(134)*	
α-tubulin	Stable	ATP required for release	No effect	No change*(134)*	
E. coli succinyl-CoA synthetase	No complex observed			No effect on refolding	*(57)*

[a]The abbreviations used are: N.D., not determined.

Table 5
Effect of Other Molecular Chaperones and Folding Catalysts on Protein Refolding

Chaperone or catalyst	Effect	Reference
Chaperones		
DnaK-DnaJ-GrpE	In the presence of ATP, the refolding of firefly luciferase increases from 10 to 50–70% through multiple cycles of binding and release	*(43,44,47,203)*
	In the presence of ATP, the *E. coli* UmuC protein partially refolds and regains DNA binding activity. Full activity recovery requires further addition of the GroEL-GroES system	*(42)*
Hsp70-Hdj1	These human homologs of DnaK and DnaJ improve the refolding of beta-galactosidase from 0 to 50% and of firefly luciferase from 12 to 50% in the presence of ATP	*(139,204)*
Hsp70-Ydj1p	These yeast homologs of DnaK and DnaJ improve the refolding of firefly luciferase from 2 to 15–30% in the presence of ATP	*(203)*
DnaK	ATP and an 80-fold excess of DnaK increase the refolding yields of the B3(Fv)-PE38KDEL immunotoxin twofold	*(135)*
Hsc70	Hsc70 reactivates heat inactivated DNA polymerases alpha and epsilon from calf thymus, as well as *E. coli* RNA polymerase and DNA polymerase III in the presence of ATP; substrate specificity appears to be broader than that of DnaK	*(205)*
Hsp90	Bovine Hsp90 increases citrate synthase refolding from 15 to 40% in an ATP-independent process	*(137)*
Small hsps	Murine Hsp25, human Hsp27 and bovine alpha-B-crystallin improve the refolding of citrate synthase from 8 to 25% and that of alpha-glucosidase from 18 to 40%; Pea Hsp17.7 and Hsp18.1 improve the refolding of citrate synthase from 15 to 40%, and that of lactate dehydrogenase from 10 to 35%	*(206,207)*
alpha-crystallin	alpha-crystallin increases the refolding yields of gamma-crystallin from 20 to 95%	*(208)*
Foldases		

PDI	PDI increases the renaturation yields of the B3(Fv)-PE38KDEL immunotoxin twofold if present at equimolar amounts and threefold at a 10:1 molar excess; The effect of PDI and DnaK are additive in the presence of ATP	*(135)*
	PDI improves the refolding of a murine MAK33 Fab fragment from 0 to 50% under certain redox conditions but does not affect the refolding of the oxidized denatured Fab	*(144,209)*
	PDI improves the refolding of bovine pancreatic trypsin inhibitor (BPTI) from 13 to 50% and increases the rate of reaction 27-fold	*(210)*
	At a 5–10 molar excess, PDI increases the refolding yields of hen egg white lysozyme from 50 to 100% by preventing the formation of disulfide-linked aggregates. When present in substoichiometric amounts, PDI reduces the recovery yields to about 17% through an anti-chaperone activity	*(141)*
	PDI increases the refolding of rhodanese (which contains four cysteine residues but no disulfide bonds) from 6.5 to 19% by reducing aggregation	*(211)*
	At a 10 : 1 excess, PDI increases the refolding yields of D-glyceraldehyde-3-phosphate dehydrogenase (which contains no disulfide bonds) from 10 to 38% by reducing aggregation	*(212)*
DsbA	Improves the refolding yields of reduced, denatured RNase from 15 to 40% in redox buffer; improves the refolding of scrambled RNase from 4 to 18%	*(213)*
	Reduced DsbA converts 65% of incorrectly disulfide-bonded insulin-like growth factor I (IGF-I) to the correct isomer without a need for a redox buffer; oxidized DsbA catalyzes the refolding of denatured, reduced IGF-I into 65% correct and 35% incorrect disulfide-bonded species	*(142)*
	DsbA slightly improves the refolding of BPTI (from 13 to 20%) in redox buffer and accelerates the reaction rate 1.5-fold	*(214)*
DsbC	DsbC improves the refolding of BPTI from 13 to 56% in redox buffer and accelerates the reaction rate fivefold	*(108)*

partially folded protein substrates bind within the central cavity of one ring of the GroEL toroid, usually at a stoichiometry of one molecule per chaperonin tetradecamer *(21, 23, 28)*. This operation arrests any legitimate refolding that would take place in the absence of GroEL, but also efficiently suppresses unproductive aggregation side reactions. Binary complexes between GroEL and partially folded proteins may be stable for days but are easily disrupted upon addition of adenine nucleotides to the mixture. Most proteins binding with high affinity to GroEL require hydrolyzable ATP to be released from the chaperonin (Table 4). However, other polypeptides can be discharged with lower efficiency by nonhyrolyzable ATP analogs, ADP (Table 4), or even shifts to low temperatures *(129, 130)*. Once ejected from the chaperonin, the protein may reach a native conformation, become trapped in an inactive or assembly-incompetent form or aggregate (*see* Table 4)

The cochaperonin GroES, a homoheptamer of seven 10-kDa subunits, is known to bind to one end of GroEL in the presence of adenine nucleotides, and has been shown to cap the polypeptide-bound end of the GroEL toroid if the substrate size is smaller than 50–60-kDa *(59,60)*. The presence of GroES in the refolding mixture may or may not be essential for productive GroEL-mediating refolding, depending on whether or not the environment is conducive to the correct folding of polypeptides that have been released from the chaperonin *(131)*. It is nevertheless clear that GroES can accelerate the rates of adenine nucleotide-mediated protein release from GroEL (Table 4). Furthermore, in those cases in which it is not necessary for progression to the native state, GroES typically improves the overall refolding yields (Table 4). Interestingly, both β-galactosidase *(132)* and glucose-6-phosphate dehydrogenase *(130)* experience an increase in refolding yields in the presence of GroEL alone. This unusual behavior is likely to be related to the fact that both proteins exhibit a low affinity for GroEL and are progressively and spontaneously released from the chaperonin in an environment that is conducive for proper folding. In most cases however, the use of a complete system consisting of GroEL, Mg-ATP, and GroES is recommended.

Table 4 shows that, in spite of the fact that a wide range of refolding buffers, protein concentrations, and temperatures have been used, the GroE chaperonins increase the recovery yields of many proteins several-fold relative to refolding buffer alone. It is however important to note that the GroEL-GroES system does not always have a beneficial effect on protein refolding. For instance, the GroE chaperonins are known to interact with the small protein barnase, but this protein refolds spontaneously with a yield of 100% in the absence of the chaperonins *(133)*. Similarly β-actin and α-tubulin form stable complexes with GroEL, but the refolding of these proteins is not facilitated by chaperonins *(134)*. Finally, denatured succinyl-CoA synthetase appears to be completely unable to interact with

GroEL *(57)*. In such cases, the use of alternative folding modulators should be considered.

Use of Other Folding Modulators In Vitro

Compared to the GroEL-GroES system, little is known about the effect of other folding modulators on in vitro protein refolding. Table 5 shows that DnaK alone or the complete DnaK-DnaJ-GrpE system are able to improve the refolding yields of certain chemically denatured polypeptides if hydrolyzable ATP is present in the mixture. Hsc70 or Hsp70, the constitutive and stress-inducible eukaryotic homologs of DnaK, respectively, are also beneficial to protein refolding either individually or in combination with the DnaJ homologs Hdj1 and Ydj1p in ATP-supplemented buffer. A landmark study by Buchner and coworkers *(135)* showed that the refolding of a single chain immunotoxin could be increased twofold in the presence of GroEL-GroES-ATP or DnaK-ATP, and almost threefold in the presence of a molar excess of PDI (Tables 4 and 5). More importantly, the effects of PDI and DnaK-ATP on the renaturation of the immunotoxin were additive *(135)*, demonstrating the usefulness of combining molecular chaperones and folding catalysts to facilitate in vitro protein refolding.

Hsp90s (the eukaryotic homologs of *E. coli* HtpG) are important proteins in higher organisms because of the fact that they associate with unliganded steroid receptors and newly synthesized protein kinases (reviewed in *136*). Hsp90s have been shown to efficiently prevent the aggregation of model polypeptides such as citrate synthase *(137,138)* and β-galactosidase *(139)*. In addition, their presence increases the refolding yields of citrate synthase (but not β-galactosidase) in an ATP-independent process *(137,139)*. Small hsps and their lens analog, α-crystallin, exhibit a similar behavior (Table 5). To date, however, it has not been demonstrated whether the *E. coli* small hsp homologs, IbpA and IbpB, have a chaperone function in vivo or in vitro. The Clp ATPases, the *E. coli* homologs of the Hsp100 family, have also been shown to exhibit molecular chaperone activity towards specific substrates in vitro, although their ability to improve protein refolding yields remains to be examined *(68)*. Additional studies will be necessary to evaluate the potential of Hsp90s, Hsp100s, and small hsps for in vitro protein refolding.

PDIs can have a significant positive effect on the refolding yields of disulfide-bonded proteins by preventing the formation of intermolecular disulfide bridges that lead to the formation of high molecular weight aggregates (Table 5). Eukaryotic PDIs also appear to possess a chaperone function since they can enhance the refolding yields of proteins that do not contain disulfide bridges *(140)*. Since it has been shown that substoichiometric concentrations of PDI promote the aggregation of hen egg white lysozyme (antichaperone activity *[141]*), care should be taken to

perform refolding operations in the presence of a molar excess of PDI. The effect of the *E. coli* periplasmic oxidoreductases DsbA and DsbC on in vitro refolding have been examined for a few substrates (Table 5). It appears that DsbC has a more powerful disulfide isomerase activity than DsbA but that it is a less efficient catalyst of disulfide bridge reshuffling than PDI *(108)*. Joly and Swartz *(142)* have demonstrated that reduced DsbA can efficiently convert an incorrectly disulfide-bonded isomer of insulin-like growth factor I to the correct molecule in the absence of redox agents. Whether DsbC also exhibits this useful property remains to be determined. However, it is likely that both proteins could find applications in biopharmaceutical manufacturing. Interestingly, DnaJ has recently been shown to exhibit protein disulfide isomerase activity in vitro *(143)*. Finally, PPIases such as cyclophilin and FK506 binding protein (FKBP) have been found to increase the rate at which a Fab fragment folds although they only slightly improved the overall recovery yields *(144)*. Thus, the practical usefulness of these folding catalysts may be more limited, despite the fact that a chaperone function has also been attributed to PPIases *(145)*.

Practical Aspects of In Vitro Chaperone Use

Just as refolding protocols must be optimized on a case-by-case basis, the choice of an appropriate folding modulator system will likely depend on the substrate polypeptide. Since molecular chaperones do not change protein folding pathways but increase recovery yields by preventing aggregation side-reactions, it is clear that they should be present in the refolding buffer in at least equimolar concentrations before addition of the denatured substrate. Similarly, PPIases may be more useful when present in buffer prior to initiation of refolding. In contrast, the catalysis of disulfide bond formation and isomerization by oxidoreductases and PDIs requires that appropriate cysteine residues be in close proximity or that non-native disulfide bridges be present in the protein. Thus, it may be more useful to add these enzymes after a proper tertiary or quaternary structure has been reached.

Two major limitations associated with the routine use of folding modulators are their cost and the need to separate them from the desired gene product following completion of refolding. Thus, the feasibility of using chaperones and folding catalysts in refolding schemes may depend upon our ability to retain and reuse them. Although only a few reports have addressed these questions, the results appear rather promising. Buchner et al. *(135)* have shown that DnaK immobilized on BioRad Affi-Gel beads could double the refolding yields of a recombinant immunotoxin in the presence of ATP, and that the matrix could be removed by centrifugation, washed, and reused up to five times without significant loss of activity.

Similarly, Phadtare et al. *(146)* found that GroEL immobilized on a matrix derivatized with anti-GroEL antibodies was capable of discharging properly folded tubulin and glutamine synthetase upon incubation with GroES and ATP.

Monomeric variants of *E. coli* GroEL retain their ability to bind nucleotides but are unable to suppress the aggregation or promote the refolding of model proteins *(137)*. However, truncated versions of the GroEL protomer can function as ATP-independent "minichaperones," albeit with lower efficiency than the oligomeric structure *(147,148)*. Virtually full-length monomers prepared from the *T. thermophilus* GroEL homolog remain capable of increasing the refolding yields of lactate dehydrogenase and rhodanese in a process that does not require adenine nucleotides *(149)*. It was further shown that the same monomers facilitate the ATP-independent refolding of rhodanese when immobilized on a TSK matrix *(149)*. This behavior is reminiscent of the mechanism of action of small hsps and Hsp90. Since no additives or cofactors are required to improve refolding, the use of immobilized chaperonin monomers from *T. thermophilus*, truncated fragments from GroEL or small hsps and Hsp90 coupled to a solid matrix may find applications in in vitro protein refolding.

Artificial Chaperone Systems

Recently, several techniques that facilitate the in vitro refolding of recombinant proteins by mimicking the basic functional features of chaperones have been developed. Rozema and Gellman have used detergents and cyclodextrins to duplicate the two-step mechanism of action of the GroEL system in the refolding of carbonic anhydrase B, citrate synthase, and lysozyme *(150–152)*. In the first step of this "artificial chaperone" strategy, a detergent (e.g. CTAB, STS, POE(10)L, or Triton X-100) captures the non-native protein upon dilution from denaturing conditions. This operation prevents partially folded intermediates from interacting with each other and aggregating, presumably by shielding solvent-exposed hydrophobic patches. Since this process also prevents any "legitimate" refolding, it is analogous to the trapping of non-native proteins within the central cavity of the GroEL toroid. In a second step, the detergent is stripped by addition of β-cyclodextrin to allow productive folding to take place. This is superficially similar to the effect of interactions between GroEL·polypeptide complexes and ATP/GroES that stimulate the release of bound proteins to permit their correct isomerization. Although detergents *(153)* and α-cyclodextrin alone *(154)* can improve refolding yields, the artificial chaperone method appears to be more efficient at high protein concentrations *(150,151)*. Another approach that mimics the aggregation suppression function of molecular chaperones is the "temperature leap" tactic developed by Xie and Wetlaufer to refold carbonic anhydrase II *(155)*. In this case,

unfolded proteins are diluted in a refolding buffer held at low temperature to suppress interactions between hydrophobic segments in the protein chain and favor the conversion of aggregation-prone folding intermediates into partially-folded but non-aggregating species. Shifting the refolding mixture to higher temperatures allows productive folding to occur. Finally, Stempfer and Rudolph have documented the successful refolding of α-glucosidase fused to a hexa-arginine extension upon noncovalent immobilization on polyionic matrices *(156)*. This process is likely to create a microenvironment that favors proper folding since matrix-bound folding intermediates are prevented from interacting with each other to give rise to aggregation reactions. The "infinite dilution" model for GroEL mode of action relies on a similar principle *(23)*. Considering the high economic stakes associated with the refolding of proteins of pharmaceutical interest, it is likely that more methodologies inspired by the mechanism of action of molecular chaperones will be forthcoming.

CONCLUSION

The discovery of molecular chaperones and folding catalysts has radically changed our perception of in vivo protein folding. It is now clear that an increase in the intracellular concentration of folding modulators can significantly improve the recovery yields of many—but not all—aggregation prone recombinant proteins in *E. coli*, as well as in other hosts *(157–159)*. Yet, our understanding of cellular protein folding remains sketchy and the mechanism of action of two of the most extensively characterized systems, DnaK-DnaJ-GrpE and GroEL-GroES, has not yet been unambiguously elucidated. The identification of small hsps, the Clp proteins and Hsp90 as molecular chaperones has opened new avenues of applied and fundamental research. The role of these proteins in host and heterologous protein folding and the interplay of molecular chaperones and folding catalysts are among the many questions that remain to be answered. While folding modulators also hold great promise in assisting and catalyzing protein refolding in vitro, the practical use of these polypeptides in refolding operations will require the development of cost-effective schemes for their production and continuous use. More importantly, a thorough understanding of the mechanism of action of molecular chaperones will give us the ability to the develop artificial chaperone systems that will mimic their function at a fraction of their cost.

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PATENTS

A selection of US patents relevant to the use of folding modulators in protein folding and refolding is presented below. Abstract may have been edited for clarity and conciseness. Copies of the patents can be obtained from the Commissioner of Patents, US Patent and Trademark Office, Box 9, Washington, D.C. 20231. For a recent review of international patents dealing with molecular chaperones, see reference *(160)*.

Pigiet, V. P., Rusche, J. P, and Schuster, B. J.
Thioredoxin shufflease and use thereof
US Pat. 4,904,602, February 27, 1990
Assignee: Repligen Corporation

The subject invention concerns a novel enzyme named thioredoxin shufflease, means for preparing the same, and procedures for using thioredoxin shufflease to fold proteins containing disulfide crosslinks. Thioredoxin shufflease is a generic term to define enzymes which have the following characteristics: (a) contain a single reactive thiol group; (b) catalyze the exchange of disulfides in a protein undergoing the refolding process; and (c) are not consumed in the oxidation/refolding process. Specifically exemplified is a thioredoxin shufflease produced from an *E. coli* thioredoxin gene.

Rudolph, R. and Fischer, S.
Process for obtaining renatured proteins
US Pat. 4,933,434, June 12, 1990
Assignee: Boehinger Mannheim GmbH

The present invention provides a process for the renaturation of denatured proteins in solution in a renaturation buffer, wherein a solution is prepared of the protein to be renatured in the critical concentration is a selected buffer and, after formation of the folding intermediate, further protein to be renatured is added in the amount of necessary for the achievement of the critical concentration.

Creighton, T. E.
Process for the production of a protein
US Pat. 4,977,248, December 11, 1990

A method for the renaturation of unfolded proteins comprises reversibly immobilizing the denatured protein on a solid phase and inducing folding by progressively reducing the concentration of a denaturing agent in the solvent in contact with the solid phase. The proteins can be folded and recovered in high yield in a small volume of buffer.

Keck, P. C., Cohen, C. M., Huston, J. S., and Ridge, R. J.
Leader sequences for the production of recombinant proteins
US Pat. 5,215,896, June 1, 1993 (see also US Pat. 5,302,526, April 12, 1994; US Pat. 5,330,902, July 19, 1994)
Assignee: Creative BioMolecules, Inc.

Disclosed is a novel polypeptide useful as a leader or trailer peptide moiety in recombinant DNA protein production techniques involving fused protein methodology. The polypeptide comprises an amphiphilic helix. When DNA encoding the helix is attached to a gene encoding a protein of interest, high level expression is achieved and inclusion bodies are spontaneously formed. The inclusion bodies may be collected and purified easily. After purification, the fused protein is cleaved to separate the amphiphilic helix from the product.

Hayano, T., Katou, S., Maki, N., Nobuhiro, T., and Masanori, S.
Peptidly propyl-cis. trans-isomerase
US Pat. 5,284,762, February 8, 1994 (see also USA Pat.5,416,015, May16,1995)
Assignee: Tonen Corporation

Isolated genes and plasmids compromising said genes encoding specified yeast and *E. coli* PPIases acting on and isomerizing the bond X(aa)-Pro (where X(aa) stands for any amino acid and Pro stands for L-proline).

Neupert, W. and Hartl, F.-U
Process for the biocatalytic, correct chain folding of denatured recombinant fusion proteins
US Pat. 5,302,518, April 12, 1994
Assignee: Hoechst

Denatured recombinant fusion proteins are correctly folded in the presence of a heat shock protein which acts according to the functional principle of the bacterial GroEL or of the equivalent mitochondrial component Hsp60, and of ATP. After cleaving off the foreign sequence, biologically and medically interesting proteins are obtained in the correct conformation.

Taylor, R. K. and Peek, J. A.
TcpG gene of vibrio cholerae
US Pat. 5,382,660, January 17, 1995
Assignee: University of Tennessee Research Corporation

A method for increasing the yield of recombinant gene products from bacteria comprising inserting a gene into the genetic material of the bacteria whereby the inserted gene is coexpressed with a desired recombinant gene product and aids the arrangement of the gene product into the proper final conformation (*tcpG* encodes a periplasmic protein homologous to DsbA *[161]*.

Trent, J. D. and Horwich, A. L.
Archaebacterial chaperonin-mediated protein folding
US Pat. 5,428,131, June 27, 1995
Assignee: Yale University

TF55 is a homooligomeric complex of two stacked rings, closely resembling the quaternary structure of the chaperonins GroEL, Hsp60 and Rubisco-binding protein. Most rings of TF55 contain nine radially arranged members. The TF55 complex binds unfolded polypeptides in vitro, preventing aggregation at elevated temperatures and exhibits ATPase activity. These features are consistent with its function as a molecular chaperone.

Mascarenhas, D.
Methods and vectors for overexpression of ubiquitin fusion proteins in host cells
US Pat. 5,459,051, October 17, 1995
Assignee: Celtrix Pharmaceuticals

The invention is directed to recombinant DNA vectors and methods of use of thereof. The vectors allow overexpression of proteins in bacterial host cells. The vectors allow the construction of gene fusions between ubiquitin and a protein of interest and encode a cytoplasmic peptidyl-prolyl cis/trans isomerase. Coexpression of the first and second genes allow overexpression of the protein of interest. In some cases, the degree of solubility of the protein of interest is also increased.

Jakob, U., Buchner, J., Wiech, H., Zimmerman, R., and Rainer, R.
Method for the stabilization of proteins using heat shock protein Hsp90
US Pat. 5,474,892, December 12, 1995

The present invention concerns a method for the stabilization of proteins in an aqueous solution which is characterized in that one or several members of the heat shock protein (Hsp90) family are added to the aqueous solution containing the protein.

Yukio, Y., Asami, O., Sugiyama, H., Idekoba, C., Hoshino, F., Hirai, M., Kajino, T., Imaeda, T., and Sarai, K.
Polypeptide from *Humicola insolens* possessing protein disulfide isomerase activity, gene encoding the same
US Pat. 5,496,719, March 5, 1996
Assignee: Kabushiki Kaisha Toyota Chuo Kenkyusho

A highly thermostable polypeptide possessing protein disulfide isomerase (PDI) activity, a gene coding for the polypeptide and a process for producing the polypeptide are provided. Since it has a higher thermostability and exhibits a stable activity over wider dithiothreitol concentrations range compared to conventional PDI, it is possible to advantageously use this polypeptide for the refolding of certain proteins. Furthermore, a

process which enables the polypeptide possessing PDI activity to be efficiently produced using *Humicola insolens* or a transformant harboring an expression vector containing the above-described gene is also provided.

Baneyx, F., Gatenby, A. and Kalbach, C.
Process for enhancing the production of heterologous protein in biologically active conformation in a transformed *E. coli dnaK* mutant host cell
US Pat. 5,552,301, September 3, 1996
Assignee: E. I. Du Pont de Nemours & Co.

Yield of biologically active proteins from recombinant *E. coli* has been increased 2–4 fold by a novel use of the *dnaK* mutation. The presence of the full-length DnaK mutant protein results in elevated levels of biologically active foreign proteins.

Yoshida, M., Taguchi, H. and Konoshi, J.
Methods and compositions for promoting protein folding
US Pat. 5,561,221, October 1, 1996
Assignee: Nippon Oil Company Limited

Disclosed are 1) compositions comprising isolated monomeric subunits of chaperonin-60 or truncated fragments thereof that promote the folding of a polypeptide chain in vitro, 2) monomeric subunits of chaperonin-60 or truncated fragments thereof, immobilized on a solid surface, that also promote the folding of a polypeptide chain in vitro, 3) methods for preparing an/or immobilizing the monomeric subunits of chaperonin-60 or truncated fragments thereof, and 4) methods for folding polypeptide chains, specifically polypeptide chains expressed in a heterologous expression system, in vitro using the monomeric subunits of chaperonin-60 or truncated fragments thereof.

Gellman, S. H. and Rozema, D. B.
Method for refolding misfolded enzymes with detergent and cyclodextrin
US Pat. 5,563,057, October 8, 1996
Assignee: Wisconsin Alumni Research Foundation

A method for refolding an enzyme from a misfolded configuration to a second native and active configuration is presented. The method comprises adding a linear alkyl detergent to a misfolded enzyme to form an enzyme–detergent complex. Then the enzyme–detergent complex is contacted with a cyclodextrin to allow the enzyme to assume a second active conformation.

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