Biotechnology and Molecular Biology Review Vol. 1 (2), pp. 66-75, June 2006
Available online at http://www.academicjournals.org/BMBR
ISSN 1538-2273 © 2006 Academic Journals

Standard Review

Molecular Chaperones involved in Heterologous Protein Folding in *Escherichia coli*

E. BETIKU

Department of Chemical Engineering, Obafemi Awolowo University,Ile-Ife, Osun State, Nigeria

Accepted 30 May, 2006

The Gram-negative bacterium *Escherichia coli* is one of the most attractive host employed in the heterologous production of proteins. However, these target proteins are deposited as insoluble aggregates known as inclusion bodies (IBs) and hence are biologically inactive. The ubiquitous molecular chaperones, a group of unrelated classes of polypeptides help in the mediation of proper folding of the target protein. However, the choice of chaperone(s) is still based on a trial-and-error procedure. Wrong choice of chaperone(s) will affect both the host micro-organism and product stability, negatively. Recent advances in the mechanisms and substrate specificities of the major chaperones and their roles in the chaperone-network now gives some ideas for more rational choice of the chaperone(s) for co-expression. Here, the functions and mechanisms of interactions between the major molecular chaperones are presented.

Key words: molecular chaperones, inclusion bodies, heterologous, aggregates, protein folding

Table of Content

1.0 Introduction
2.0 Methods for preventing or decreasing protein aggregation.
2.2 Use of fusion proteins
2.3 Mutations in the target protein
2.4 Optimisation of cultivation conditions
2.5 Co-expression of molecular
3.0 Molecular chaperones
3.1 The Trigger Factor (TF)
3.2 The DnaK System
3.3 Cooperation of DnaK system with other Chaperones
3.4 The Small Heat-Shock Proteins (sHsps)
3.5 The GroEL System
4.0 Conclusion
5.0 Acknowledgement
6.0 References

1.0 INTRODUCTION

The heterologous production of proteins in the bacterium host *Escherichia coli* is a widely used techniques both in research and for commercial purposes. However, a fraction of these proteins are deposited in insoluble form. These proteins form aggregates that accumulate into inclusion bodies (IBs). IBs are refractile protein aggregates with porous structures (Taylor et al. 1986; Rinas et al. 1992; Carrió and Villaverde 2001). They have high density (Hwang 1996) and are known to be in non-native form and hence biologically inactive (Goloubinoff et al. 1999; Hoffmann and Rinas 2004). The need to

*Correspondence author. E-mail: ebetiku@oauife.edu.ng, Tel.: +234-836602988, Fax: +234 (36) 232401.

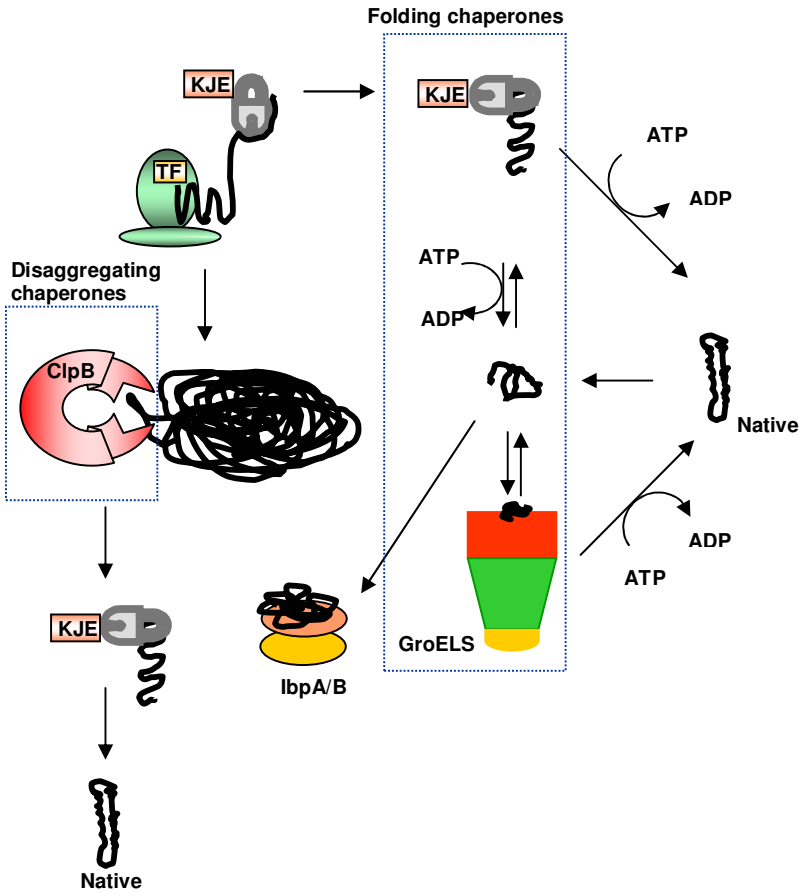

Figure 1. A model of molecular chaperone-mediated protein folding in the cytoplasm of the bacterium *Escherichia coli*. Newly synthesized polypeptides first interact with Trigger Factor or DnaKJE. The intermediate formed may reach native protein or interacts with GroELS before reaching native form. The intermediate may also form aggregates known as inclusion bodies (IBs), which may need to interact with ClpB for disaggregation before reaching native form after interaction with DnaKJE. IbpAB binds partially folded proteins until disaggregating chaperone ClpB becomes available.

avoid formation of aggregates during heterologous production of proteins in *E. coli* is not only informed by the increase demand for cellular "quality control" machinery (Hoffmann and Rinas 2004) which, may lead to low productivity but also has to do with the involvement of aggregates in some unrelated diseases such as Alzheimer's disease, bovine spongiform encephalopathy and type II diabetes (Haper and Lansdury 1997; Azriel and Gazit 2001).

Molecular chaperones are ubiquitous and highly conserved proteins that shepherd other polypeptides to fold properly and are not themselves components of the final functional structures (Hartl 1996; Baneyx and Palumbo, 2003). There are ~ 20 families of this class of proteins which have different molecular weights, structures, cellular locations and functions (Radford, 2000). They were originally identified by their increased abundance as a result of heat shock (Bukau and Horwich, 1998). Molecular chaperones work as networks in protein folding in the cytoplasm of *E. coli* (Figure 1).

2.0 Methods for preventing or decreasing protein aggregation.

Several methods have been suggested or shown to prevent or decrease aggregation during overproduction of recombinant protein in the host cell. Some of these methods include: rate of synthesis, fusion proteins, mutations in the target protein, cultivation conditions and coexpression of molecular chaperones.

2.1 Control of the rate of synthesis of expressed proteins

Typically, the more rapid the intracellular product accumulation, the greater the probability of product aggregation. The expression rate and the correct folding of the product are among other parameters determined by the level of gene induction, promoter strength, the efficiency of translation initiation and mRNA stability (Swartz 2001). Best results are usually obtained by low

cultivation temperature (18-25°C) and application of low gene dosage (Kopetzki et al. 1989; Swartz 2001). Hence, high soluble protein yield depends on low specific protein synthesis rate and sustained production period (Kopetzki et al. 1989; LaVallie et al. 1993).

2.2 Use of fusion proteins

Unrelated proteins originally were constructed together (at genetic level) to facilitate protein detection/purification and immobilization (Uhlen et al. 1983), and to couple the activity of enzymes acting in a single metabolic pathway. However, expression of a set of foreign genes e.g. protease domain of human urokinase UKP as a fusion to ubiquitin gene in yeast showed improved yield of recombinant protein (Butt et al. 1989). Some fusion partners often employed include prokaryotic *Staphylococcus* protein A (Abrahmsén et al. 1986); maltose-binding protein (Sachdev and Chirgwin 1998), thioredoxin (Lavillie et al. 1993) and DsbA (Winter et al., 2000) from *E. coli*. The order of fusion partners often determines the solubility level of the target protein (Sachdev and Chirgwin 1998). As a periplasmic protein, maltose-binding protein directs by its native signal peptide the whole fusion to the periplasmic space of the cells. This positive influence of maltose-binding protein can be attributed to both its molecular characteristics and its interaction with the target proteins. Although, most protein fusions are soluble but the target proteins are not always correctly folded (Sachdev and Chirgwin 1998) and IBs can still be formed (Strandberg and Enfors 1991). The disadvantages of fusion-protein technologies include: liberation of the passenger proteins requires expensive proteases (such as Factor Xa), cleavage is rarely complete leading to reduction of yields, additional steps may be required to obtain an active product e.g. formation and isomerisation of disulfide bonds (Banyex 1999).

2.3 Mutations in the target protein

Many reports have been presented to show the effects of mutations in target proteins overproduced in *E. coli*. King and co-workers employed genetic techniques to identify second-site suppressor mutations of temperature sensitive folding mutants of the P22 tailspike protein, which when placed in a wild-type background, give the phenotype of decreased IBs content compared to wild-type (Fane and King 1991). Mutations in the hFGF-2 gave different results; no soluble hFGF-2 was formed when cysteines 26 and 93 were replaced with serines, while a single substitution of cysteine 70 by serine decreases the fraction of soluble hFGF-2 significantly (Rinas et al. 1992). Recombinant production of interferon gamma protein (IFN-γ) in *E. coli* at 37°C results in over 90% of the total accumulated gene product into IBs and in addition, mutations in the protein show mutants that retain high biological activity and are localized almost entirely in the soluble fraction (Wetzel et al. 1991). Observations in a series of mutations in the human interleukin-1 beta (IL-1β) show no strong correlations between extent of IB formation and either thermodynamic or thermal stability (Chrunyk et al. 1993). Replacement of Lys^{97} by Val produces substantially more IL-1β in IBs than in wild type despite generating a protein more thermodynamically stable than wild type (Chrunyk et al. 1993).

2.4 Optimisation of cultivation conditions

IB formation during high-level recombinant production may be reduced or avoided by optimising culture conditions. Growth temperatures have been shown to affect formation of IBs e.g. β-lactamase (Valax and Georgiou 1993) and hFGF-2 (Squires et al. 1988). High cultivation temperature leads to recombinant protein aggregation (Schein and Noteborn 1988; Schein 1989). Low temperatures can greatly reduce the formation of IBs (Chalmers et al. 1990). This is corroborated by the work of Piatak et al. (1988), soluble and fully functional Ricin A chain was produced at 37°C, but the one produced at 42°C was aggregated. pH of the culture medium also affects inclusion body formation. Formation of IBs also depends on the level of induction. By using 0.01 mM IPTG for induction of alkaline phosphate when produced in *E. coli*, more than 90% of the product could be recovered from the periplasm in soluble form, whereas when induction was made at 1 mM IPTG, most of the secreted alkaline phosphate formed IBs (Choi et al. 2000). The expression of α-glucosidase depends upon the inducer concentration as well as on the period of induction (Kopetzki et al. 1989). The type of medium employed for recombinant production has influence on the level of IBs formed. It has been shown that growth on glycerol (Kopetzki et al. 1989) or on complex medium (Winter et al. 2000) can be advantageous for solubility and folding of the recombinant product. The ratio of soluble to aggregated β-lactamase can be increased by growing cells in the presence of certain non-metabolizable sugars (Bowden and Georgiou 1988) and it was also shown that the inhibition of aggregation depends on the concentration of the sugar in the growth medium (Bowden and Georgiou 1990). Wunderlich and Glockshuber (1993) reported a five-fold increase in correctly folded target protein after adding reduced and oxidized glutathione to the growth medium. Glycine also influences the folding of aggregation-prone proteins (Kaderbhai et al. 1997). However, optimization of these various parameters (such as pH, host strains, media, temperature) is required for prevention of aggregation and for the production of soluble and active products (Kopetzki et al. 1989; Winter et al. 2000).

Table 1. Enhancing soluble production by co expression of molecular chaperones

Chaperone	Protein product	Results	References
GroEL/GroES	Rubisco	Increased production of assembled and active Rubisco proteins from various species is observed.	Goloubinoff et al., 1989
	Protein-tyrosine kinase P^{50csk}	>50% of P^{50csk} is soluble following GroELS overexpression.	Amrein et al., 1995
Trigger Factor (TF)	Endostatin	>80% of Endostatin is soluble following TF overexpression.	Nishihara et al., 2000
DnaK	Human growth factor	Co expression of DnaK inhibits human growth factor IB formation and increases the amount of soluble product from 5% to >85%.	Blum et al., 1992
GroEL/GroES and TF	ORP150	86% of ORP150 is soluble following GroELS/TF overexpression.	Nishihara et al., 2000
GroEL/GroES and DnaK	Cryj2	Co expression of GroELS/DnaK resulted in marked stabilization and accumulation of Cryj2 without extensive aggregation	Nishihara et al., 1998

Source: Betiku, 2005

2.5 Co-expression of molecular chaperones

Anfinsen's observation that all information necessary for a protein to adopt the unique three-dimensional structure is contained in the amino acids sequence (Anfinsen 1973) remains unchallenged, in the last decade this view of cellular protein folding has changed considerably. Protein folding in the vicious and crowded environment of the cell is very different from *in vitro* processes in which a single protein is allowed to refold at low concentration in an optimised buffer (Baneyx and Palumbo 2003). The initial folding of proteins and assembly of multiprotein complexes can be helped and sometimes required the participation of chaperones. By binding exposed hydrophobic patches on the protein, they prevent proteins from aggregating into insoluble, non-functioning IBs and help them reach their stable native state (Wickner et al. 1999). Chaperones do not provide specific steric information for the folding of the target protein, but rather inhibit unproductive interactions (Walter and Buchner 2002).

3.0 Molecular chaperones

The major chaperones implicated in *de novo* protein folding are the trigger factor (TF), and the DnaK and the GroEL chaperone systems (Horwich et al. 1993; Bukau et al. 2000). Other chaperones involved in folding of recombinant proteins include the AAA+ chaperone ClpB and IbpA/IbpB. These molecular chaperones have been reported to enhance soluble production of recombinant proteins in *E. coli* (Table 1).

3.1 The Trigger Factor (TF)

TF was originally identified by its activity to stimulate membrane translocation of the precursor of the outer-membrane protein A (preOmpA) *in vitro* (Crooke and Wickner 1987). In the *E. coli* cytosol, nascent polypeptides interact first with TF (Valent et al. 1995; Hesterkamp et al. 1996). TF has both peptidyl-prolyl cis-trans isomerase activity and chaperone-like function (Crooke and Wickner 1987; Hesterkamp et al. 1996). The enzymatic mechanism of TF follows the Michaelis-Menten kinetic (Scholz et al. 1997). TF binds to the ribosome at proteins L23/L29 near the polypeptide exit site (Kramer et al. 2002). TF's peptidyl-prolyl cis-trans isomerase activity is not essential for protein folding in *E. coli* (Kramer et al. 2004). It is composed of three domains: an N-terminal domain, which mediates association with the large ribosomal subunit; a central substrate binding and peptidyl-prolyl cis-trans isomerase (PPIase) domain with homology to FKBP [(FK506 Binding Protein)(FK506 is a macrolide lactone, Tacrolimus also called Fujimycin)]; and a C-terminal domain of unknown function (Hesterkamp and Bukau 1996; Hesterkamp et al. 1997). TF affinity for substrate is very low compared to most chaperones and is ATP independent, suggesting that rapid binding to and release from TF may be critical for elongating polypeptide chains (Maier et al., 2001). The binding motif of TF has been identified as a stretch of eight amino acids, enriched in aromatic residues and with a positive net charge (Patzelt et al. 2001). TF cooperates with the DnaK system in folding of nascent polypeptides. They share an overlapping substrate pool (Teter et al. 1999; Deuerling et al. 1999, 2003). Both chaperones help in multidomain

protein folding but at the expense of folding speed (Agashe et al. 2004). They can compensate for one another; however, their combined deletion is lethal at temperatures above 30°C (Deuerling et al. 1999; Teter et al., 1999). Overproduction of GroEL chaperone system could efficiently suppress the growth defect as a result of *tigdnak* deletion (Genevaux et al. 2004). TF function together with GroEL-GroES in selective degradation of certain polypeptides (Kandror et al. 1995) and *in vivo*, TF associate with GroEL to promote its binding to certain unfolded proteins (Kandror et al. 1997). TF prevents the aggregation of recombinant proteins either in combination with the chaperonin GroEL-GroES or alone (for example, lysozyme, Nishihara et al. 2000).

3.2 The DnaK System

The DnaK is the most general molecular chaperone. It is also known as heat shock protein 70 (Hsp70). The structural and mechanistic features of the *E. coli* DnaK chaperone system have been reviewed (Bukau and Horwich 1998). DnaK works in cooperation with its cochaperones – DnaJ and GrpE (Liberek et al. 1991). Structural features of DnaK are required for interaction with DnaJ (Suh et al. 1999). The importance of these features for substrate binding has been shown by mutational analysis (Mayer et al. 2000). The rate of ATP hydrolysis is accelerated by DnaJ (Laufen et al. 1999). This stimulation is disrupted by mutation of conserved leucine residues of DnaK located in the linker between substrate binding and ATPase domains, resulting in considerable loss of chaperone activity (Han and Christen 2001). DnaJ also targets the substrates to DnaK (Liberek et al. 1995), and substrates with low affinity for DnaK are not able to stimulate the ATPase and chaperone activity of DnaK without DnaJ (Mayer et al. 2000). The cochaperone GrpE accelerates the exchange of ADP with ATP, resulting in the release of the unfolded substrate and completion of the chaperone cycle (Packschies et al. 1997). Besides the promiscuous binding of aggregation-prone substrate proteins, DnaK – targeted by DnaJ (Liberek et al. 1995) – specifically recognizes a “region C” of the heat-shock sigma factor σ^{32} (Nagai et al. 1994). Abundant free DnaK-DnaJ inhibits σ^{32}-dependent gene expression (Tatsuta et al., 1998). Furthermore, the C-terminal part of σ^{32} becomes accessible to the protease FtsH, resulting in rapid degradation of σ^{32} (Blaszczak et al. 1999), therefore, DnaK negatively regulates the heat-shock response. Under stress conditions, misfolded proteins withdraw DnaK from σ^{32}, which regains activity and stability, resulting in enhanced transcription of σ^{32}-dependent heat-shock genes, including *dnaK*, until sufficient amounts of DnaK accumulate to bind both the misfolded proteins and σ^{32} (Bukau 1993). The increase in the level of σ^{32} is accelerated, when, additional to the titration of DnaK by misfolded proteins, high temperatures stimulate translation of the *rpoH* mRNA (Morita et al. 2000). The DnaK chaperone acts on different levels: *de novo* folding of protein (Bukau and Horwich 1998), rescue or degradation of denatured proteins and reversion of aggregation (Hoffmann and Rinas 2004). In addition to the role in ATP-dependent unfolding, DnaK can prevent aggregation by longterm binding to thermolabile substrates when higher temperatures reduce the affinity of DnaK for both DnaJ and GrpE (Diamant and Goloubinoff 1998), thereby preventing aggregation or stabilizing the substrates for refolding by the GroEL chaperone system (Buchberger et al. 1996). DnaK binds preferentially newly synthesized proteins in the size range of 16-167 kDa with an enrichment of proteins larger than 60 kDa (Deuerling et al. 2003).

3.3 Cooperation of DnaK system with other Chaperones

Beside TF, DnaK also cooperates with the *E. coli* Hsp31 (“holdase”) in management of protein misfolding under severe stress conditions (Mujacic et al. 2004). *In vitro* and *in vivo* experiments show that cooperation between DnaK and the AAA+ chaperone ClpB is needed for prevention and reversion of aggregation in prokaryotes (Mogk et al. 1999; Zolkiewski 1999). Heat-inactivated proteins released by the DnaK-ClpB bichaperone system are recognized as non-native folding intermediates by the chaperonins system (Watanabe et al. 2000). The disaggregating activity of the ClpB-DnaK chaperone network exhibits broad substrate specificity; at least 75% of thermally aggregated *E. coli* proteins in cell extract are solubilised (Mogk et al. 1999). The mechanism of solubilisation and refolding of protein aggregates by this bichaperone network is sequential (Goloubinoff et al. 1999). It has been proposed that ClpB interacts directly with protein aggregates prior to the DnaK on protein substrates (Weibezahn et al. 2003). Schlee et al. (2004) have shown that a specific interaction between ClpB and DnaK exists, and the affinity of the complex formed is weak and their interaction is nucleotide-dependent (Schlee et al. 2004). Hsp104/ClpB was first described as a heat-inducible protein conferring thermo-tolerance to yeast (Sanchez and Lindquist 1990). ClpB is ATP-dependent (Woo et al. 1992) and belongs to the Hsp100/Clp family of AAA+ (ATPase associated with a variety of cellular activities) and is composed of an N-terminal domain and two AAA domains that are separated by a “linker” region (Schirmer et al. 1996). The AAA domains mediate ATP binding and hydrolysis and are essential for ClpB oligomerization (Mogk et al. 2003a). The function of the N domain and the “linker” segment are currently unknown. While the N domains are dispensable for the disaggregating activity of ClpB, the linker region has an essential function in this process (Mogk et al. 2003a).

3.4 The Small Heat-Shock Proteins (sHsps)

The small heat-shock proteins (sHsps) are ATP-independent proteins, grouped as a family of heat-shock proteins based on a low degree of homology in a core region of about 85 amino acids (the α-crystallin domain), their ability to be induced by cellular stress, and their low protomer molecular weight, which usually ranges between 15-30 kDa (Shearstone and Baneyx 1999). The *E. coli* homology is IbpA/IbpB with molecular weight of 14- and 16-kDa, respectively, co-transcribed during stress by the bacterial heat shock transcription factor σ^{32} (Allen et al. 1992). IbpB consists mainly of β-pleated secondary structure (Shearstone and Baneyx 1999). In *E. coli*, IbpA and IbpB are found associated with endogenous proteins that aggregate intracellularly during heat shock (Laskowska et al. 1996) and with non-native recombinant proteins in Inclusion bodies (Allen et al. 1992). However, they are not found in inclusion bodies of partially soluble proteins (Valax and Georgiou 1993; Hoffmann and Rinas 2000). Over-production of IbpA/IbpB can increase stress tolerance in *E. coli* (Thomas and Baneyx, 1998). Despite the high sequence homology between IbpA and IbpB, the two proteins behave differently upon over-expression in *E. coli*; whereas IbpA is found in the insoluble S-fraction, IbpB is mainly soluble when produced in the absence of IbpA, but co-migrates to the aggregated fraction upon co-production with IbpA (Kuczyńska-Wiśnik et al. 2002). Generally, sHsps bind substrate proteins exposing hydrophobic surfaces and for refolding, a transfer to ATP-dependent chaperones is required (Hoffmann and Rinas 2004). IbpA/IbpB cooperate with the bichaperone (DnaK and ClpB) both *in vivo* and *in vitro*, in reversing aggregated proteins, and they become essential at 37°C if DnaK levels are reduced (Mogk et al. 2003b).

3.5 The GroEL System

The GroEL-GroES system (i.e. chaperonins) are currently the molecular chaperone system, for which there is the most structural and mechanistic information (Braig et al. 1994; Rye et al. 1997; Sigler et al. 1998). They are essential for cell viability at all temperatures (Fayet et al. 1989; Horwich et al. 1993). During cellular stress, 30% of newly translated polypeptides depend on the GroEL chaperone (Horwich et al. 1993). GroEL is also known as heat shock protein 60 (Hsp60) and is a homo-oligomer of 14 subunits, each of relative molecular mass of 57 kDa, arranged into two heptameric rings, forming a cylindrical structure with two large cavities (Braig et al. 1994). Substrate protein, with hydrophobic amino acid residues exposed, binds in the central cavity of the cylinder, engaging the hydrophobic surfaces exposed by the apical GroEL domain (Fenton et al. 1994). Folding usually occurs with the aid of GroES, a dome-shaped ring containing seven subunits of 10 kDa (Hunt et al. 1996). Binding of GroES to the polypeptide-containing ring of GroEL in an ATP-dependent reaction results in the displacement of polypeptide into an enclosed cage, defined by the GroEL cavity and the dome of GroES, in which aggregation is prevented and folding to native state is possible (Weissman et al. 1994). After the GroEL-bound ATP has been hydrolysed to ADP, ATP binding to the opposite ring of GroEL results in the dissociation of GroES and folded protein from GroEL (Figure 2). Some proteins require multiple chaperonin cycles for folding (Hartl 1996; Sigler et al. 1998). GroEL preferentially interacts with newly synthesized polypeptides with the size range between 10-55 kDa (Ewalt et al. 1997), but most GroEL substrates are larger than 20 kDa (Houry et al. 1999). GroEL substrates consist of two or more domains with αβ-folds, which contain α-helices and buried β-sheets with extensive hydrophobic surfaces (Houry et al. 1999). The oligomeric structure of GroEL-GroES is required for biologically significant chaperonin function in protein folding (Weber et al. 1998) and the maximum size of substrate protein that can be encapsulated in the GroEL-GroES cavity is ~ 57 kDa (Sakikawa et al. 1999). GroEL-GroES can also mediate folding of substrate protein, which are too large to be enclosed within this cavity (Chaudhuri et al. 2001). Co-production of GroEL-GroES can increase solubility of some recombinant proteins (Goloubinoff et al. 1989; Amrein et al. 1995). GroEL-chaperone system cooperates with other molecular chaperones, for example DnaK (Buchberger et al. 1996; Nishihara et al. 1998, 2000), and TF (Nishihara et al. 2000) in increasing solubility of certain recombinant proteins. Betiku (2005) show that GroELS can prevent inclusion bodies formation during recombinant production of human basic Fibroblast Growth Factor (hFGF-2) in *E. coli*. GroELS of *E. coli* are the rate-limiting cellular determinant of growth at lower temperatures (Ferrer et al. 2003).

4.0 CONCLUSION

Several methods have been suggested or shown to prevent or decrease aggregation during overproduction of recombinant protein in the host cell. Some of these methods include: controlling the rate of synthesis, use of fusion proteins, mutations in the target protein, optimisation of cultivation conditions and co-expression of molecular chaperones. The use of chaperones is just evolving and more studies are still needed to understand how they function. Hitherto, the use of chaperones in improving recombinant production of protein is by trial-and-error procedure. Future research should be focused on the interactions/cooperation between these chaperones and the various target protein. When there is enough data, it will then be possible to choose the right combination of molecular chaperones to co-express with the individual target protein in order to avoid formation of aggregation and hence, increase the yield.

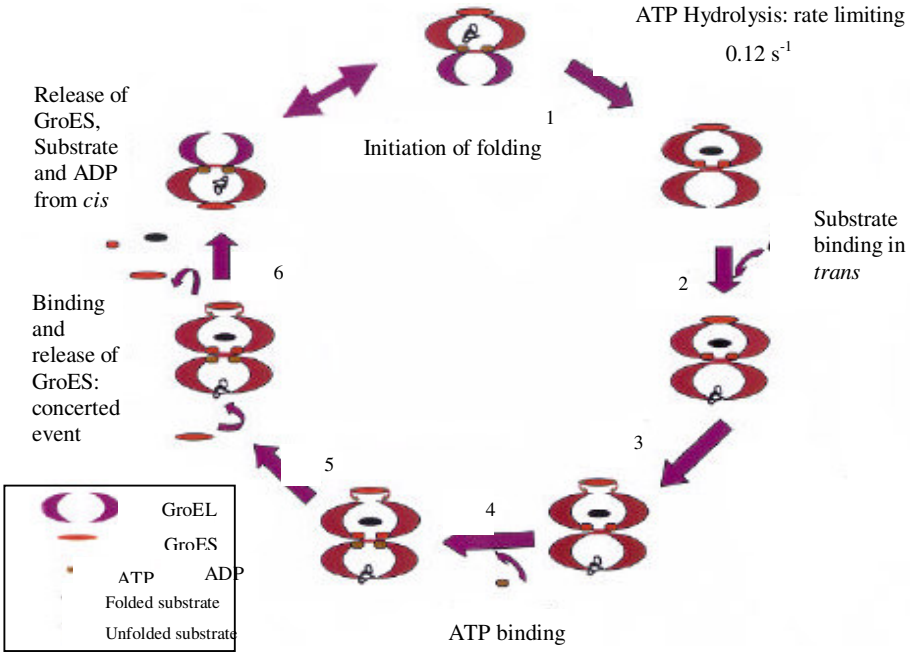

Figure 2. The GroEL-GroES reaction cycle. (1) Binding of substrate protein stimulates ATP and GroES binding in *cis*, which leads to the substrate protein being released in the cavity, and initiation of folding. (2) Substrate protein binds to the *trans* ring only after ATP hydrolysis takes place in the *cis* ring. (3) In the presence of substrate in the *trans* ring, there is a fast structural rearrangement in the ADP and GroES-bound *cis* ring that primes it for releasing GroES. (4) The binding of substrate protein in the *trans* ring stimulates ATP binding in *trans*. (5) The subsequent binding of GroES to the *trans* ring is simultaneous with the release of GroES from the *cis* ring. (6) The GroES- and ATP- bound *trans* ring causes structural rearrangements in the *cis* ring leading to release of ADP and substrate protein. Upon completion of one folding cycle, the next cycle is initiated in the alternate ring (adapted from Bhutani and Udgaonkar, 2002).

5.0 ACKNOWLEDGEMENT

The author wishes to acknowledge the financial support for this work by DAAD. This work was carried out at the National Research centre for Biotechnology, Braunschweig, Germany.

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