Model for ER Chaperone Dynamics and Secretory Protein Interactions

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Expression of proteins in eucaryotic systems is often the only way to ensure the correct folding and processing necessary for protein function. Heterologous proteins, however, are commonly retained in the secretory pathway, so that secreted product yield is low despite a high level of transcription. A major limiting step in protein secretion is protein folding in the lumen of the endoplasmic reticulum. This process is assisted by accessory macromolecules resident in this compartment, including chaperones such as the hsp70 homologue binding protein (BiP). Although induction of foreign proteins in yeast initially elicits a transient increase in local chaperone concentration, long-term protein expression lowers both chaperone and secreted product. A mechanistic model that can account for the experimentally observed role of BiP in secretion and the effects of BiP overexpression on the secretory pathway is described here. The model predicts that equimolar synthesis of chaperone and foreign protein should optimize protein secretion.

Introduction

Many products in the biotechnology industry depend on the successful expression, folding, and assembly of foreign proteins within genetically engineered host cells. Use of eucaryotic cell host systems for this purpose is often the only way to ensure the intracellular molecular processing necessary for proper protein function. At the same time, high levels of production and secretion into the extracellular medium is typically desirable. However, expression of foreign genes at high levels does not always lead to corresponding levels of protein production (Smith et al., 1985). Indeed, foreign proteins are commonly retained in the secretory pathway, so that little secreted product is obtained despite substantial rates of synthesis.

Within the first organelle of the secretory pathway, the endoplasmic reticulum (ER), a secreted protein must fold into its compact, biologically active conformation before continuing in the pathway (Hurtley and Helenius, 1989). In the ER, at least three classes of proteins are known to assist the protein folding process. Foldases such as protein disulfide isomerase (PDI) and peptidyl prolyl isomerase catalyze modifications that may be required for correct folding of some secreted proteins (Freedman, 1989; Harding et al., 1989; Freedman, 1992). The precise role of chaperones, such as the hsp70 homologue binding protein (BiP), in the folding of secreted proteins has yet to be determined. However, the interaction of BiP with secreted proteins, both transient and stable, are well established (Gething et al., 1986; Dorner et al., 1987; Hendershot et al., 1988; Kassenbrock et al., 1988; Bole et al., 1989; Hendershot, 1990; Blount and Merlie, 1991; Suzuki et al., 1991; Ng et al., 1992).

Many studies of secretion of native proteins have shown that the limiting step is transit from the ER to the Golgi, the second organelle in the secretory pathway (Lodish et al., 1983; Shuster, 1991). In both mammalian cells and yeast, BiP has been found to associate reversibly with secreted proteins, both native and foreign (Dorner et al., 1987; Machamer et al., 1990). It has been proposed that incorrect or inefficient interactions of foreign proteins with chaperones may be preventing the protein product from leaving the cell (Robinson and Wittrup, 1995).

BiP has been observed both in transient and stable interactions with secreted proteins (Gething et al., 1986; Dorner et al., 1987; Hendershot et al., 1988; Kassenbrock et al., 1988; Bole et al., 1989; Hendershot, 1990; Blount and Merlie, 1991; Suzuki et al., 1991; Ng et al., 1992). The evidence supports a transient interaction with normal proteins and more stable interactions with misfolded or mutant proteins. BiP is an es-

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sential gene in eucaryotic yeast cells, i.e., deletion of the chromosomal copy is lethal to the cell (Rose et al., 1989).

However, Dorner and his colleagues have suggested that BiP protein is detrimental to recombinant protein secretion in mammalian cells (Dorner et al., 1988). When the level of BiP is increased by overexpression in mammalian CHO cells, secretion of the human proteins von Willebrand factor and factor VIII are reduced (Dorner et al., 1992). CHO cells with reduced levels of BiP, through introduction of an antisense BiP gene, resulted in increased secretion of tPA (Dorner et al., 1988). In contrast, high-level recombinant protein expression in the yeast *Saccharomyces cerevisiae* reduces native BiP levels, at the same time decreasing secretion (Robinson nd Wittrup, 1996). Other evidence indicates that genetically decreasing the levels of BiP below normal levels reduces the levels of secreted human granulocyte colony stimulating factor and *S. pombe* acid phosphatase in the yeast *S. cerevisiae* (Robinson et al., 1996). The mechanism behind the differences in the interactions and observed role of BiP in regulating secretion is unclear.

We have therefore developed a mechanistic model in order to gain insight into the dynamics of the interactions between BiP and secreted proteins. The importance of cellular parameters in altering protein secretion are not well understood, but may become clearer through this type of modeling. BiP likely performs multiple functions in the lumen of the ER, where it has been found in association with translocation complexes (Sanders et al., 1992). However, given the importance of protein folding in determining secretion levels, and the established bichemical evidence for BiP interactions with unfolded proteins, the mechanisms described in this model involve molecular parameters governing secretion. The model was designed to reproduce existing experimental data on BiP interactions with secreted proteins in yeast. The qualitative results of the model are also likely to reflect those interactions for mammalian cells, as there are many strong similarities in protein composition and organelle function between the secretory pathways of each cell type.

Mathematical models of the eucaryotic secretory pathway constructed by other investigators have focused on the overall secretory pathway and transport between the secretory organelles, treating each organelle as a well-mixed compartment (Park and Ramirez, 1989; Bibila and Flickinger, 1991; Sambanis et al., 1991). The model described here focuses on folding steps in the lumen of the ER, based on the notion that these steps are limiting for secretion (Pelham, 1989; Gething and Sambrook, 1990; Bibila and Flickinger, 1991). Protein folding *in vivo* has been modeled previously, but the presence of chaperones was neglected (Kiefhaber et al., 1991). In an earlier model, BiP concentration was fixed at a constant level (Robinson and Wittrup, 1993). That model did not reflect the cell's regulation of BiP levels, and so was unable to capture all of the dynamics in secretion.

Our model incorporates the processes of folding, aggregation, and binding to chaperone that occur when a secretory protein is first translocated across the ER membrane. It further allows chaperone synthesis to be regulated by native protein synthesis rates as well as induction of foreign protein synthesis. Also included are entrapment of both chaperone and protein by aggregates formed within the ER. The model predicts that an equimolar rate of chaperone synthesis is required for optimal secretion of foreign protein at any given level of foreign gene expression in yeast. The model additionally predicts that a higher affinity of chaperone for the unfolded protein can have a positive effect of protein secretion, contrary to elementary intuition.

Background

Since the early 1950s there have been numerous studies on the nature of the forces driving proteins to attain their ordered, biologically active form. Anfinsen's seminal studies with staphylococcal ribonuclease showed that all the information necessary for a protein to reach its native, folded state is contained within its amino acid sequence (Anfinsen, 1973). Similar results have been obtained with many small proteins and a few more complex proteins (reviewed by Kim and Baldwin, 1990). Many other proteins have been recalcitrant to refolding or reversible unfolding.

Within a cell total protein concentration is much higher than the conditions studied *in vitro* (Freedman, 1992). This may lead to differences in folding rates or pathways because of the tendency of proteins to aggregate at high concentrations (Zettlmeissl et al., 1979). Since in principle folding of proteins can start as soon as the *N*-terminal region is free of the ribosome, folding *in vivo* may occur before synthesis of the protein is complete (Freedman, 1992). Therefore a protein folding pathway *in vivo* may be different from the refolding pathway described *in vitro*. The discovery of chaperone proteins, named for their role in preventing improper molecular interactions (Ellis, 1987), has led to a continually escalating interest in protein folding *in vivo*.

The first of these chaperones was discovered through studies of heat shock of cells (Craig and Gross, 1991). (The term heat shock describes a sudden increase in temperature from the normal growth temperature.) Under conditions of heat shock, levels of mRNA coding for a small number of proteins within the cell increase up to tenfold, and the synthesis of these heat shock proteins (hsp's) escalates (Gething and Sambrook, 1990; Craig and Gross, 1991). Among the best characterized members of this class is the hsp70 family (Lindquist and Craig, 1988). Chaperone proteins within the hsp70 family dissociate aggregates, keep nascent chains competent for translocation, and uncoat clathrin-coated vesicles.

The hsp70 protein BiP was originally identified in CHO cells, in association with misfolded μ heavy chains, a subunit of IgM antibodies (Haas and Wabl, 1984). Since its identification, BiP has been found in both stable and transient interactions with a number of secreted proteins (Gething et al., 1986; Dorner et al., 1987; Hendershot et al., 1988; Kassenbrock et al., 1988; Bole et al., 1989; Hendershot, 1990; Blount and Merlie, 1991; Suzuki et al., 1991; Ng et al., 1992). BiP-heavy chain complexes are very stable and can be dissociated with the addition of ATP *in vitro* or by the coexpression of IgM-light chains within the cells (Hendershot et al., 1988; Kassenbrock et al., 1988; Bole et al., 1989; Hendershot, 1990). ER-retained T-cell antigen receptor α chain (TCR-α) is also found in a stable complex with BiP (Suzuki et al., 1991).

In addition to these stable interactions with misfolded or unassembled proteins, transient association of BiP with newly translocated proteins has also been demonstrated in several instances (Gething et al., 1986; Dorner et al., 1987; Bole et

al., 1989; Hendershot, 1990; Blount and Merlie, 1991; Ng et al., 1992). Overall, the evidence points to a transient association of the chaperone with normal proteins and a more stable interaction with mutant or misfolded forms of a protein. As a result, BiP may play a dual role in solubilizing folding precursors and preventing transport of unfolded and unassembled proteins.

Although the exact mechanism of BiP function is unclear, there exists a great deal of evidence about BiP's role in the cell. BiP production can be induced by glucose starvation, heat shock, treatments that inhibit glycosylation, and the presence of misfolded secreted proteins (Kozutsumi et al., 1988; Machamer et al., 1990; Blount and Merlie, 1991; Earl et al., 1991). Studies of the peptide binding of BiP has led to a general model for binding to hydrophobic residues in the unfolded protein chain (Flynn et al., 1989; Gething et al., 1994). Because of its location in the ER, and its association with secreted proteins, BiP may play a proofreading role in secretion of folded proteins. In other words, BiP may bind to incompletely folded or misfolded proteins and either target them for degradation, retain them in the ER, or catalyze their folding. In an effort to facilitate accurate predictions about the effects of altering BiP concentrations or BiP–protein binding, we have developed a mechanistic model based on observations from our laboratory and others, as well as some new experiments to complement the existing database of BiP effects.

Experimental Methods

Cultures of BJ5464 (*α ura3-52 trp1 leu2-Δ1 his3-Δ200 pep4::HIS3 prbl-Δ1.6R can1 GAL*) of *Saccharomyces cerevisiae* were obtained from the Yeast Genetic Stock Center (Berkeley, CA). The parent strains were stored at −70°C and revived onto rich media (YPD) plates prior to introduction of a recombinant expression system (Sherman, 1991). All transformations were performed by electroporation using a Bio-Rad Gene-Pulser (Becker and Guarente, 1991). Transformants were grown on selective media lacking tryptophan. This technique enables a genetic selection for BJ5464 cells bearing a plasmid-borne copy of TRP1, a gene necessary for biosynthetic production of the essential amino acid tryptophan.

BJ5464 cells were transformed with pKEαFScEPO for transient expression of human erythropoietin (EPO) as described previously (Robinson and Wittrup, 1995). Cells overexpressing BiP were obtained by transformation with pGalKar2-Leu (Robinson and Wittrup, 1995). For transient EPO expression, freshly transformed colonies were inoculated into 2 mL of a synthetic minimal media (SD + AA − Trp) in culture tubes (Sherman, 1991). Following growth to saturation at 30°C, cells were transferred at an optical density at 600 nm (OD_{600}) of approximately 0.1 into 250-mL baffled erlenmeyer flasks holding 50 mL of synthetic minimal media containing galactose instead of glucose (SG + AA − Trp) to begin induction of EPO synthesis. At designated times, one OD_{600}·mL of cells was collected by centrifugation for 5 min at 5,000 × *g* and lysed by sodium dodecyl sulfate–glass bead lysis as described previously (Robinson et al., 1994). Supernatants from the centrifugation step were collected and used for EPO detection as described below.

For long-term studies of high-level EPO expression, plates of freshly transformed colonies of BJ5464 + pGalKar2-Leu + pKEαFScEPO were stored at 4°C. Individual colonies were selected at the times indicated on the figure and inoculated into 2 mL of selective media (SD-CAA) in culture tubes. Following growth to saturation, cells were transferred at an OD_{600} of 0.1 into 250-mL baffled erlenmeyer flasks containing 50 mL of selective media (SG + AA-Trp) and samples were taken during the exponential phase of growth at an OD_{600}·mL of 0.5–1.0, collected, and lysed as previously.

For analysis of BiP protein levels, dilutions of equal OD_{600}·mL of extract were collected on nitrocellulose (0.2 μm) using a Slot-Blot manifold (BioRad). BiP protein was visualized by Lumiphos (Boehringer Mannheim) Western analysis using a 1:10,000 dilution of polyclonal rabbit IgG antisera raised to a *C*-terminal fragment of BiP (Rose et al., 1989) and secondary detection with a 1:2000 dilution of HRP-conjugated goat antirabbit antibody (Sigma). Quantitation of Westerns was performed as described previously (Robinson and Wittrup, 1995). Briefly, the linearity of the Western was determined by dilutions of protein extract, with an error based on both sampling error and measurement error of 8%. Only dilutions in the linear range were included in the determination of protein levels. At least three points in the linear range were averaged for each time point indicated on Figures 1 and 2.

Secretion of EPO protein was detected in supernatant using dilutions of supernatants collected on nitrocellulose (0.2 μm) using a Slot-Blot manifold (BioRad). Lumiphos (Boehringer Mannheim) Western detection was then performed using a monoclonal antibody, SY14 at 1:2,000 dilution (gift of S. Elliot, Amgen), and HRP-conjugated goat antimouse secondary antibody at 1:1000 (Sigma). EPO protein levels were compared to an EPO standard generously given by S. Elliot at Amgen.

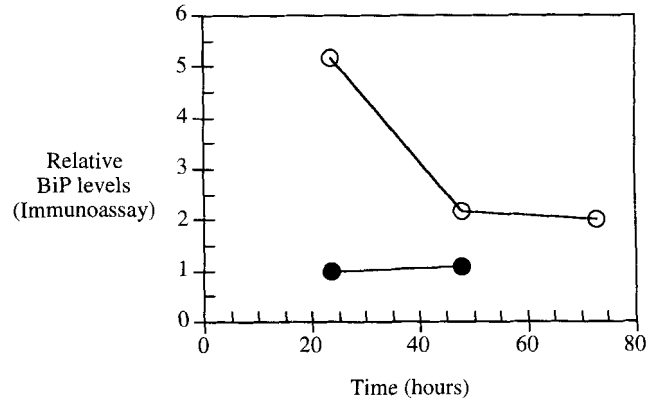

Figure 1. Protein induction leads to a transient increase in BiP protein levels.

Wild-type yeast (BJ5464) was transformed with an expression vector for overexpression of erythropoietin, pKEαFScEPO (EPO; ○) under the control of an inducible galactose promoter or with a control vector (WT; ●). EPO expression was induced at time zero by transfer of the cells from glucose to galactose-containing medium. Samples of cell culture were removed at the indicated times, and cellular extracts were analyzed for BiP protein through Western immunoassay. BiP protein levels were quantitated as described in Experimental Methods. At least three dilutions of cellular extract for each time point were in the linear range of the assay, and error for each point is less than 8%.

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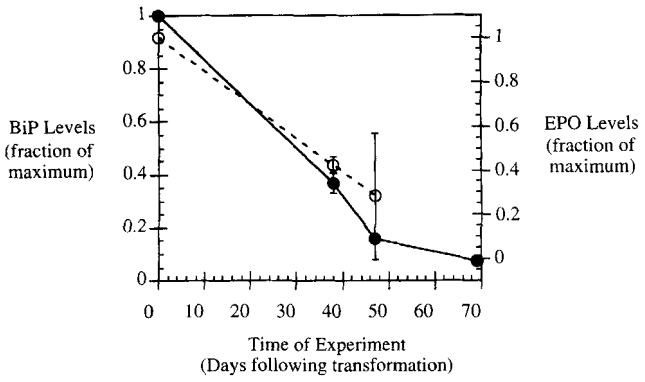

Figure 2. Effect of cellular aging on protein and chaperone expression levels.

Yeast BJ5464 cells were transformed with pGalKar2-LEU+ pKEαFScEPO (Gal-regulated overexpression of BiP and EPO). Transformants were stored at 4°C and inoculated into flesh flasks at the indicated times following transformation. Culture were grown at 30°C, and samples were taken at mid-exponential growth. BiP levels (●) are compared to untransformed BJ5464 cells described in Figure 1 and Experimental Methods. Error bars indicate the 8% error in measurement as described in Experimental Methods. For each time point, a standard curve for EPO was generated using at least three dilutions of standard. Secreted EPO protein levels were determined by interpolation using one or more dilutions of supernatant samples. Error bars indicate the error in the slope of the standard curve.

Experimental Results

Other investigators have shown previously that BiP message levels are increased following induction of foreign or mutant protein production (Kozutsumi et al., 1988; Watowich et al., 1991; Tokunaga et al., 1992). Previously, we have reported that transient expression of EPO in *Saccharomyces cerevisiae* raises BiP protein levels (Robinson, 1994; Robinson and Wittrup, 1995). After an initial rise in BiP protein in response to this cellular stress, the levels of BiP appear to fall to wild type or below. A representative experiment from Robinson and Wittrup (1995) is shown in Figure 1, where BiP protein falls to wild-type levels within about 40 h following EPO induction. Constant high-level expression of foreign proteins in yeast leads to reduced levels of BiP, with a concomitant decrease in secretion (Robinson and Wittrup, 1995). This effect is enhanced when yeast cells are stored either at 4°C or at −70°C following foreign protein expression.

When yeast cells transformed with an expression system for transient foreign protein production are stored on plates at 4°C and then cultured and assayed at normal growth temperature (30°C), the levels of both cellular chaperone and secreted foreign protein decrease as the duration of storage time increases. We have observed this trend in more than a dozen separate stocks, with four different proteins (Robinson, 1994). Figure 2 shows measured levels of secreted human EPO as a function of storage time at 4°C. Intracellular BiP levels drop to undetectable levels after 70 days; secreted EPO drops to 30% of its original level after 45 days. We proposed earlier (Robinson and Wittrup, 1995) that chaperone entrapment caused by the aggregates formed from unfolded proteins was one possible source of this reduction. This entrapment mechanism has been incorporated into the model, assuming first-order binding of aggregated material to both chaperone and protein. In personal communications, other investigators have described anecdotal evidence consistent with Figures 1 and 2. However to date, no mechanistic explanations for decreases in chaperone and protein losses have been offered, nor have strategies been developed to deal with them, beyond using fresh transformants.

Model Development

Figure 3 shows the interactions of BiP and secreted proteins included in the model. Chaperone (C) and protein (P) are synthesized by mRNA translation and translocated into the ER at the rates V_c and V_p, respectively. Chaperone synthesis can be increased, "induced," by cellular stress, and is included in the term $V_{c0}\eta(1-e^{-[P]/[P]_I})$. Chaperone can bind newly synthesized protein ($k_b[C][P]$) or be trapped by aggregates ($k_e[A][C]$) or undergo degradation ($k_t[C]$). Newly translocated protein can be depleted by four competitive processes: self-folding (k_N), binding to chaperone ($k_b[C][P]$), aggregation ($k_e[A][P]$, $k_a[P]^2$), or undergo degradation ($k_t[P]$). The chaperone–protein bound complex (B) is formed by chaperone–protein binding ($k_b[C][P]$), and depleted by protein release ($k_h[B]$). Nonproductive folding is assumed to lead to aggregation. In this model, aggregation (A) is assumed to be irreversible and is reduced only by turnover ($k_d[A]$) or dilution by growth ($\mu[A]$). Chaperone, protein, and the bound chaperone–protein complex (B) also can be diluted by growth ($\mu[C]$, $\mu[P]$, and $\mu[B]$, respectively). Folded protein (F) rapidly exits the ER and the cell through secretion, and therefore dilution by growth is neglected. Folded protein is formed from either self-folding ($k_N[F]$) or release from BiP accompanied by folding ($k_h[B]$).

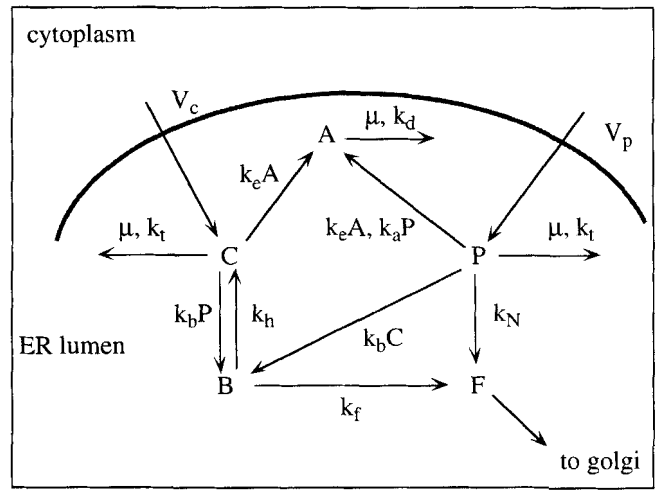

Figure 3. Model schematic encompassing entrapment and BiP regulation.

C = chaperone; P = newly translocated protein; A = aggregate; B = bound P–C complex; F = folded protein. V_c is the rate of chaperone synthesis; V_p is the rate of protein synthesis; k_e is the rate of entrapment; μ is the growth rate of the cells; k_t, k_d are degradation, or turnover, rates; k_a is the rate of aggregation; k_h is the rate of release of the complex; k_b is the rate of chaperone binding; k_N is the rate of self-folding; k_f is the rate of chaperone-assisted folding. Values for the rates are in Table 1. The membrane between the endoplasmic reticulum and the cytoplasm is depicted as a thick line.

In the following paragraphs, we provide estimates for the parameters used in the model, based on published experimental data. Baseline values were chosen to best approximate currently known system behavior. In the Model Behavior and Model Predictions sections, we present parametric studies of the effects of changing the most sensitive and biologically important parameters. Contributions of parameters and sensitivity of the model behavior to parameter values is described in the "Discussion" section.

The stoichiometry of BiP binding to unfolded nascent chains is taken to be 1:1 in the present model. Palleros et al. observed approximately 1:1 stoichiometry of binding of cytosolic hsp73 to reduced carboxymethylated α-lactalbumin *in vitro* (Palleros et al., 1991). Suzuki et al. immunoprecipitated a truncated T-cell receptor α-chain from CHO cell extracts complexed with BiP at stoichiometric ratios between 1:1 and 2:1 (Suzuki et al., 1991). Thus, 1:1 binding is a reasonable approximation.

The binding rate of chaperone and protein has not been determined in a cellular system. However, based on optimal binding rates of short, linear proteins, or peptides, to BiP *in vitro* (Flynn et al., 1989) a value of 30 $M^{-1}\cdot s^{-1}$ was chosen for the initial simulations.

Since ATP hydrolysis is likely to be rate-limiting for the release of BiP-bound proteins, an estimate of the rate of dissociation of the BiP-protein complex (k_h) can be obtained from the reported ATPase activity, 0.003 s^{-1}, from *in vitro* binding studies (Flynn et al., 1989). However, ATP hydrolysis by DnaK, another heat shock protein, is enhanced in the presence of accessory factors GrpE and DnaJ to rates as high as 0.35 s^{-1} (Liberek et al., 1991). DnaJ homologues have been found in yeast (Blumberg and Silver, 1991; Caplan et al., 1991; Luke et al., 1991). Therefore, the rate of release *in vivo* in the presence of accessory factors may be enhanced, and could fall in the range of 0.003–0.3 s^{-1}. An initial value of $k_h = 0.1$ s^{-1} is assumed. In the initial set of simulations, release of protein from the chaperone by ATP hydrolysis was also assumed to be accompanied by folding of secreted protein.

Individual protein characteristics are not considered in this model, and therefore all secreted proteins are considered together. Rothman has estimated the concentration of nascent chains within the ER at approximately 50 μM (Rothman, 1989). Cotranslational translocation was assumed to occur at approximately the rate of protein translation in eucaryotes ($\approx$ 2 amino acids/s) (Freedman, 1992). Given an average secretory protein size is 400 amino acids, the rate of appearance of new protein within the ER (V_p) is then 250 nM/s.

The native synthesis rate of chaperone (V_c) was not determined experimentally but was assumed to be one-tenth of all protein synthesis, or 25 nM/s, unless otherwise stated. The model additionally incorporates feedback on BiP synthesis from the level of unfolded proteins present. Experimentally, this corresponds to the increases in BiP synthesis when the cell is subjected to stress, such as increases in temperature, changes in carbon source, and accumulation of unfolded proteins. This feedback step, or "chaperone induction," is modeled as an exponential term based on the ratio of the unfolded protein concentration to the protein induction level (P_I), where BiP synthesis is increased in response to unfolded proteins. The value for P_I was originally chosen as ten times the level of nascent protein concentration in the endoplasmic reticulum, i.e., 500 μM (Rothman, 1989), but was also tested over several orders of magnitude.

As reviewed by Ptitsyn and Semisotnov (1991), the dominant folding time constant for proteins varies from 1 to 1,000 s. Self-folding, independent of chaperone (k_N) was neglected in the initial set of simulations. A value of 0.01 s^{-1} was chosen for folding (k_f) following release from the BiP-protein complex.

Aggregation is believed to result from hydrophobic interactions between unfolded, or misfolded, protein chains. In this model, aggregation is considered to be irreversible, leading eventually to degradation of the aggregated polypetides (Stafford and Bonifacino, 1991). Aggregation is not limited to a second-order binding between two unfolded proteins, but an aggregate of two or more proteins can also bind additional unfolded or partially folded proteins, leading to higher order aggregation. For ease of calculation and without likely loss of generality, aggregation is assumed to be second order in this model. A second-order rate constant of 1×10^5 $M^{-1}\cdot s^{-1}$ for aggregation of unfolded proteins has been measured *in vitro* (Zettlmeissl et al., 1979). However, *in vivo* many factors can alter this rate, including the possibility of chaperone-assisted dissociation of aggregates. It has been shown that DnaK can stimulate refolding of aggregated RNase *in vitro* (Skowyra et al., 1990), and that aggregates of influenza hemagglutinin are disassembled by an ATP-dependent mechanism that presumably involves BiP (Braakman et al., 1992). Therefore, second-order aggregation was assumed to occur at a lesser extent *in vivo*, with a rate constant of 1×10^3 $M^{-1}\cdot s^{-1}$.

Intracellular concentrations of the chaperone, unfolded protein, aggregate and bound complex decreased in the model through both dilution by growth and by degradation. The specific cell growth rate, μ, was taken as 1.4×10^{-4} s^{-1}, based on a doubling time of 2 h (Sherman, 1991). Degradation was assumed to occur at the same rate for chaperone, protein, and aggregate (k_t, k_d). Turnover based on a 30-min half-life was used in the initial set of simulations, and was varied over two orders of magnitude.

Differential equations were derived from the interactions described earlier, and shown schematically in Figure 3, where C is the chaperone, P is the newly translocated protein, A is aggregated protein, B is the bound protein–chaperone complex, and F is the folded protein destined for secretion. Standard values of the model parameters determined from the ranges tested are listed in Table 1. Figure captions indicate where parameter values deviate from those listed in the table.

$$\frac{d[C]}{dt} = V_{c0}[1+\eta(1-e^{-[P]/[P]_I})] - k_b[C][P] + k_h[B] - k_t[C] - \mu[C] - k_e[A][C] \quad (1)$$

$$\frac{d[P]}{dt} = V_p - k_a[P]^2 - k_b[P][C] - k_e[A][P] - \mu[P] - k_d[P] - k_N[P] \quad (2)$$

$$\frac{d[A]}{dt} = k_a[P]^2 + k_e[A][C] + k_e[A][P] - \mu[A] - k_d[A] \quad (3)$$

$$\frac{d[B]}{dt} = k_b[P][C] - k_h[B] - \mu[B] \quad (4)$$

Table 1. Model Parameters

Parameter	Definition	Std. Value	Range Tested	Reference
V_{c0}	Synthesis rate of chaperone	equal to V_p	0.1–10 V_p	
V_p	Synthesis rate of protein	2.5×10^{-7} M s^{-1}		Freedman, 1992
η	Induction ratio	10	0–10,000	
P_I	Protein concentration where induction occurs	5 μM	0.5–500 μM	Based on concentration of nascent chains (Rothman, 1989)
k_b	Rate of chaperone binding	30 M$^{-1}\cdot$s^{-1}	0.3–300 M$^{-1}\cdot$s^{-1}	Flynn et al., 1989
k_h	Rate of ATP hydrolysis (release by chaperone)	0.1 s^{-1}	0.01–10 s^{-1}	Flynn et al., 1989; Liberek et al., 1991)
k_t	Protein turnover rate	4×10^{-4} s^{-1}	4×10^{-3}–4×10^{-5} s^{-1}	30-min half-life
μ	Growth rate	1.4×10^{-4} s^{-1}	Doubling time of 2h	Sherman, 1991
k_a	Rate of aggregation	10^3 M$^{-1}\cdot$s^{-1}	1–10^5 M$^{-1}\cdot$s^{-1}	Zettlmeissl et al., 1979
k_e	Rate of entrapment	1 M$^{-1}\cdot$s^{-1}	1–10^5 M$^{-1}\cdot$s^{-1}	
k_N	Rate of self-folding	Initially 0	0–0.1 s^{-1}	Ptitsyn and Semisotnov, 1991
C_0	Initial chaperone concentration	2.0×10^{-4} M	10^{-3}–10^{-9} M	Based on 1% of cellular protein, yeast containing 6 pg/cell (Sherman, 1991)

$$\frac{d[F]}{dt} = k_h[B] + k_N[P]. \tag{5}$$

The linked differential equations were solved transiently using an adaptation of Newton's method, based on the Jacobian matrix (LSODE).

Model Behavior

The ability of the model to capture chaperone induction in the context of new protein synthesis was investigated. The initial intracellular concentrations for unfolded protein, bound complex, aggregate, and folded protein were set at zero. The initial chaperone value (C_0) was set at 0.5% of cellular protein. In yeast, where total protein in a haploid yeast cell is 6 pg/cell (Sherman, 1991) the molecular weight of BiP is 78 kDa, a doubling yeast cell is approximately a 2-μm sphere, and the ER is approximately 20% of the total volume, this yields a local (ER) concentration of 50 μM. The degree of cellular feedback on BiP synthesis as a result protein induction is modulated by the parameter η. Changing the value of η affected mainly the height and width of the peak in chaperone expression, but not the final chaperone concentrations following the induction (Figure 4). When η is very high, that is, $\eta > 100$, the time for induction drops. At this value, chaperone has been induced to over thirty times wild-type concentrations. In general, the observed increases have been on the order of two- to tenfold. Therefore, for the remaining simulations, η was assigned a value of 10.

The protein concentration where induction occurs, P_I, had a negligible effect on both the height and timing of the chaperone peak, as well as the final outcome of the simulation (data not shown). The rate of entrapment, k_e, strongly altered the rate of increase in chaperone levels and the breadth of the chaperone peak (Figure 5). The value of k_e was set at 1.0 M$^{-1}\cdot$s^{-1}, to mimic the experimental results shown in Figure 1. The model reproduced the experimental observa-

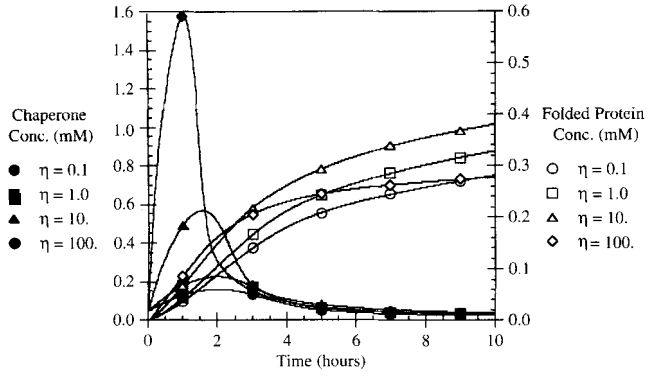

Figure 4. Induction ratio, η, alters the extent of chaperone induction.

A simulation of growth and protein secretion was run given an initial foreign protein concentration of 0 mM and an initial chaperone concentration of 0.2 mM. Time steps of 60 s in a 2500-step simulation are plotted as smooth lines, and symbols indicate every 500th step. Chaperone (C, open symbols) and folded protein (F, closed symbols) are given in units of local (intracellular) concentration (mM). Circles, $\eta = 0.1$; squares, $\eta = 1.0$; triangles, $\eta = 10.0$; diamonds, $\eta = 100$.

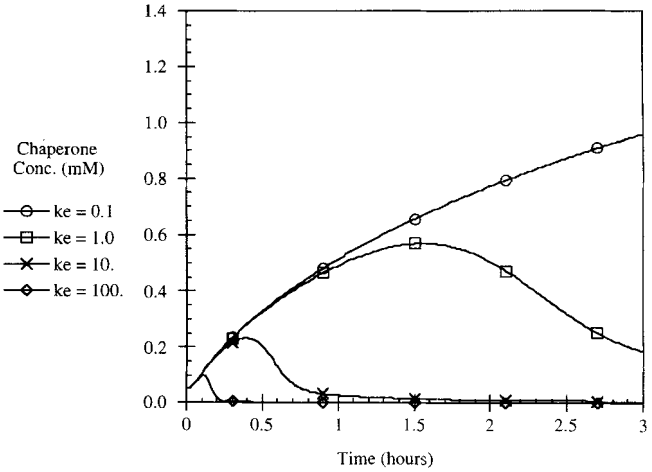

Figure 5. Rate of entrapment, k_e, determines the rate and breadth of chaperone induction.

The rate of entrapment k_e was varied over four orders of magnitude while other parameters were maintained at standard values. Time steps of 60 s in a 2500-step simulation are plotted as smooth lines, and symbols indicate every 500th step. Values for k_e are as follows: Circles, 0.1 M^{-1}s^{-1}; squares, 1.0 M^{-1}s^{-1}; crosses, 10.0 M^{-1}s^{-1}; diamonds, 100.0 M^{-1}s^{-1}. Chaperone concentration is given as the local, intracellular concentration.

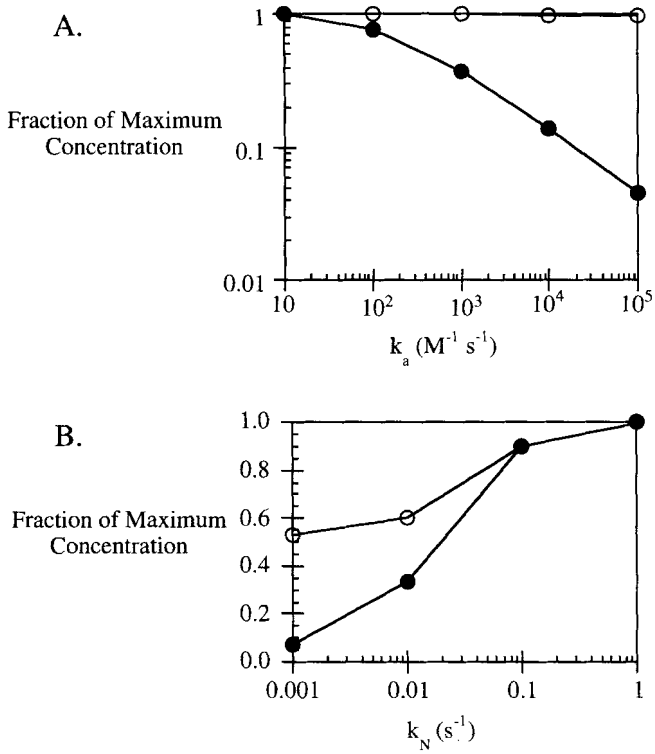

Figure 6. Protein secretion and chaperone levels are effected by the rate of aggregation (k_a) and the rate of protein self-folding (k_N).

Chaperone and protein concentrations in M are determined from the final steady-state results following a simulation run for a time length of 48 h, in 60-s time steps, and are compared to the maximum value for the set of simulations. (A) The rate of aggregation (k_a) was varied over five orders of magnitude while other parameters were maintained at the standard values. Open circles, chaperone; closed circles, secreted protein. (B) The rate of self-folding (k_N) was varied over four orders of magnitude while other parameters were held constant. The steady-state results are indicated by the open circles for chaperone; closed circles for secreted protein.

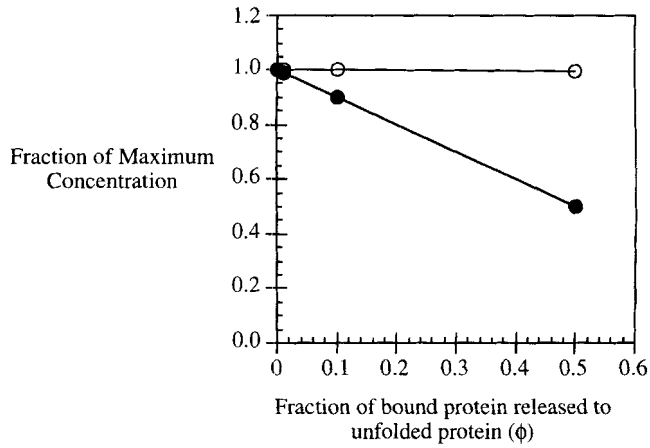

Figure 7. Fraction of protein that is released to the unfolded state alters the secreted protein level.

Chaperone and protein concentrations in M are determined from the final steady-state results following a simulation run for a time length of 48 h, in 60-s time steps, and compared to the maximum value for the set of simulations. The fraction (ϕ) of chaperone-bound protein that returns to the unfolded state U vs. that which folds to F was varied from 0 to 0.5. The steady-state results are indicated by the open circles for chaperone; or closed circles for secreted protein.

tion of a transient increase in chaperone concentration following protein induction.

The qualitative transient behavior of the model was largely insensitive to changes in all other parameters. However, several of the parameters had a measurable effect on the steady-state values of a transient solution, as described in detail in the following paragraphs and in Figures 6–11.

The rate of aggregation, k_a, has minimal effects on chaperone induction, but a major effect on the final value of folded protein (F) with little change in the final chaperone concentration (C) (see Figure 6A). The results of the simulation are also altered by varying the self-folding rate (k_N) (see Figure 6B). As the self-folding rate increases, both the intracellular chaperone concentration and the secreted protein concentration increase.

Initially in the model simulations, dissociation of the protein–chaperone complex resulted in the release of only folded protein and free chaperone. Within the cell, the outcome of dissociation is likely to be free chaperone and both unfolded and folded protein. Therefore, we examined the effect of varying ϕ, the fraction of protein that returns to unfolded protein. When more bound protein is released as unfolded protein, the lower the secreted protein (Figure 7).

The model was used to examine the experimental observation that decreases in intracellular chaperone and secreted protein occur when cells are allowed to age (Figure 2). To test the ability of the model to account for the data, an "aging" simulation was run using values for μ (growth rate) and for k_d (degradation) at 1/100 and 1/1,000 their normal (30°C) values to reflect slower growth and decreased enzymatic activity at 4°C. The final values for C, P, and A for given lengths of aging (from hours to weeks) were then set as the initial values for a "growth" simulation run under standard rate conditions. This represents a period of very slow growth at 4°C (aging) followed by inoculation and growth at 30°C.

Figure 8 shows the effect of the length of aging on values of chaperone and secreted protein concentrations. The only parameter that shows a strong effect on aging is k_d, the degradation rate chosen during the "growth" simulation (Figure 8). At the conclusion of aging, the level of aggregation is high, and a simulation at normal conditions produces a drop in aggregation due to turnover and dilution through growth. Therefore, the degradation rate seems to be a strong controlling factor in the aging phenomena, and higher levels of degradation lead to less dramatic aging effects. Intuitively, the faster the cell is able to turn over its contents, the better it is able to adapt to its new environment. Thus the model accurately reproduces the experimental observations, confirming that aggregation and entrapment can lead to a loss of chaperone and secreted protein, and that loss is increased by "aging" of the cells.

Model Predictions

Of exceptional interest was whether molecular parameters that can be altered can change secreted protein concentrations. It is particularly desirable to improve protein secretion

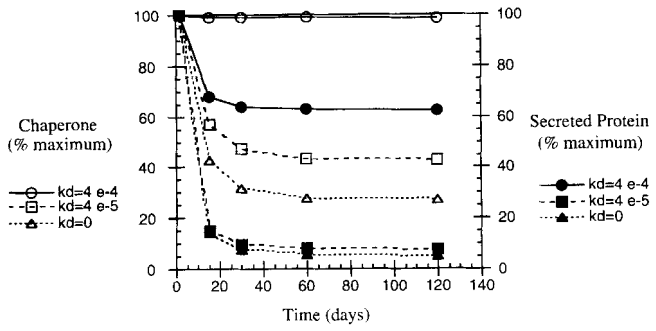

Figure 8. Effect of varying the degradation rate, k_d, on the extent of aging.

"Aging" of culture was simulated using the following initial conditions: $C = C_0$; $P = 0$; $B = 0$; $A = 0$; $F = 0$. During aging, μ (growth rate) and k_d (degradation) were used at 1/100 and 1/1000 their optimal values for induction. The final steady-state values obtained upon aging were then introduced into a simulation run for a time length of 48 h, in 60-s time steps, using standard parameter values listed in Table 1 with varying k_d. The steady-state results of this run were compared to the maximum value for the set of simulations, and are indicated by the open symbols, which designate chaperone levels, and the closed symbols, which designate protein levels. Circles, $k_d = 4 \times 10^{-4}$ s^{-1}; squares, $k_d = 4 \times 10^{-5}$ s^{-1}; triangles, $k_d = 0$ s^{-1}.

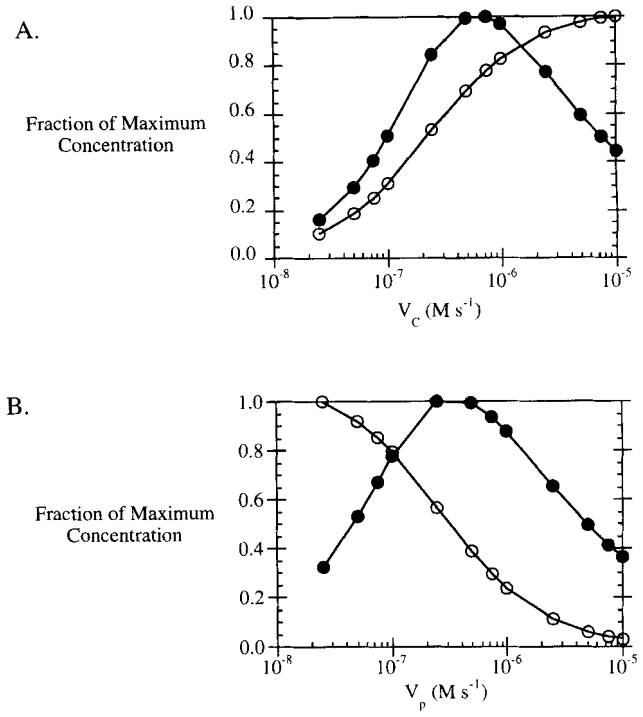

Figure 9. Protein secretion and chaperone concentration are affected by the rates of chaperone synthesis (V_{c0}) and protein synthesis (V_p).

Chaperone and protein concentrations in M are determined from the final steady-state results following a simulation run for a time length of 48 h, in 60-s time steps, and compared to the maximum value for the set of simulations. (A) The protein synthesis (V_p) rate was varied over four orders of magnitude while other parameters were maintained at standard values. Open circles, local chaperone concentration; closed circles, secreted protein concentration. (B) The rate of chaperone synthesis (V_{c0}) was varied over four orders of magnitude while other parameters were maintained at standard values. Steady-state results of the simulation are indicated by the open circles, chaperone concentration; or closed circles, secreted protein concentration.

for industrial and biochemical applications. The rate of chaperone synthesis (V_{c0}) and protein synthesis (V_p) are parameters that can be engineered in a cell through recombinant DNA technology. When all other parameters are fixed, chaperone synthesis (V_{c0}) and protein synthesis (V_p) both show an optimum for protein secretion (F) (Figure 9).

To investigate the possibility of an optimum ratio between chaperone and protein synthesis, both parameters were varied independently over four orders of magnitude (Figure 10). This analysis shows that protein secretion is best when the synthesis rates of chaperone and protein are equal, and has a maximum at the highest level tested (2.5×10^{-6} Ms^{-1}).

Other molecular parameters that were examined are the binding rate of the chaperone to protein (k_b) and the release of protein following ATP hydrolysis (k_h). These parameters could be altered experimentally through mutagenesis of the chaperone gene, although generally only the off-rate can be changed in an enzymatic reaction to yield increased affinity. In both cases the chaperone concentration was relatively unaffected. Increases in the rate of ATP hydrolysis led to decreases in the amount of secreted protein, while increases in the binding rate led to improved secretion (Figure 11). This indicates that chaperone binding can be limiting for secretion.

Discussion

A mechanistic model for the role of BiP in secretion has been developed. The model incorporates both increases in BiP synthesis by the presence of newly synthesized protein, and entrapment of both chaperone and protein by aggregates. The experimental observations of BiP function have been reproduced by the model. The increase in BiP synthesis following protein induction seen experimentally (Figure 1) is reflected in the model (Figure 4). Although our predictions are designed to reproduce experimental data from yeast, our results are likely more broadly applicable to higher eucaryotes, due to the high degree of functional similarity for BiP across species.

The model shows that chaperone decreases during long-term protein expression can be accounted for by including a term for nonspecific entrapment by protein aggregates. The rate of chaperone synthesis is strongly affected by this rate of entrapment. The rate of aggregation has a small effect on chaperone synthesis, but the duration of chaperone elevation, and the rate of depletion of chaperone following induction are most dramatically affected by entrapment (Figure 5). This indicates that proteins that are more likely to aggregate are less likely to be secreted.

The tendency of proteins to form aggregates or inclusion bodies *in vivo* is a phenomenon that has been extensively documented (for review, see Freedman, 1992), yet the factors or determinants for protein aggregation are largely unknown. Entrapment of chaperones into inclusion bodies has been reported during overexpression of foreign proteins in *E. coli* (Allen et al., 1992). Based on the functional similarities in heat shock proteins across species, the existence of a similar phenomenon in yeast cells seems likely. This idea is sup-

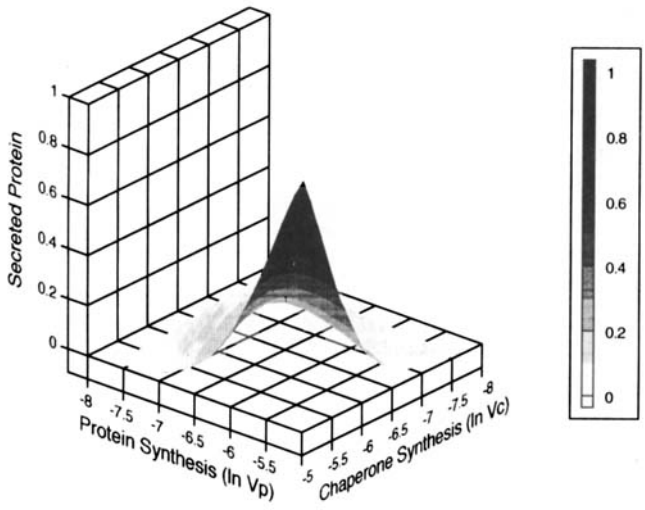

Figure 10. Optimum protein secretion is determined for different rates of chaperone synthesis (V_{c0}) and protein synthesis (V_p).

Folded protein concentrations (M) were determined from the final steady-state results following a simulation run for a time length of 48 h, 60-s time steps when V_p and V_{c0} parameters were varied from 2.5×10^{-8} to 2.5×10^{-6} Ms^{-1}, and compared to the maximum value for the set of simulations. The highest values for protein secretion are indicated by white (100% of maximum), black = 0%, grayscale is linear between 0 and 100%.

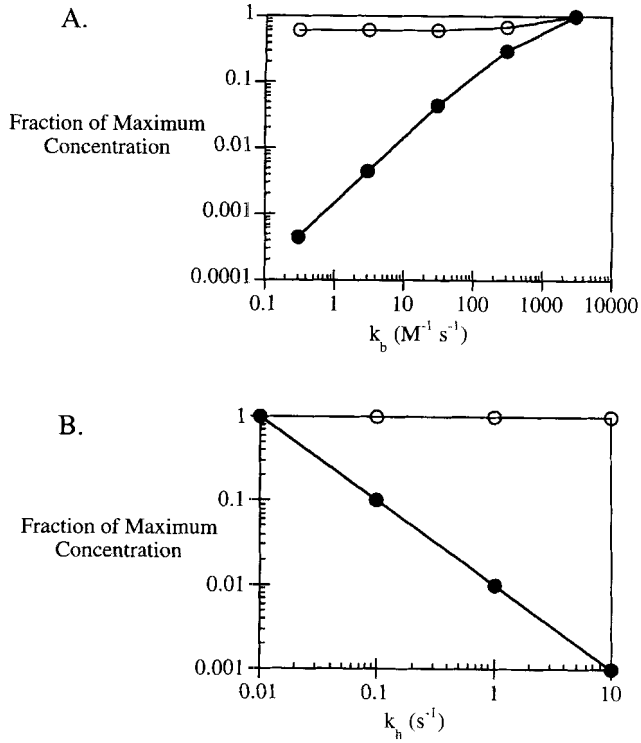

Figure 11. Protein secretion and chaperone concentration are affected by the rate of binding of protein to chaperone (k_b) and the rate of protein release (k_h).

Chaperone and protein concentrations (M) are determined from the final steady-state results following a simulation run for a time length of 48 h, in 60-s time steps, and compared to the maximum value for the set of simulations. (A) The rate of binding of protein to chaperone (k_b) was varied over five orders of magnitude while other parameters were maintained at standard values. (B) The rate of protein release following ATP hydrolysis (k_h) was varied over four orders of magnitude while other parameters were maintained at standard values. Steady-state results of the simulation are indicated by the open circles, chaperone concentration; or closed circles, secreted protein concentration.

ported by studies with temperature-sensitive ATPase mutants of yeast BiP. When yeast cells are shifted to the nonpermissive temperature, resulting in nonfunctional BiP, folding and transport of yeast carboxypeptidase Y (CPY) is inhibited, and CPY is sequestered in BiP-associated aggregates (Simons et al., 1995).

The effect of long-term protein expression on experimental decreases in chaperone and protein levels (Figure 2) are convincingly explained by the entrapment term included in model simulations (Figure 8). Interestingly, the turnover rate of chaperone, protein, and aggregate has the strongest effect on the magnitude of the decreases in both chaperone and protein concentrations. Contrary to intuition, as the turnover rate increases, protein secretion improves. This result can be explained by considering the mechanism for the disappearance. Examination of the kinetics of the disappearance during a model simulation (data not shown) indicates that aggregation followed by entrapment causes the decrease. Since in our model aggregation is second order, and folding is first order, successful folding of newly synthesized peptide chains is only possible when folding rates are competitive with aggregation. Degradation provides an opportunity for the cell to reduce the level of aggregation, thereby increasing final folding yields.

Clearly, aggregation is a dominating factor in determining folding yields. Increasing the rate constant for aggregation (k_a) by three orders of magnitude ($10\ M^{-1} \cdot s^{-1}$ to $10^4\ M^{-1} \cdot s^{-1}$) decreases final secreted protein yields following a 48-h batch growth simulation 100-fold (Figure 6A). The competition between the aggregation and folding rates is determined by the effectiveness of productive folding. Two avenues for folding exist: self-folding and folding while chaperone-bound. As the rate of self-folding increases, the level of secreted protein also increases (Figure 6B). However, the increase in yields is only marginal, that is, a tenfold rate of enhancement of self-folding yields a twofold rate of increase in protein concentration. These results imply that it is very important to limit off-pathway rates, that is, aggregation, and not as important to address self-folding, as increasing rates have only a marginal effect on secretion.

Another important parameter is the branching ratio between chaperone-bound protein that returns to the unfolded state vs. that which is released in the folded state. As expected, higher fractions of protein released to the unfolded state result in a decrease in secretion (Figure 7). Increased release leads to additional pools of unfolded protein that follow the aggregation pathway.

Of particular interest were model predictions for varying experimentally controllable parameters. Chaperone synthesis (V_{c0}) and protein synthesis (V_p) are attractive candidates for experimental changes, since varying plasmid copy number or gene-promoter strength are straightforward methods for controlling these parameters. When protein synthesis is fixed, low

rates of chaperone synthesis lead to a high concentration of unfolded protein within the cell (Figure 9A). As chaperone synthesis increases, the concentration of bound protein–chaperone complex increases, and secretion is improved. However, when the chaperone synthesis rate is very high (tenfold higher than protein synthesis) the release of protein from the chaperone limits the secretion rate. Analogously, as protein synthesis is increased, the amount of secreted protein increases (Figure 9B). However, high rates of protein synthesis lead to high aggregation, which ultimately lowers secretion. Varying both synthesis levels independently shows that optimal secretion is obtained at equimolar rates of synthesis (Figure 10). This suggests that both rates are important, and that for any given rate of protein synthesis from a native promoter, the rate of chaperone synthesis may need to be altered in order to optimize secretion. Improving production may therefore require *either* raising *or* lowering the chaperone synthesis rate.

Another attractive candidate for cellular engineering is the specificity of chaperone to protein binding, which is controlled by the binding rate of protein–chaperone complex (k_b) and the release rate from the chaperone (k_h). This specificity can be altered through mutagenesis of the chaperone, followed by careful *in vitro* studies to examine the effects of these changes on binding and ATP hydrolysis. Model results suggest that increased binding and decreased hydrolysis increase secretion (Figure 11). This supports the intuitive notion that increased chaperone affinity improves secretion; however, it simultaneously suggests that rapid release is also extremely important. Therefore optimal chaperone interaction requires a tight but transient interaction. Prolonged interaction actually decreases the final product yield.

One difficulty with exploring genetic changes in transcription rates and BiP interactions experimentally is the ramifications these effects can have on other cellular processes. In general, altering BiP levels experimentally has proved to be more complicated than previously thought (Robinson and Wittrup, 1995). By deleting the chromosomal copy of BiP, we have been able to vary BiP levels in cells expressing foreign proteins from 10% to 300% of control cells, leading to a better understanding of BiP function (Robinson et al., 1996). Post- translational regulation of BiP by degradation or inactivation may be more difficult to control. However, protein synthesis rates and protein interactions with BiP are attractive targets of molecular and cellular engineering that have not been fully explored.

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Parameters

k_r = rate of release of bound chaperone–protein complex, B, due to ATP hydrolysis, s^{-1}
t = time, s

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