Protein Folding Stability Can Determine the Efficiency of Escape from Endoplasmic Reticulum Quality Control*

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A fraction of each secreted protein is retained and degraded by the endoplasmic reticulum (ER) quality control apparatus that restricts export to correctly folded proteins. The intrinsic biophysical attributes that determine efficiency of escape from this proofreading process have been examined by expressing mutants of bovine pancreatic trypsin inhibitor (BPTI) in yeast. Secretion efficiency is strongly correlated with thermodynamic stability for a series of six point mutations of BPTI. No correlation of secretion efficiency with either oxidative folding or refolding rates in vitro is found; both the rapidly folded Y35L BPTI mutant and the slowly unfolded G36D BPTI mutant exhibit low secretion efficiency. Elimination of cysteines 14 and 38 by mutagenesis does not increase secretion efficiency, indicating that intramolecular thiol/disulfide rearrangements are not primarily responsible for retention and degradation of destabilized BPTI variants. Mutant yeast strains with diminished ER-associated degradation do not secrete BPTI more efficiently, indicating that retention and degradation are separable processes. These data support a model for ER quality control, wherein protein folding is functionally reversible and the relative rates of folding, unfolding, vesicular export, and retention determine secretion efficiency.

The quality control system of the endoplasmic reticulum allows export of only correctly folded and assembled proteins (1.) The components of the quality control system include the calnexin/glucosylation cycle (2–4), persistent binding to endoplasmic reticulum (ER)1 -retained chaperones such as BiP or GRP94 (5–7), and exclusion of large aggregates from transport vesicles budding off from the ER (8–9). In addition to complete retention of grossly misfolded proteins, some fraction of every secreted protein is retained and degraded by the quality control system. Secretion efficiency varies markedly among proteins and can be particularly low for overexpressed heterologous, secreted proteins (10–15).

It is not obvious what intrinsic biophysical properties determine a given protein’s secretion efficiency. Folding rate could predominate, if folded protein rapidly escapes from the proofreading apparatus by exiting the ER promptly upon achieving a folded conformation. Alternatively, thermodynamic folding stability could determine secretion efficiency by defining a rapidly equilibrated partition between conformations subject to two irreversible processes: degradation of the unfolded form versus export of the folded form. Yet another possibility is that the varying strength of currently ill-defined ER export signals affects ER residence time (16), hence exposing proteins to potential retention and degradation for varying lengths of time. In all likelihood, each of these properties influences secretion efficiency to some extent, with particular attributes accentuated for particular proteins.

It was recently shown that secretion efficiency in yeast for bovine pancreatic trypsin inhibitor mutants lacking disulfide bonds is correlated with thermodynamic stability but not with reported in vitro folding rates (17). Because removal of cysteines by site-directed mutagenesis qualitatively alters the oxidative folding pathway, the linkage between stability and secretion efficiency was tested further in the present study by examining six point mutants of BPTI that destabilize the surface 14–38 disulfide (18). For these mutants, secretion efficiency is strongly correlated with the destabilization free energy of the 14–38 disulfide but is not predicted by in vitro folding or unfolding rates. These results support a model for BPTI quality control in which a variable fraction of the protein is degraded while the folded form awaits export from the ER, implying that folding of even relatively stable proteins is functionally reversible in the ER.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—Cultures of S. cerevisiae strain BJ5464 (α ura3–52 trp1 leu2Δ his3Δ200 pep4::HIS3 prb1Δ6.1′ GAL can1 GAL) were obtained from the Yeast Genetic Stock Center (Berkeley, CA). BJ5464 is deficient in vacuolar proteases, reducing proteolytic artifacts during cell extract preparation (19). Strain YVH10 for overexpression of PDI was derived from the parent strain BJ5464 (20). It contains an integrated copy of the yeast PDI gene under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, resulting in 15-fold overexpression of yeast PDI. Strains W303-BA (MATα ura3::URA3 ura3–1 his3–11,15 leu2–3, 112 trp1–1 ade2–1α can1–1100), W303-CQ (MATα prc1–1 ubc7::LEU2 ura3–1 his3–11,15 leu2–3, 112 trp1–1 ade2–1α can1–100), and W303-1b (MATα ura3–1 his3–11,15 leu2–3, 112 trp1–1 ade2–1α can1–100) were gifts of D. Wolf. All yeast transformations were performed by electroporation using a Bio-Rad Gene-Pulser (21). Transformants were selected on minimal medium containing casamino acid supplement (SD-CAA + uracil) buffered with sodium phosphate buffer (50 mM, pH 6.0) (22). Independent transformant colonies were grown in culture tubes with 5 ml of liquid SD-CAA + uracil media for 96 h prior to measurement of BPTI secretion.

Plasmids and Mutant Construction—The plasmid used to express EA-BPTI in Saccharomyces cerevisiae was constructed by subcloning the expression cassette from the vector pUC-G-BPTI (23) into the multiple yeast shuttle vector YEplac112 (24) as an EcoRI-BamHI fragment. The resulting plasmid is referred to as YE112-GPD-BPTI, BPTI is expressed using the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase promoter. A synthetic preproleader is used to target BPTI to the ER (23). The preregion contains a dibasic Lys-Arg site at its N terminus to allow cleavage by the Kex2p protease. This results in a
Glut-AAla extension at the N terminus of the secreted, mature BPTI. The construction of the C14/38A BPTI plasmid has been described previously (17). This plasmid is referred to as YE112-GPD-BPTI-C14/38A.

Mutations to YE112-GPD-BPTI and YE112-GPD-BPTI-C14/38A plasmids were introduced using an oligonucleotide-directed mutagenesis kit (Morph site-specific plasmid DNA mutagenesis kit, 5 Prime–3 Prime, Inc.). Each oligonucleotide introduced the indicated amino acid change, as well as a silent mutation that introduced a restriction site to aid in screening of the mutant colonies. The following oligonucleotides were used for each mutant: Y35F, CCTTGGTTTTCGGTGGTTGTAGGGCTAAGAGAAACAACTTCAAGTCCGC; P13S, TTATCAG; G36D, GGCTGGTTTGTGTCAAACGTTCGTTTACGAGCTAAG, G37A, 9.3 ± 1.5; A16V, GGAACCACCGTATACGGGCCCATGTAAGGTTAGAAG; Y35L 1.9 ± 0.6; G36A 19.1 ± 0.4; ND 25.6 ± 0.3; P13S + C14/38A 8.1 ± 0.6; Y35L + C14/38A 2.1 ± 0.5.

TABLE I

| Form of BPTI expressed | BJS546 (wild-type yeast) | YVH10 (wild-type isogenic overexpression) | W303–1b (wild-type isogenic to BD and CQ) | W303–BD Δ DER1 | W303–CQ Δ UBC7 |
|------------------------|-------------------------|------------------------------------------|------------------------------------------|----------------|----------------|
| WT BPTI                | 23.6 ± 1.4              | 7.5 ± 0.9                                | 24.0 ± 0.5                               | 12.6 ± 9.2     | 13.7 ± 6.6     |
| Y35F                   | 15.8 ± 0.4              | 6.2 ± 0.1                                | ND                                       | ND             | ND             |
| P13S                   | 11.6 ± 1.2              | 4.0 ± 0.3                                | 14.1 ± 0.6                               | 13.2 ± 1.2     | 11.3 ± 1.0     |
| G37A                   | 9.3 ± 1.5               | 4.2 ± 0.5                                | ND                                       | ND             | ND             |
| A16V                   | 12.7 ± 1.3              | 4.8 ± 0.6                                | ND                                       | ND             | ND             |
| G36D                   | 10.4 ± 0.5              | ND                                       | ND                                       | ND             | ND             |
| Y35L                   | 1.9 ± 0.6               | 0.9 ± 0.5                                | 8.5 ± 0.3                                | 7.0 ± 0.4      | 6.3 ± 0.5      |
| G36A                   | 19.1 ± 0.4              | ND                                       | ND                                       | ND             | ND             |
| P13S + C14/38A         | 8.1 ± 0.6               | 2.5 ± 0.3                                | ND                                       | ND             | ND             |
| Y35L + C14/38A         | 2.1 ± 0.5               | 0.4 ± 0.6                                | ND                                       | ND             | ND             |

RESULTS

Yeast Secretion Efficiency of Six BPTI Point Mutants—A series of point mutations that destabilize the 14–38 disulfide of BPTI by 0.6–3.7 kcal/mol (18) were selected for examination of secretion efficiency from yeast. These mutations were introduced into a yeast expression and secretion vector for BPTI (23), and BPTI secretion levels were determined (Table I.) The observed variation in secreted BPTI was not because of variable stability in the culture medium because secreted BPTI mutant activities were stable for at least one week in the growth culture under these experimental conditions, in the presence or absence of 1 mg/ml bovine serum albumin as a carrier protein.

To confirm that the observed variation in secreted BPTI levels is attributable to variable secretion efficiency, pulse-chase radiolabeling experiments were performed for WT and two mutant forms of BPTI. Yeast cultures expressing wild-type BPTI, P13S BPTI, or Y35L BPTI were labeled with $^{35}$S-met-
cultures expressing and secreting BPTI were labeled with \(^{35}\text{S}\) methionine for 1 min, and BPTI secretion kinetics were quantified by SDS-PAGE and autoradiography as described under "Experimental Procedures." Levels of secreted BPTI were normalized by the amount of WT BPTI secreted following 4 h of chase time. Similar levels of each construct were synthesized during the labeling pulse, as a percentage of total protein: WT (1.4 ± 0.3%), P13S (1.4 ± 0.3%), Y35L (1.4 ± 0.3%), and Y35L (1.4 ± 0.3%).

The rate of appearance of radiolabeled BPTI in the culture supernatant is shown in Fig. 1. Two h following the radiolabeling pulse, levels of secreted radiolabeled P13S and Y35L BPTI relative to wild-type BPTI parallel total secretion levels in Table I. Wild-type BPTI continues to accumulate intracellularly for each construct as a percentage of total protein: 1.4 ± 0.3% for wild-type BPTI, 1.2 ± 0.3% for P13S BPTI, and 1.4 ± 0.3% for Y35L BPTI. This result indicates an absence of substantial variation in pre-ER processes such as plasmid stability, transcription, translation, or ER membrane translocation. These percentages are in quantitative agreement with earlier measurements for C14/38A, C30/51A, and C5/55A BPTI in the same expression system under the same experimental conditions (17).

The rate of appearance of radiolabeled BPTI in the culture supernatant is shown in Fig. 1. Two h following the radiolabeling pulse, levels of secreted radiolabeled P13S and Y35L BPTI relative to wild-type BPTI parallel total secretion levels in Table I. Wild-type BPTI continues to accumulate in the medium 30 min following the radiolabeling pulse, whereas the less stable mutant P13S BPTI reaches its maximum secretion level within 30 min. Similar secretion kinetics were observed previously for wild-type BPTI relative to the destabilized mutants C14/38A and C30/51A BPTI (17), with the less stable mutants ceasing accumulation in the medium by 1 h of chase time, while WT BPTI continues to accumulate.

Secretion levels are strongly correlated with the reported extent of destabilization of the 14–38 disulfide (Fig. 2). Secretion efficiency is not predicted by the \textit{in vitro} reduction rates because G36D BPTI actually displays a slower \textit{in vitro} reduction rate for the 14–38 disulfide (18) yet is secreted less efficiently than WT BPTI, while each of the other five mutants have faster reduction rates than WT BPTI. Secretion efficiency is also not predicted by the \textit{in vitro} oxidative folding rate because Y35L BPTI has been shown to fold at an order of magnitude faster than wild-type BPTI \textit{in vitro} (27). The correlation between thermodynamic stability and secretion shown in Fig. 2, together with the absence of such a correlation with \textit{in vitro} oxidative folding or reduction rate, supports the hypothesis that a pseudo-steady-state equilibration between the folded and unfolded states determines the efficiency of escape from the ER quality control system for this series of BPTI mutants.

Secretion Efficiency Does Not Depend on Intramolecular Thiol/Disulfide Exchange with Cysteines 14 and 38—Removal of cysteines 14 and 38 by mutagenesis indicates that folding instability rather than destabilization of the 14–38 disulfide \textit{per se} is responsible for reduced secretion efficiency. Following reduction of the 14–38 disulfide, BPTI unfolding can proceed both by intramolecular attack on the buried 30–51 or 5–55 disulfides by the newly liberated cysteine 14 or 38 thiols, or by intermolecular attack of the buried 30–51 and 5–55 disulfides by a reducing agent (28). To determine whether an increased presence of free cysteine 14 and 38 thiols is responsible for the observed reduction in secretion efficiency of P13S and Y35L BPTI, we introduced the C14/38A mutations into these expression constructs. If the intramolecular rearrangement pathway were primarily responsible for misfolding, retention, and degradation of P13S and Y35L BPTI, then removal of cysteines 14 and 38 should produce a marked stabilization of these forms, leading to increased secretion efficiency. This is not the case, as indicated in Table I and Fig. 3. Loss of the 14–38 disulfide reduces secretion of wild-type, P13S, and Y35L BPTI by similar proportions. These results indicate that destabilization of the folded conformation rather than the increased presence of Cys-14 and Cys-38 thiols is primarily responsible for reduced secretion. As discussed in further detail below, thermodynamic linkage between disulfide redox equilibria and folding stability have been established previously for other mutants of BPTI, DsbA, and thioredoxin.

Increased PDI Levels Decrease Secretion of All Mutants Proportionally—Overexpression of PDI in yeast can increase secretion of some proteins by as much as an order of magnitude (20, 29), yet overexpression of PDI reduces secretion of BPTI (Ref. 17, Table I, Fig. 4). If either oxidative folding or reductive unfolding rates alone determined the efficiency of quality con-
trol escape for these BPTI mutants, then increased PDI levels might be expected to exert differential effects among the mutants. However, PDI overexpression reduces secretion of wild-type, Y35F, A16V, P13S, G37A, Y35L, G36D, P13S + C14/38A, Y35L + C14/38A BPTI proportionally (Fig. 4). The correlation of secretion efficiency with thermodynamic stability therefore persists under substantially altered conditions for redox folding catalysis in the ER. Of course, a redox folding catalyst such as PDI accelerates the oxidative folding and unfolding kinetics of a protein without altering its underlying thermodynamic

FIG. 3. Negligible effect on Y35L, P13S, and WT BPTI secretion upon mutation of cysteines 14 and 38 to alanines. If intramolecular thiol/disulfide rearrangements with cysteines 14 and 38 were involved in ER retention, the C14/38A mutation would increase secretion efficiency.

FIG. 4. PDI overexpression reduces secretion of WT & 8 BPTI mutants (Y35F, A16V, P13S, G37A, Y35L, G36D, P13S + C14/38A, Y35L + C14/38A) by a constant proportion. The correlation between thermodynamic stability and secretion efficiency persists under conditions in which the rates of oxidative folding and reductive unfolding are accelerated by increased PDI. The mechanism for reduced secretion efficiency upon PDI overexpression is unknown.

FIG. 5. Secretion efficiency is not increased upon genetic diminishment of ER-associated degradation. Secretion of WT, P13S, and Y35L were measured in yeast strains in which the genes encoding Der1p and Ubc7p, key components of this proteolytic pathway, are deleted. Open bars, W303–1b, wild-type control strain; shaded bars, W303–BD (ΔDER1); striped bars, W303–CQ (ΔUBC7).

ER-associated Degradation Mutations Do Not Increase BPTI Secretion Efficiency—Because the extent of retention and proteolysis of unfolded protein determines secretion efficiency (Fig. 1), one might expect that loss of key components of the degradation pathway might increase secretion efficiency. This was not found to be the case however (Fig. 5). Secretion of wild-type, P13S, and Y35L BPTI was unaffected by loss of function of the DER1 and UBC7 genes, which are required for efficient ER-associated degradation (32–33). Thus, BPTI secretion efficiency is not increased in the absence of a fully functional ER-associated degradation apparatus. It is interesting that greater clonal variation of WT BPTI secretion occurred in the ER degradation-deficient mutant strains; this is because of the presence of several cultures in which secretion dropped precipitously to ~ 5 mg/liter. Such variation was not observed for P13S or Y35L BPTI secretion in these strains.

DISCUSSION

The data presented here supports a model for ER quality control whereby thermodynamic stability can determine the efficiency of escape from retention and degradation. Such a correlation was also observed for mutants of BPTI with cysteine/alanine replacements (17). A kinetic model for this pathway is represented schematically in Fig. 6. If folded protein were promptly exported from the ER by vesicular transport, one would expect secretion efficiency to be determined chiefly by the rate of folding. However, BPTI mutants that fold more rapidly than wild-type in vitro (Y35L and C30/51A BPTI) are secreted less efficiently than wild-type (Table I, Fig. 2; Ref. 17). The ER export rate for folded protein is determined by an ill-characterized process of protein cargo concentration into vesicles budding from the ER membrane (16). The nature of the required export signals and possible cargo receptors for soluble proteins are still uncertain, although Emp24p is a candidate yeast cargo receptor for some proteins (34–35). If a protein folds rapidly but is delayed in export from the ER because of slow or inefficient cargo loading into transport vesicles, then a

2 J. M. Kowalski, R. N. Parekh, J. Mao, K. D. Wittrup, manuscript in preparation.
greater fraction of protein may be unfolded and degraded while awaiting export. Consistent with this scenario, the half-time for appearance of WT BPTI in the growth medium is approximately 30 min (Fig. 1) whereas the half time for folding, as assayed by binding to trypsin-Sepharose, is 2–5 min.2 BPTI therefore persists intracellularly with a half time of approximately 30 min following completion of folding, during which time it may be partially unfolded, retained, and degraded, with the extent of such losses correlated with stability.

Retention and degradation are represented as separable processes in Fig. 6 because loss of components of the degradation pathway (Der1p and Ubc7p) does not shift the balance toward secretion (Fig. 5). ER retention could result either from physical binding to an ER-resident component of the quality control apparatus or exclusion from export vesicles. A constant fraction of WT, P13S, and Y35L BPTI are retained by the quality control apparatus even in the absence of a degradation sink for the retained material (Fig. 5), indicating that any retention receptors are not saturable at this level of multiplicity expression. Calnexin has been shown to serve as a retention receptor for glycosylated proteins; however, BPTI is not glycosylated. Furthermore, deletion of the yeast calnexin homolog CNE1 does not increase the secretion efficiency of wild-type, P13S, or Y35L BPTI (data not shown). The identity of a quality control receptor responsible for BPTI retention has not been determined.

It should be emphasized that although secretion is correlated with thermodynamic stability, true equilibrium between the folded and unfolded forms cannot exist in the ER because of the presence of irreversible rate processes: degradation and ER export. Rather, a pseudo-steady-state partition between folded and unfolded proteins is attained. Of course, if the rates of degradation and export are much slower than the rates of folding and unfolding, then the pseudo-steady-state ratio of folded to unfolded protein could closely approximate the equilibrium value. Given wild-type BPTI's extreme stability (Tm = 105 °C, Ref. 36), even the 3.7 kcal/mol of 14–38 disulfide destabilization produced by the Y35L mutation should yield a protein that is mostly folded at equilibrium at 30 °C. The precipitous drop in secretion efficiency for Y35L BPTI would be difficult to explain strictly on the basis of the small fraction of unfolded protein at equilibrium in vitro. However, the presence of high concentrations of protein folding chaperones such as BiP and PDI in the ER are likely to shift the folding equilibrium toward the unfolded state by Le Châtelier’s principle, stabilizing partially unfolded conformers through chaperone binding free energy. Chaperones as structurally and functionally diverse as GroEL and SecB have been shown to catalyze deuterium exchange with normally protected amides proteins in folded proteins (37–38). Such “unfoldase” activity would be strongly present in the ER lumen given the high concentration of protein folding chaperones in this organelle. Therefore, the unfolded protein fraction vulnerable to retention and degradation would be expected to be larger than the equilibrium unfolded fraction in vitro.

There are several precedents for thermodynamic stability determining the half-life of proteins in vivo. Mutants of the bacteriophage λ cro protein with varying stability exhibit a correlation between increasing stability and decreasing degradation in the cytoplasm of Escherichia coli (39). Degradation and resulting expression yield in the E. coli periplasm also correlates with thermodynamic stability of mutants of barnase (40). The metabolic stability in E. coli of T4 lysozyme mutants correlates roughly with their thermal stability with several clear exceptions (41); the same general correlation between thermal and metabolic stability with several distinct exceptions is also observed for stability of radiolabeled T4 lysozyme mutants injected into HeLa cells (42).

The available data indicate that secretion efficiency depends on the stability of the BPTI fold itself, rather than directly on the stability of the 14–38 disulfide. Because removal of cysteines 14 and 38 by mutagenesis does not itself substantially reduce secretion efficiency of otherwise wild-type BPTI (Table I and Fig. 3, Ref. 17), destabilization of this disulfide per se is unlikely to directly reduce secretion efficiency. Furthermore, misfolding because of intramolecular thiol/disulfide rearrangements with free cysteines 14 and 38 cannot be responsible for reduced secretion of P13S or Y35L BPTI because the C14/38A mutation does not increase secretion of these mutants (Table I, Fig. 3). Destabilization of the 14–38 disulfide therefore must correspond to destabilization of the folded form of BPTI in the absence of the 14–38 disulfide. Such thermodynamic linkage between disulfide stability and folding stability has been demonstrated for several BPTI mutants previously (43). This linkage has also been demonstrated for mutants of DbA (44) and thioredoxin (45).

The kinetic model presented in Fig. 6 leads to several alternative limiting regimes depending on a given protein’s rates of folding and unfolding relative to the rates of export and retention. For BPTI and the mutants examined to date, it appears that the folding and unfolding rates in vivo are substantially more rapid than export and retention rates, leading to a rapid equilibrium relative to the irreversible processes leading to export or degradation. For proteins that fold more slowly or are exported more rapidly following folding, such equilibration would not occur and the folding rate would be expected to determine secretion efficiency. The intrinsic biophysical attributes of folded BPTI that lead to its slow export kinetics from the ER are currently under investigation.

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Secretion Efficiency Correlates with Folding Stability

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