Secretion Efficiency in *Saccharomyces cerevisiae* of Bovine Pancreatic Trypsin Inhibitor Mutants Lacking Disulfide Bonds Is Correlated with Thermodynamic Stability†

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ABSTRACT: Bovine pancreatic trypsin inhibitor (BPTI) has been widely used as a model protein to investigate protein structure and folding pathways. To study the role of its three disulfide bonds in folding, proofreading, and secretion of BPTI in an intact eucaryotic cell, BPTI was expressed and secreted from a synthetic gene in the yeast *Saccharomyces cerevisiae*. Site-directed mutagenesis was used to create all possible single and pairwise cysteine to alanine BPTI mutants, and the effect of these mutations on secretion efficiency was determined. The 5−55 disulfide bond is found to be essential for secretion—loss of either Cys5, Cys55, or both prevents secretion. Removal of the 14−38 disulfide bond results in a small reduction of secretion, but individual Cys14 or Cys38 replacements reduce secretion efficiency by 30%. Cys30 and Cys30−51 mutants are secreted at half the level of wild-type BPTI, while secretion of the Cys51 mutant is reduced by 90%. BPTI containing only a single disulfide bond (5−55) is not secreted. No relationship is observed between secretion efficiency and in vitro folding or unfolding rates, but mutant BPTI secretion is directly correlated with the in vitro unfolding temperature T_m and the free energy of stabilization provided by each of the three disulfides. These results indicate that structural fluctuations rather than the time-averaged structure observed by NMR or X-ray crystallography may determine recognition of a protein as misfolded and subsequent retention and degradation.

The rate-limiting step in protein secretion is often protein folding in the lumen of the endoplasmic reticulum (ER),[1] since proteins exit the ER only after folding and subunit assembly (Gething & Sambrook, 1990; Helenius et al., 1992; Hammond & Helenius, 1995). Most secreted eucaryotic proteins contain disulfide bonds that stabilize the native structure. The kinetics of disulfide bond formation and interchange can effectively determine the rate and yield of folding and, hence, secretion (Freedman, 1995; Creighton 1992; Bardwell, 1994; Gilbert, 1994; Wittrup, 1995). The biological function of disulfide bonds includes thermodynamic aspects (stabilization of protein structure) as well as kinetic aspects (determining the pathway and efficiency of protein folding). Misfolded secretory proteins are retained in the ER by a conformational proofreading apparatus and eventually degraded (Bonifacino & Lippincott-Schwartz, 1991). Although it is clear that grossly misfolded proteins are retained by the proofreading pathway, the limits of detection by this system are not yet known. For example, a marginally stable protein will equilibrate between the folded conformation and an ensemble of unfolded states that could potentially be recognized as unfolded, causing the protein to be retained and degraded. By shifting the equilibrium toward the folded state, disulfide bonds may increase the efficiency of escape from the retention/degradation pathway.

Disulfide formation kinetics can play a decisive role in determining the rate and efficiency of protein folding in the ER. Loss of a native disulfide bond generally reduces stability, but this modification can also affect the kinetics of protein secretion in unpredictable ways, ranging from extensive aggregation and ER retention to dramatic acceleration of secretion. The role of disulfide bonds in exit from the ER has been examined in a number of studies in which disulfide bonds were eliminated by site-directed mutagenesis. The role of each of the four disulfide bonds of human lysozyme in folding and secretion in yeast has been extensively studied by Kikuchi and co-workers (Taniyama et al., 1988; Omura et al., 1992). Individual removal of three disulfide bonds (6−128, 30−116, 65−81) essentially eliminates folding and secretion. By contrast, alanine replacement of cysteines 77 and 95 results in an 8-fold increase of secreted lysozyme in yeast and also accelerates secretion kinetics in mammalian cells. The folding pathway of human chorionic gonadotropin β subunit (hCGβ) in vivo and the consequences of replacing alanines for each cysteine pair in hCGβ, which possesses six disulfide bonds, have been examined (Suganuma et al., 1989; Bedows et al., 1993). The loss of disulfides that form early in the folding pathway retard secretion, while removal of two of the disulfides that form late in the pathway results in no substantial effect. Interestingly, removal of the 26−110 disulfide, which forms late in the pathway, substantially increases the secretion rate ($t_{1/2}$ = 34 min as compared to $t_{1/2}$ = 150 min for wt). Elimination of three of the six disulfides in influenza hemagglutinin reveals a critical role for these bonds, since loss of any one

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[1] Abbreviations: ER, endoplasmic reticulum; BPTI, bovine pancreatic trypsin inhibitor; wt, wild type.

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of these disulfides results in misfolding, aggregation, and defective transport (Segal et al., 1992). These results highlight the important role of some disulfide bonds in promoting intramolecular folding reactions relative to intermolecular aggregation.

The global significance of disulfide formation in secretory protein processing has been demonstrated by treatment of whole cells with reducing agents such as DTT (Braakman et al., 1992; Lodish & Kong, 1993; Simons et al., 1995). Proteins requiring disulfide bonds are retained in the ER after synthesis in the presence of DTT and are post-translationally folded and secreted upon re-establishment of ER oxidative homeostasis following a change to DTT-free growth medium.

We have expressed and secreted bovine pancreatic trypsin inhibitor (BPTI) in *Saccharomyces cerevisiae* as a model system to study the interplay between disulfide formation and the ER conformational proofreading pathway. BPTI is a small (58 aa) polypeptide with a single domain structure and three disulfide bonds: Cys14−Cys38, Cys5−Cys55, and Cys30−Cys51. The depth of structural, kinetic, and thermodynamic knowledge available concerning BPTI folding in vitro makes this an attractive model protein. The three-dimensional structures of the native protein and many cysteine replacement mutants have been characterized in detail using 2-D NMR and X-ray crystallography (van Mierlo et al., 1991a,b, 1993; Schulman & Kim, 1994; Staley & Kim, 1994; Pan et al., 1995; Kim & Woodward, 1993; Berndt et al., 1993). BPTI lacking any individual disulfide possesses essentially native structure and inhibits trypsin stoichiometrically. BPTI possessing only the 5−55 disulfide is also nativelike and inhibits trypsin. The apparent structural dispensability of each individual disulfide raises the following question: what biological function exerted evolutionary selection pressure for the presence of all three?

The oxidative in vitro folding pathway of BPTI has been studied in depth in terms of accumulation of disulfide bonded intermediates during folding (Creighton, 1992; Weissman & Kim, 1991; Goldenberg 1992). The two-disulfide intermediates that accumulate during refolding are commonly referred to as N′ (30−51, 14−38), N* (5−55, 14−38), and $N^{SH,SH}$ (30−51, 5−55). The rate-limiting step in folding in vitro is the rearrangement of N′ to $N^{SH,SH}$, which is then quickly oxidized to the native three-disulfide form. In vitro, at neutral pH, about half the molecules become trapped as N*, which is a dead- end intermediate that is stable for weeks. N* and to a lesser extent N′ have a nativelike structure that buries free cysteine thiols and inhibits formation of mixed or non-native disulfides necessary for formation of the second buried disulfide (Weissman & Kim, 1995; Creighton & Goldenberg, 1984). In vitro refolding rates for BPTI molecules lacking two of the three disulfide bonds have been reported. C30/51A BPTI folds more rapidly than wild-type (wt) BPTI, presumably because the 30−51 bond cannot sterically hinder formation of the 5−55 bond (Marks et al., 1987a). The in vivo function of the (30−51) disulfide is not apparent from in vitro thermodynamic and kinetic data, since its loss negligibly perturbs BPTI structure and function and actually accelerates folding. C14/38A BPTI folds more slowly in vitro than wt BPTI since neither of the two surface cysteines are available for formation of non-native disulfide intermediates (Marks et al., 1987b).

BPTI folding has also been studied in microsomes (Creighton et al., 1993; Zapun et al., 1992) and in the *E. coli* periplasm (Ostermeier & Georgiou, 1994, Ostermeier et al., 1996). In both cases, folding is significantly more rapid than in vitro, though apparently following essentially the same pathway as elucidated in vitro. Protein disulfide isomerase (PDI) activity can account for the observed folding rate acceleration in microsomes, and PDI expression in the bacterial periplasm increases BPTI folding yield severalfold. Although the bacterial and microsomal systems demonstrate the significance of foldases such as PDI and DsbA in BPTI folding, they cannot reproduce the conformational proofreading process that ultimately determines whether a molecule is retained in the ER or secreted.

The present study was performed to examine how BPTI mutants with known structure, stability, and in vitro folding kinetics interact with the folding and conformational proofreading machinery in an intact eucaryotic secretory pathway. We have previously reported development of a system to express and secrete BPTI in *S. cerevisiae* (Parekh et al., 1995). Site-directed mutagenesis was used to express single and pairwise cysteine to alanine mutants of BPTI in *S. cerevisiae*, and secretion levels were measured by both activity assay and densitometry of Coomassie-stained SDS−PAGE. Antiserum against unfolded forms of BPTI was used to probe intracellular accumulation by Western blot. Pulse−chase radiolabeling kinetic studies verify that secretion levels vary due to differing efficiency of transport through the secretory pathway following translation. Observed secretion efficiencies correlate with mutant stability in vitro but are not predicted by in vitro folding or unfolding kinetics. This correlation implies that structural fluctuations are responsible for discrimination among the mutant BPTI forms by the proofreading apparatus, since no gross structural differences are apparent that would explain the substantial variation in retention. Since proteins are generally promptly transported from the ER to the Golgi upon folding, one might expect that minimizing ER retention time by maximizing folding rate would produce maximal secretion efficiency. The results presented here indicate that nominally folded proteins may nevertheless be retained and degraded by the eucaryotic secretory proofreading apparatus.

MATERIALS AND METHODS

Yeast Strains and Growth Media. Cultures of *S. cerevisiae* strain BJ5464 (α *ura3−52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R 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 (Jones, 1991). Strain YVH10 for overexpression of PDI was derived from the parent strain BJ5464 (Robinson et al., 1994). 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. All yeast transformations were performed by electroporation using a Bio-Rad Gene-Pulser (Becker & Guarente, 1991). Transformants were selected on minimal medium containing casamino acid supplement (SD-CAA + uracil) buffered with sodium phosphate buffer (50 mM, pH 6.0) (Sherman, 1991). Independent transformant colonies were grown in culture

Table 1: Oligonucleotides Used for Mutagenesis

	5'----------3'
Cys 14 Ala	CACGGGCCCAGCTAAGGCTAGAATTATCCGGTACTTCTAC
Cys 38 Ala	GTCAAACCTTCGTATACGGTGGTGCTAGGGCTAAG
Cys 30 Ala	GGCTGGTTTGGCTCAAACGTTCGTTTACGGTGGTTG
Cys 51 Ala	CTTCAAGTCAGCTGAAGACGCTATGAGAACCTGTGG
Cys 5 Ala	GAGAGAAGCTAGGCCTGACTTCGCTTTGGAACCACCATAC
Cys 55 Ala	CGGAAGACTGCATGCGCACCGCTGGTGGTGC

tubes with 5 mL of liquid SD-CAA + uracil media for 96 h prior to measurement of BPTI secretion.

Construction of BPTI Mutants. The plasmid used to express wt BPTI in *S. cerevisiae* was constructed by subcloning the expression cassette from vector pUC-G-BPTI (Parekh et al., 1995) into the multicopy yeast shuttle vector YEPlac112 (Gietz & Sugino, 1988) as an *Eco*RI−*Bam*HI fragment. BPTI is expressed using the constitutive yeast glyceraldehyde-3-phosphate dehydrogenase promoter and a synthetic pre−pro leader is used to target BPTI to the ER (Parekh et al., 1995). The pro region contains a dibasic Lys-Arg site at its C terminus for cleavage by the Kex2p. The resulting plasmid is referred to as YE112-GPD-BPTI. Various single or pairwise cysteine to alanine mutations were carried out using an oligonucleotide-mediated mutagenesis kit from 5′ to 3′ (Morph mutagenesis kit). The oligonucleotides used for mutagenesis are listed in Table 1. Each oligonucleotide introduced a single mutation for Cys to Ala as well as a silent mutation that introduced a restriction site to aid in screening for mutant colonies. The various mutations were confirmed by sequence analysis (Automated Applied Biosystems Sequencer, UIUC Biotechnology Center). Plasmid manipulations were carried out according to standard procedures (Sambrook et al., 1989).

BPTI Trypsin Inhibition Assays. The level of BPTI secreted in the supernatant was measured as described (Parekh et al., 1995). Briefly, the transformants were grown in selective growth medium (SD-CAA + uracil) in 5-mL test tube cultures for 96 h to an OD_{600} of approximately 10.0. Saturated OD_{600} did not vary with expression of different mutants, and quintuplicate replicates varied less than 5% in OD_{600}. Densitometry of Coomassie Blue stained tricine−SDS−PAGE gels was performed with an 8-bit black and white scanner and Image 1.44 software (NIH). Increase in intensity of L-BAPA (*N*-α-benzoyl-arginine-*p*-nitroanilide; Sigma), a synthetic trypsin substrate, at 405 nm was used to measure trypsin activity. A standard curve was obtained for a given level of trypsin (36 μg) and varying amounts of standard native BPTI (0−10 μg) obtained from Worthington Biochemicals. Trypsin inhibition obtained with the supernatant was compared to the standard curve to estimate the level of BPTI present in the culture. Since BPTI−trypsin binding is essentially irreversible, BPTI activity is equal to the difference between the added and detected trypsin activities.

Cell Extracts and Western Blots. Cell extracts were prepared by a modification of the procedure developed by Hann and Walter (1991). One OD_{600} × milliliter of cells was washed once with ice-cold water and resuspended in 0.5 M iodoacetamide to block all free thiols. Cells were blocked for 20 min and then resuspended in 100 μL of trichloroacetic acid (TCA) buffer (20 mM TrisCl, pH 7.9, 50 mM ammonium acetate, 2 mM EDTA) and added to 600 μL of zirconium oxide beads containing 100 μL of 20% TCA. Cells were disrupted with two 50-s cycles of agitation (Bead Beater, Biospec). The supernatant and two 500-μL washes (1:1 TCA buffer:20% TCA) of the beads were combined and centrifuged at 10000*g* for 5 min. The pellet was resuspended at 100 °C for 5 min in resuspension buffer (3% SDS, 100 mM Tris base, pH 11). The cell extracts were stored at −70 °C.

Equal volumes of resuspended cell extracts were separated by tricine−sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Schägger & von Jagow, 1987), allowing resolution of proteins between 1 and 70 kDa. A Novex minigel apparatus was used to separate and transfer of proteins to nitrocellulose (Bio-Rad). Anti-native-BPTI primary IgG (gift of G. Georgiou) and anti-reduced-BPTI-primary IgG (Wittrup lab) were used at a dilution of 1:5000 and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase at 1:2000 (Sigma). Detection of the antigen−antibody complex was performed with enhanced chemiluminescence (ECL, Amersham), and images were recorded on preflashed film (Hybond ECL, Amersham).

Pulse−Chase Radiolabeling Kinetic Experiments. For metabolic labeling of cells, an exponentially growing culture of BJ5464 in selective minimal media was used. The cells were transformed with either the expression plasmid YE112-GPD-BPTI or with YE112-GPD-BPTI with cysteine to alanine mutations that removed a single disulfide bond. The cell cultures were switched to minimal media lacking methionine 2 h before labeling. Cells were pulsed with 50 μCi of L-[^{35}S]methionine (Redivue, Amersham) per one OD_{600} unit of cells for 1 min. For the chase, methionine was added to a final concentration of 10 mM. Intracellular samples were taken immediately following the addition of unlabeled methionine to determine initial BPTI synthesis. Supernatant samples were taken for times between 0 min and 2 h to record the appearance of secreted BPTI. During labeling and chase periods, the cells were incubated at 30 °C with shaking at 14 000 rpm using an Eppendorf Thermomixer.

For the intracellular samples, one OD_{600} unit was pelleted by centrifugation at 16000*g* for 15 s. The supernatant was removed, and 100 μL of ice-cold stop buffer was rapidly mixed with the pellet [20 mM Tris-HCl (pH 8.0), 50 mM ammonium acetate, 500 mM iodoacetamide, 20 mM sodium azide, 0.45 mM cycloheximide, 5 mM EDTA]. The sample was then frozen in a dry ice ethanol bath.

Cell extracts were prepared as described previously (Robinson et al., 1996). Triplicate samples were thawed by the addition of ice-cold cell extract buffer and vortexing [final concentrations: 10 mM Tris-HCl (pH 8.0), 25 mM ammonium acetate, 500 mM iodoacetamide, 20 mM sodium azide, 0.45 mM cycloheximide, 20 mM EDTA, 10% TCA, 4 mM AEBSF, 1 mM PMSF, 1 μg/mL pepstatin A, 3 μg/mL leupeptin, 1 μg/mL antipain). This suspension was transferred to a tube containing 600 μL of zirconium oxide beads (BioSpec Products, Bartlesville, OK). The cells were mechanically lysed by two 50-s cycles in a Bead-Beater (BioSpec Products) with cooling on wet ice between cycles. The supernatant was transferred to a new tube, and the beads were washed twice with 500 μL of wash buffer [10 mM

Tris-HCl (pH 8.0), 25 mM ammonium acetate, 2 mM EDTA, 10% TCA]. The washes were combined with the original supernatant, and the mixture was centrifuged for 5 min at 16000*g*. The pellet was resuspended in 40 μL of TCA resuspension buffer [3% SDS, 100 mM Tris (pH 11), 10 mM DTT] and boiled for 5 min. After being boiled, iodoacetamide was added to a final concentration of 50 mM, then 160 μL of dilution buffer was added [60 mM Tris (pH 7.4), 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100]. Ten microliters of Omnisorb (CalBiochem) was added, and the mixture was incubated with rotation for 10 min. This mixture was centrifuged at 16000*g* for 5 min, and the supernatant was prepared for electrophoresis. In control immunoprecipitations, the bands corresponding to intracellular and secreted BPTI were identified. Immunoprecipitation was not necessary for the quantitation presented here because no abundant cellular proteins were present in the molecular weight range of BPTI and pro-BPTI.

Supernatant samples were concentrated 10-fold in a Microcon-3 concentrator (Amicon) and prepared for electrophoresis. The sample was mixed with an equal volume of 2× tricine sample buffer (Novex, San Diego, CA) with DTT added to a final concentration of 65 mM and boiled for 5 min. This mixture was then loaded onto a 10−20% gradient tricine gel (Novex, San Diego, CA) and was electrophoresed at 125 V for 90 min using tricine running buffer (10 mM Tris, 10 mM tricine, 0.05% SDS, pH 8.3).

The gel was placed on 3 MM Whatman paper and covered with plastic wrap. The gel was then dried on a gel dryer (Hoeffer) for 2 h at 80 °C and allowed to cool for 30 min under vacuum only. The dried gel was placed in a PhosphorImager exposure cassette (Molecular Dynamics) for 24−72 h. The cassette was scanned and analyzed using a Molecular Dynamics PhosphorImager with ImageQuant software.

RESULTS

Secretion Efficiency of BPTI Mutants in Yeast. We have previously constructed and characterized an expression system for BPTI secretion in *S. cerevisiae*, with BPTI targeted to the ER by a synthetic pre pro region (Parekh et al., 1995), utilizing a 21 amino acid synthetic pro region based on the pro region of yeast α mating hormone (QPVISTTVGSAAEGSLDKREA) (Clements et al., 1991). Although it is formally possible that this hydrophilic peptide will alter BPTI's folding pathway in the ER, a 13 amino acid pro region (TPGADTSNQAKAQ) has been shown previously to exert no detectable effect on in vitro folding kinetics relative to mature BPTI (Weissman & Kim, 1992a). Furthermore, the addition of 4−6 amino acid peptides to the N terminus of BPTI was found to result in negligible perturbation of BPTI structure and stability (Lauritzen et al., 1992).

Single and pairwise cysteine to alanine mutants of BPTI were constructed by site-directed mutagenesis. To study the consequences of unpaired cysteines for processing and secretion, each of the six cysteines in BPTI were individually replaced with alanine (C5A, C14A, C30A, C38A, C51A, C55A). The in vivo significance of each of the three disulfides was determined by expression of pairwise cysteine-alanine replacements (C14/38A, C30/51A, C5/55A). BPTI with only one disulfide bond (C14/38/30/51A) was also expressed, since this variant has been shown to have nativelike structure and to inhibit trypsin stoichiometrically (Staley & Kim, 1992). It has been argued that cysteine replacement with serine better mimics partially ionized cysteine present at pH 8.7 (van Mierlo et al., 1991a). For BPTI mutants, cysteine to serine mutations have been found to be more destabilizing than cysteine to alanine replacements (Staley & Kim, 1992). BPTI with all four cysteines except 5−55 replaced by serine has a melting temperature of 10 °C, while replacement of cysteines with alanine yields a melting temperature of approximately 40 °C. To decouple the consequences of losing a disulfide bond from the effect of requiring burial of a hydrophilic serine in the folded state, alanine replacements were chosen for the present work.

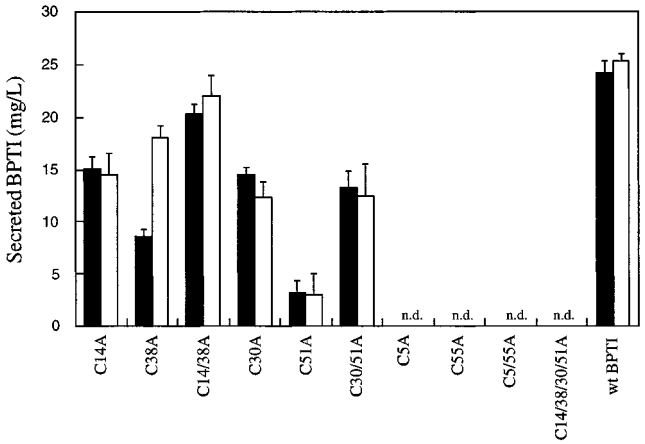

FIGURE 1: Secretion from yeast of BPTI mutants with cysteine/alanine replacements. The filled bars represent activity in a trypsin inhibition assay (Materials and Methods). Open bars represent densitometry of bands on Coomassie-stained SDS−PAGE gels. For those mutants marked "n.d.", neither trypsin inhibition activity nor a Coomassie-stained band were detected.

Plasmids were individually transformed into *S. cerevisiae* strain BJ5464, and the level of secreted BPTI was detected in supernatants by trypsin inhibition assay as described in Materials and Methods. Five colonies from each transformation were selected to account for clonal variation. To account for differences in specific activity of the mutants, the intensity of bands on Coomassie Blue stained SDS−PAGE gels were determined by densitometry and compared to the results obtained by trypsin inhibition assay. Figure 1 shows the level of BPTI in the culture supernatant after 96 h of growth on selective media. For the mutations that resulted in no measurable secreted activity (C5A, C55A, C5/55A, C14/38/30/51A), no BPTI is detected in the supernatant by SDS−PAGE densitometry. Secretion was highest for wt BPTI (25 mg/L), while for several mutant BPTI molecules secretion was completely abolished. The 5−55 disulfide is essential for efficient folding and secretion, while removal of the 14−38 disulfide bond results in a small reduction in secretion efficiency. Removal of the 30−51 disulfide bond results in 50% loss in secretion. It is also evident that a free cysteine can result in lower secretion efficiency than when both cysteines are removed. This is observed most strikingly for the C51A mutant, which is secreted at a level of 2.5 mg/L while BPTI lacking the 30−51 disulfide bond is secreted at 12.5 mg/L. Free cysteine thiols may participate in intermolecular disulfides and retard exit from the ER;

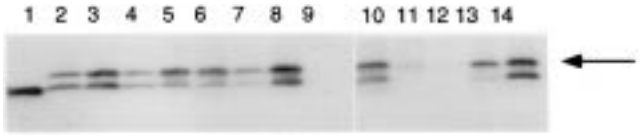

FIGURE 2: Total accumulated intracellular BPTI, detected by Western blot with antisera against reduced BPTI. Cell extracts were reduced as described in Materials and Methods prior to electrophoresis. The arrow indicates a pro-BPTI fusion resident in the ER, while the mature BPTI band is in a post-ER compartment. Lane 1, BPTI standard (20 ng); lane 2, C14A; lane 3, C38A; lane 4, C14/38A; lane 5, C30A; lane 6, C51A; lane 7, C30/51A; lane 8, wt BPTI; lane 9, control yeast strain not expressing BPTI; lane 10, C5A; lane 11, C55A; lane 12, C5/55A; lane 13, C14/38/30/51A; lane 14, wt BPTI.

alternatively, free cysteines may accelerate unfavorable intramolecular disulfide rearrangements (Mendoza et al., 1994). The close agreement between SDS−PAGE analysis and trypsin inhibition activity indicates that secreted BPTI is essentially native in conformation, as expected. Thus, any misfolded BPTI is retained and degraded by the proofreading apparatus.

The densitometry results are consistent with the activity data for all mutations except C38A, for which densitometry indicates roughly 50% higher BPTI levels than measured by trypsin inhibition. Although the 14−38 disulfide bond is not essential for activity, binding of the free Cys14 to a small thiol molecule can perturb trypsin binding. In fact, analysis by electrospray mass spectrometry indicates that essentially all secreted C38A BPTI is present as a mixed disulfide with glutathione (data not shown). A mixed disulfide with glutathione has been observed previously in a mutant of human lysozyme possessing a free cysteine when it is secreted in yeast (Taniyama et al., 1990). Approximately half of the secreted C14A BPTI is a mixed disulfide with glutathione, with the remainder at a molecular weight consistent with a mixed disulfide with cysteine (data not shown). It is not clear why C38A BPTI does not also form mixed disulfides with cysteine. Although glutathione has been determined to be the predominant small molecule redox buffer of the endoplasmic reticulum (Hwang et al., 1992), a mixed disulfide with cysteine has also been observed previously in secreted IgM (R. Sitia, personal communication). Cys51 of secreted C30A BPTI is a free thiol, as determined by electrospray mass spectrometry (data not shown).

Intracellular BPTI Levels. Western blots of cell extracts were performed to determine intracellular levels of each BPTI mutant. Figure 2 shows a Western blot of intracellular extracts performed with antiserum specific to reduced, unfolded BPTI. The samples were reduced with DTT by boiling in SDS and blocked with iodoacetamide before analysis, so total intracellular BPTI is detected. BPTI is targeted to the ER using a pre−pro leader peptide. The pre region is cleaved upon entering the ER, and the pro region is cleaved by the Kex2 protease which resides in the Golgi. Thus the lower band corresponds to mature BPTI, which has exited the ER, while the upper band corresponds to pro-BPTI present in the ER. Replacement of the cysteines involved in disulfide bonds 14−38 and 30−51 does not markedly alter the quantity of BPTI accumulated intracellularly (Figure 2, lanes 2−7). Quantitative precision of the Western blot technique is substantially less than that of

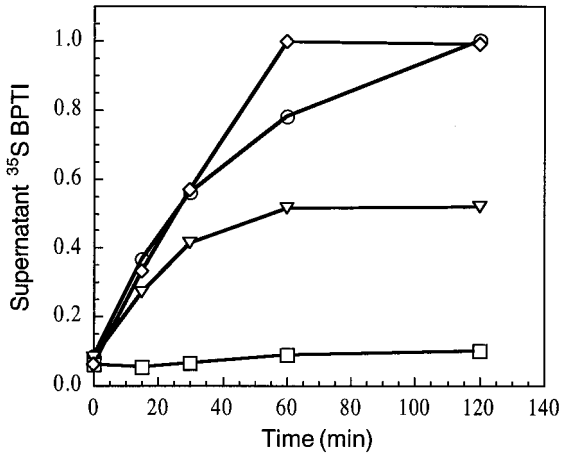

FIGURE 3: Secretion kinetics for BPTI disulfide deletion mutants. Yeast cultures expressing wt (○), C14/38A (◇), C30/51A (▽), or C5/55A BPTI (□) were pulse radiolabeled with ^{35}S methionine for 1 min followed by a chase period with excess nonradiolabeled methionine and cycloheximide. Secreted BPTI was quantified by phosphorimager autoradiography following SDS−PAGE.

extracellular trypsin inhibition assay and SDS−PAGE densitometry, so measured reductions in secretion of 10−50% would not necessarily be expected to produce differences in intracellular accumulation apparent by Western blot. However, BPTI is not detected intracellularly for the C55A and C5/55A mutations, suggesting that these mutations result in a polypeptide that is rapidly degraded. Surprisingly, the C5A and C14/38/30/51A mutants accumulate intracellularly despite the absence of secretion of either mutant. Furthermore, intracellular accumulation of an apparently Kex2 cleaved BPTI fragment is observed for these two mutants (see Figure 2, lanes 10 and 13). Since Kex2 cleavage occurs only in the Golgi, a post-ER mechanism may exist that retains and degrades the C5A and C14/38/30/51A mutants.

Secretion Kinetics. The secretion kinetics of wild type, C14/38A, C30/51A, and C5/55A BPTI were examined by pulse−chase metabolic radiolabeling. Yeast cultures were labeled for a 1-min pulse with [^{35}S]methionine, followed by a chase period of incubation with excess nonradioactive methionine and cycloheximide to stop protein synthesis. Secreted radiolabeled BPTI in the supernatant was quantified by SDS−PAGE and phosphorimager autoradiography, and the results are shown in Figure 3. The characteristic time for secretion is qualitatively similar for wt, C14/38A, and C30/51A BPTI, but the relative secreted radiolabeled BPTI after a 2-h chase period (wt = 1.0; C14/38A = 0.92; C30/51A = 0.44; C5/55A = 0.04) is similar to the ratios for total secreted BPTI following 96 h of growth (Figure 1, wt = 1.0 ± 0.04; C14/38A = 0.84 ± 0.03; C30/51A = 0.54 ± 0.07; C5/55A not detected). This result indicates that although BPTI secretion efficiency is altered by disulfide removal, the kinetics of transit through the secretory pathway are not substantially altered, indicating that a fraction of newly synthesized BPTI is rapidly (<10 min) targeted for retention and degradation.

To confirm that equivalent levels of BPTI are expressed and targeted to the ER by the wt, C14/38A, C30/51A, and C5/55A BPTI multicopy expression plasmids, the amount of BPTI synthesized and translocated across the ER membrane was quantified following a 3-min labeling pulse with [^{35}S]methionine. Intracellular BPTI was quantified by SDS−

PAGE and phosphorimager autoradiography. No pre−pro BPTI forms were observed, indicating either cotranslational ER membrane translocation or very rapid (≪3 min) post-translational translocation. As a percentage of total TCA-precipitable radioactivity, essentially identical amounts of each BPTI disulfide mutant were translocated into the ER during the 3-min pulse (wt BPTI = 1.5 ± 0.09%; C14/38A = 1.7 ± 0.1%; C30/51A = 1.9 ± 0.2%; C5/55A = 1.6 ± 0.2%). These data, taken together with those in Figure 3, indicate that variation in secreted BPTI levels among these mutants are due to processes that occur following translocation across the ER membrane but prior to release from the cell.

Further control experiments were performed to confirm that the observed differences in secretion are due to altered secretion efficiency rather than pre-ER processes (e.g., plasmid stability, altered transcription, altered translation, altered membrane translocation) or post-secretion processes (degradation in the growth medium). The expression cassettes for wt, C14/38A, C30/51A, and C5/55A BPTI were subcloned into CEN single copy plasmids, and the ratio of secreted BPTI levels expressed from the CEN plasmid (wt = 1.0 ± 0.18; C14/38A = 0.90 ± 0.22; C30/51A = 0.72 ± 0.09; C5/55A not detected) is similar to data from the multicopy 2 μm plasmid (wt = 1.0 ± 0.04; C14/38A = 0.84 ± 0.03; C30/51A = 0.54 ± 0.07; C5/55A not detected), indicating that saturation of the secretory pathway does not alter the relative secretion efficiency of these mutant forms of BPTI. Plasmid stability for the CEN expression plasmids does not vary significantly among the mutants (wt BPTI = 0.78 ± 0.07; C14/38A = 0.87 ± 0.05; C30/51A = 0.88 ± 0.05; C5/55A = 1.00 ± 0.04) and therefore does not contribute to variation in BPTI secretion levels. Secreted wild-type and mutant BPTI activity is stable in the growth medium for at least 1 week, and the addition of 1 mg/mL bovine serum albumin as a carrier protein does not increase BPTI levels detectable by Western blot. This indicates that extracellular degradation does not account for observed variation in BPTI secretion.

Overexpression of Protein Disulfide Isomerase. Protein disulfide isomerase (PDI) overexpression in yeast has been shown previously to substantially increase secretion efficiency of some proteins (Robinson et al. 1994; Schultz et al., 1994). PDI catalyzes BPTI folding in vitro, accelerating rearrangements as much as 6000-fold (Weissman & Kim, 1993; Zapun et al., 1992). The effect of PDI overexpression on secretion efficiency was determined for each of the cysteine replacement mutants and is shown in Figure 4.

PDI overexpression does not substantially alter the rank order of secretion efficiency for the mutants. Interestingly, secretion of wild-type, C14A, C38A, and C14/38A BPTI is actually decreased by 23−37% by PDI overexpression. Secretion of C30A, C51A, and C30/51A BPTI is essentially unaffected by PDI overexpression, and C5A, C55A, C5/55A, and C14/38/30/51A BPTI remain unsecreted. Overexpression of Eug1p, another yeast ER redox foldase (Tachibana & Stevens, 1992) also does not substantially affect wild-type BPTI secretion (data not shown).

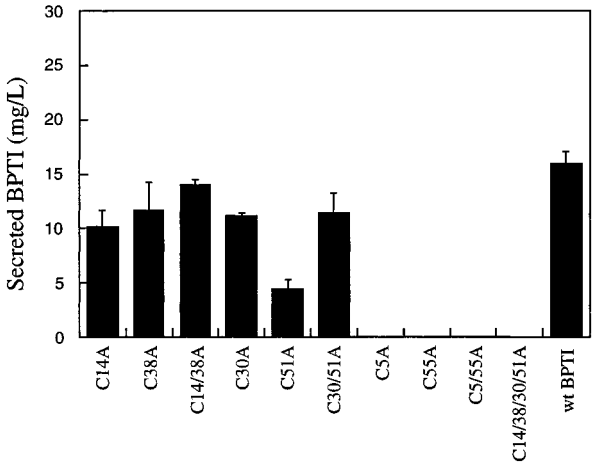

FIGURE 4: Effect of protein disulfide isomerase overexpression on secretion of BPTI and cysteine/alanine replacement mutants. PDI was overexpressed 15-fold from a single integrated gene (Materials and Methods). Filled bars represent activity in a trypsin inhibition assay, as in Figure 1.

DISCUSSION

BPTI has been expressed and secreted in yeast as a model system to study the role of disulfide bonds in eucaryotic secretory protein folding and conformational proofreading. Site-directed mutagenesis was used to express various single and pairwise cysteine to alanine mutants of BPTI. The 5−55 bond is found to be essential for secretion, removal of the 14−38 bond slightly reduces secretion efficiency, and secretion of BPTI lacking the 30−51 bond is reduced by half. A negative role for unpaired cysteines in secretory processing is indicated, since in three of four cases a free cysteine reduces secretion efficiency more than removal of both of the cysteines involved in a disulfide bond. The general agreement between trypsin inhibition activity data and SDS−PAGE/densitometry (Figure 1) indicates that secreted BPTI mutants are nativelike in structure. It is thus the retention and degradation of misfolded polypeptides by the conformational proofreading pathway that determines the observed variation in secretion efficiency among the mutants. These results are discussed below in detail for each of the disulfides.

(a) 14−38 Disulfide. The disulfide bond between Cys 14 and Cys38 is located on the surface of BPTI and links together two polypeptide segments that form the trypsin binding site of the inhibitor. The 14−38 disulfide can be readily reduced by DTT under conditions where the rest of the protein retains its folded conformation (Mendoza et al., 1994). The structure of BPTI with Cys14 and Cys38 replaced by serine has been studied by NMR and found to be very similar to the wt BPTI structure (Wagner et al., 1979).

Since Cys14 and Cys38 form transient non-native disulfides with Cys5 along the preferred pathway for in vitro refolding (Weissman & Kim, 1992b; Creighton 1992), their loss retards folding in vitro. The rate of folding of C14/38A and C14/38S BPTI is 2−5-fold slower than wt BPTI at 37 °C and at 25 °C is much slower with a pronounced lag time (Marks et al., 1987b; Goldenberg, 1988). Despite their significance for in vitro folding, Cys14 and Cys38 are unnecessary for efficient folding and secretion in yeast at 30 (Figure 1) or 25 °C (data not shown). One would expect the rate of C14/38A folding in vivo to resemble the wild-type rate in any case, since cellular factors such as PDI can provide the free sulfhydryl groups necessary for rearrange-

ment of kinetically trapped BPTI thiols and disulfides (Weissman & Kim, 1993; Zapun et al., 1992; Walker & Gilbert, 1995), obviating this role for Cys14 and Cys38.

BPTI with either Cys14 or Cys38 replaced with serine can form one of the non-native disulfides necessary for rearrangement to form wt BPTI in vitro and, hence, fold at a rate very similar to wt BPTI (Darby et al., 1992). Similar rates of refolding would be expected for alanine replacements. However the secretion efficiencies of C14A and C38A BPTI were found to be lower than wt or C14/38A BPTI (Figure 1). The presence of a particular free cysteine thiol has been shown previously to cause complete retention of IgM; removal of the cysteine by site directed mutagenesis or treatment of whole cells with β-mercaptoethanol cause IgM release (Fra et al., 1993). This result is not completely general, however, since proteins with free cysteines can also be secreted with minimal loss in efficiency (Taniyama et al., 1990; Figure 1). Surface exposed Cys14 or Cys38 may participate in mixed disulfides with ER-resident redox foldases such as PDI or Eug1p and impede exit from the ER, reducing secretion efficiency. Such a mechanism has been shown to retard secretion of Ig λ light chains with free cysteines (Reddy et al., 1996.) Alternatively, a free cysteine might cause unfavorable intramolecular rearrangements via thiol−disulfide exchange. Secreted C38A BPTI does not bind trypsin stoichiometrically, due to the presence of a mixed disulfide with glutathione. This reduction in specific activity was not observed with secreted C14A BPTI, although it too possesses mixed disulfides with glutathione and cysteine.

It has been found that a majority of BPTI molecules may form the 14−38 disulfide first when refolded in vitro (Dadlez & Kim, 1995). Since this disulfide is dispensable for efficient secretion, early formation of the 14−38 disulfide is apparently not an obligate step in the in vivo folding pathway. Given the vectorial nature of membrane translocation, however, the 14−38 cysteine pair emerges into the lumen of the ER prior to any of the other native disulfide cysteine pairs. Thus, this disulfide might also form early in the folding pathway of wild-type BPTI in vivo.

(b) 30−51 Disulfide. The 30−51 disulfide bond is buried in wt BPTI and resistant to reduction in the absence of denaturant. During in vitro folding at neutral pH, approximately half of BPTI becomes trapped as a nativelike species lacking the 30−51 disulfide bond (N*), a species that is very slow to either directly form a third disulfide or rearrange to generate other two disulfide intermediates (States et al., 1984; Creighton & Goldenberg, 1984.) BPTI lacking the 30−51 disulfide has a very nativelike structure with no major structural perturbations (Eigenbrot et al., 1990). With both Cys 30 and Cys51 replaced with alanine (C30/51A), the folding pathway funnels to an analogue of N* and ends there. Since folding of C30/51A does not require rearrangement of disulfide bonds but only the consecutive direct formation of 5−55 and 14−38, its in vitro rate of folding is faster than wt BPTI (Marks et al., 1987a). However, when the C30/51A mutant is expressed in yeast, the secretion yield is 50% of wt BPTI. The capability to fold more rapidly in vitro does not improve C30/51A BPTI's secretion efficiency, as might have been expected a priori.

Interestingly, C30A secretion efficiency is equivalent to C30/51A, but C51A secretion yield is only 10% of wt BPTI. Cys30 is the more reactive of the two cysteine thiols (Creighton & Goldenberg, 1984), and thus it is likely that Cys30 forms intermolecular disulfides in vivo that reduce the efficiency of secretion. As determined by ES−MS, secreted C30A possesses a free Cys51 thiol (data not shown). A mixed disulfide between glutathione and either Cys30 or Cys51 would presumably prevent folding due to the energetic penalty of burying a bulky, polar glutathione in the protein interior. Such a mixed disulfide would be kinetically trapped, however, by the absence of the second cysteine to complete the 30−51 bond by thiol−disulfide exchange. This mixed disulfide species would likely be recognized as misfolded and therefore retained and degraded.

(c) 5−55 Disulfide. The 5−55 disulfide bond contributes most to the stability of BPTI and is buried in the hydrophobic core. The structure of BPTI lacking the 5−55 disulfide (N′) is nativelike, but to a lesser extent than N*. NMR studies of C5/55S BPTI indicate a structure with an extended conformation in the N-terminal (residues 1−7) region and some differences in the C-terminal (residues 55−58) region (van Mierlo et al., 1991a). The remainder of the protein is substantially identical with native BPTI. The 5−55 disulfide bond is essential for secretion in yeast. Negligible intracellular accumulation of C55A and C5/55A BPTI is observed, indicating that these mutations result in accelerated degradation. It is likely that the unstructured N-terminal peptide segment in these mutants is recognized by the conformational proofreading system as unfolded, and the protein is retained and degraded.

BPTI with a Single Disulfide Bond (5−55). BPTI with a single disulfide bond (C14/38/30/51A) has a nativelike structure (Staley & Kim, 1992). When cysteines 14, 38, 30, and 51 are replaced by serines, the mutant's melting temperature is 10 °C (van Mierlo et al., 1991b), but if cysteines are replaced by alanines, the transition temperature is approximately 40 °C and the molecule retains trypsin binding activity. Since only one disulfide needs to be formed, folding of C14/38/30/51A BPTI is expected to be very rapid in vivo. However C14/38/30/51A BPTI is not secreted, demonstrating that the proofreading apparatus can distinguish wt BPTI from a variant with nativelike structure. This also indicates a key biological function for the 14−38 and 30−51 disulfides, since BPTI cannot be efficiently processed in their absence despite the existence of a thermodynamically stable structure.

Secretion Yield Correlates with Thermodynamic Stability, but Not In Vitro Folding Rate. In vitro folding rates clearly do not predict the secretion efficiency of these BPTI mutants, contrary to initial expectations. If folding proceeds irreversibly and the folded form is promptly exported from the ER, then rapid folding should result in rapid secretion and consequently greater secretion efficiency. However, C30/51A folds faster than wt BPTI in vitro but is secreted at 50% of wt BPTI levels. C14/38A BPTI folds 2−5-fold slower than wt BPTI in vitro, but is only slightly reduced in secretion efficiency. The aforementioned folding rate measurements (Marks et al., 1987a,b) were made in a redox buffer (10 mM GSH/1 mM GSSG) that approximates the ER lumenal conditions in vivo (Hwang et al., 1992). C5/55A and C14/38/30/51A BPTI should rapidly fold into nativelike structures in vitro since only one buried disulfide needs to be formed, but they are not secreted. Of course,

the folding pathway in vivo may differ qualitatively from that in vitro, particularly as it is dominated by interactions with PDI (Creighton et al., 1993). It is therefore formally possible that secretion efficiency does correlate with PDI-catalyzed folding rates in vivo. A definitive test of this hypothesis awaits the development of methodology for measuring BPTI disulfide formation kinetics in vivo, an aim currently being pursued.

With the exception of C14/38A, decreased secretion efficiency correlates with increased in vitro reductive unfolding rate. C30/51A unfolds severalfold faster than wt BPTI in vitro, while C5/55A is the most rapidly unfolded variant (Nilsson et al., 1991; States et al., 1987; Creighton et al., 1978). Thus, the in vitro reductive unfolding rates of wt, C30/51A, and C5/55A are inversely related to secretion yield. C14/38A BPTI reductively unfolds slower than wt BPTI in vitro due to the absence of nonnative disulfide rearrangements mediated by Cys14 and Cys38 (Goldenberg, 1988). PDI may substitute for Cys14 and Cys38 in this role, potentially causing C14/38A BPTI to unfold at a rate similar to or greater than that for wt BPTI in vivo.

Neither folding nor unfolding kinetics in vitro fully explain the observed trend in secretion efficiency of BPTI mutants. However, a direct relationship is observed between thermodynamic stability and secretion efficiency (Figure 5). The free energy change of folding/unfolding is a path-independent thermodynamic variable and will not be altered by the presence of ER redox foldases. Stability of BPTI variants lacking disulfide bonds due to chemical modification, mutagenesis, or trapping of folding intermediates has often been reported as a melting transition temperature, T_m (Figure 5A). Secretion of BPTI mutants increases monotonically with increasing T_m. The "effective concentration" C_{eff} of a thiol pair is another measure of the stabilization free energy contributed by a disulfide (Goldenberg, 1985), with a large C_{eff} corresponding to a large destabilization upon removal of that disulfide bond. Secretion efficiency of C5/55A, C14/38A, and C30/51A BPTI decreases with increasing C_{eff} (Figure 5B), indicating again that less stable forms of BPTI are secreted less efficiently. The free energy of stabilization afforded by a disulfide is also a function of the environment's redox potential (Goldenberg, 1985). Hwang et al. (1992) determined that the ratio of reduced to oxidized glutathione in the ER is in the range 1:1 to 3:1. It was also estimated, without direct measurement, that the reduced glutathione concentration in the lumen is 0.5−1.0 mM. Thus, the lumenal value of $[GSSG]/[GSH]^2$ may range from 1000 to 6000 M^{-1}, corresponding to the asymptotic limit of essentially irreversible disulfide formation in BPTI (Goldenberg, 1985). Assuming a C_{eff} of 10^{-2} M for unfolded BPTI, stabilization free energies are readily calculated from C_{eff} for each of the three disulfides. When these stabilization free energies are subtracted from the calorimetrically determined ΔG_{fold} of −12.3 kcal/mol for BPTI (Moses & Hinz, 1983), ΔG_{fold} at 30 °C and neutral pH can be predicted for BPTI lacking each of the three disulfides. As shown in Figure 5C, secretion efficiency increases with increasing $-\Delta G_{fold}$.

Each two-disulfide bonded form of BPTI, though predominantly nativelike in structure, is destabilized relative to wt BPTI. Given its nativelike character, one would expect C30/51A to escape conformational proofreading and leave

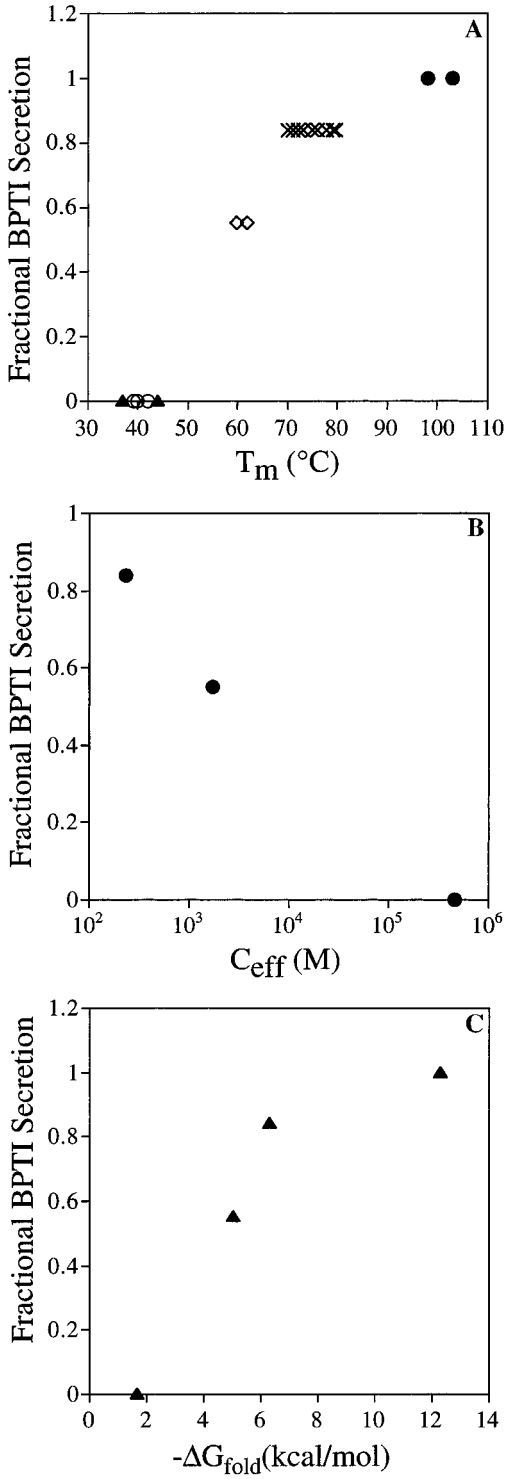

FIGURE 5: Correlation of BPTI secretion efficiency with reported measures of thermodynamic stability for BPTI lacking various disulfide bonds. (A) Melting transition temperatures T_m measured at pH > 3.8. Wild-type BPTI (●), Moses and Hinz (1983). Lacking 14−38 (×), Vincent et al. (1971); Nilsson et al. (1990); States et al. (1987); Wagner et al. (1979); Schwarz et al. (1987). Lacking 30−51 (◇), Nilsson et al. (1990). Lacking 5−55 (▲): States et al. (1987); Creighton et al. (1978). Lacking 30−51 and 14−38 (○), Yu et al. (1995); Schulman and Kim (1994); Staley and Kim (1992). (B) Effective concentration of thiol pairs in nativelike state, C_{eff} (Creighton & Goldenberg, 1984.) As C_{eff} increases, thermodynamic stability decreases. (C) Free energy of folding ΔG_{fold} at 30 °C calculated from calorimetric data (Moses & Hinz, 1983) and C_{eff} (Creighton & Goldenberg, 1984), assuming $C_{eff} = 0.01$ M for the unfolded state and effectively irreversible formation of disulfide bonds at oxidizing ER redox potential (discussed in text).

the ER; however, secretion efficiency is reduced by 50% relative to wt BPTI. C14/38/30/51A BPTI also possesses nativelike structure but is not secreted. This suggests that the proofreading machinery distinguishes among C14/38/30/51A, C30/51A, and wt BPTI on the basis of characteristics not evident from comparison of NMR structures, even at a temperature (30 °C) where the proteins are all stably folded. The fine conformational details of recognition by the ER proofreading machinery have not yet been elucidated. The present work indicates that a static or time-averaged structure such as obtained by crystallography or NMR does not in general predict functional differences in proofreading in vivo. The observed correlation of secretion efficiency with thermodynamic stability suggests instead that structural fluctuations can dominate proofreading by transiently exposing hydrophobic portions of the molecule. If the native conformation is in rapid equilibrium with an ensemble of unfolded conformations, then binding and/or proteolysis of the unfolded forms would produce a kinetic trap that reduces the native secreted fraction even at temperatures below the melting temperature. This effect might be expected to predominate at 30 °C for the C14/38/30/51A and C5/55A mutants with $T_m \approx 40$ °C, but it is somewhat surprising for the C30/51A and C14/38A mutants at temperatures over 30 °C below their T_m.

The importance of thermodynamic stability in determining secretion efficiency of BPTI is further supported by the absence of any significant effect of PDI overexpression on secretion of the disulfide mutants studied. PDI catalyzes BPTI folding in vitro (Weissman & Kim, 1993; Zapun et al., 1992) and in microsomes (Creighton et al., 1993) but cannot alter the intrinsic thermodynamic stability of the mutants studied. It is possible to functionally alter oxidative folding kinetics in vivo, since PDI overexpression increases secretion of some proteins by an order of magnitude (Robinson et al., 1994; Schultz et al., 1994). It might be argued that PDI overexpression has no effect on BPTI secretion simply because wt PDI levels are sufficient to catalyze folding of the particular amount of BPTI synthesized. However, multicopy overexpression of BPTI as studied here has been shown previously to saturate the folding capacity of the yeast ER, causing intracellular accumulation of unfolded BPTI (Parekh et al., 1995.) Overall secretion efficiency is determined by convolution of both extensive factors (availability of chaperones and foldases) and intrinsic factors (thermodynamic stability.) When chaperones and foldases are available in excess, secretion efficiency depends solely on intrinsic properties of the protein, but protein synthesis in excess of the available foldases and chaperones leads to aggregation, retention, and degradation, the extent of which will in turn depend on intrinsic protein properties. The data in Figure 4 indicate that PDI is not a limiting cellular factor in BPTI processing under the conditions examined.

Expression of BPTI and mutants lacking certain disulfides has been studied previously in *Escherichia coli*. The secretion of C14/38A and C30/51A in *E. coli* are 2−10-fold greater than wt BPTI, and secretion of C5/55A is similar to wt BPTI, in contrast with the present findings in yeast (Nilsson et al., 1991). The redox folding environment of the bacterial periplasm is qualitatively different from the ER in that conditions are more oxidizing and disulfide isomerization occurs to a lesser extent (Joly & Swartz, 1994; Ostermeier et al., 1996, Walker & Gilbert, 1994). Since secreted bacterial proteins typically have only one or two disulfides, less isomerization is required for folding by comparison to most eucaryotic proteins. The bacterial periplasm lacks the extensive chaperone cohort of the ER, such as BiP, GRP94, calnexin, and calreticulin (Helenius et al., 1992). For comparison with the results presented here, perhaps the most salient distinction between periplasmic and ER protein processing is the absence of a conformational proofreading pathway in bacteria, given that retention and degradation of BPTI is responsible for the variation in secretion efficiency observed in yeast (Figures 1−3).

It is not clear what ER components are responsible for BPTI proofreading in yeast, since it is not a glycoprotein and therefore not expected to bind calnexin or calreticulin (Ou et al., 1993). It should also be pointed out that the presence or absence of an N-linked glycosylation site in the pro region does not affect BPTI secretion (Parekh et al., 1995). Although BiP has been implicated in heterologous protein retention (Dorner et al., 1987), 5-fold BiP overexpression in yeast does not increase or decrease wt BPTI secretion, and reduced BiP levels reduce BPTI secretion (Robinson et al., 1996). PDI has been indirectly implicated as a component of the ER proofreading system (Puig & Gilbert, 1994; Otsu et al., 1994). The absence of a negative effect of PDI overexpression on secretion of all three (30−51) mutants (Figure 4) argues against a dominant proofreading role for PDI; however, PDI overexpression does reduce secretion of wt BPTI and all three mutants lacking the 14−38 disulfide.

Caution must be exercised in extrapolating these findings with a single-domain protein such as BPTI to multidomain or multisubunit proteins. The relatively rapid collapse of BPTI to an ensemble of compact folding intermediates may be responsible for the apparent irrelevance of folding rate in determining secretion efficiency. For larger proteins, the slower kinetics of subunit assembly or docking between distant folding domains could predominate over single-domain thermodynamic stability in governing interactions with the proofreading apparatus. An example of such a case is the retention of folded IgG heavy chains in the absence of IgG light chain expression (i.e., infinitely slow assembly kinetics). Disulfide formation affects both the kinetics of folding and subsequent structural flexibility, either of which could significantly impact on conformational proofreading. Direct measurement of in vivo disulfide formation kinetics for the mutants studied here will further discriminate between these two contributions to conformational proofreading, but the correlation of secretion efficiency with thermodynamic stability argues for a dominant role for flexibility in this case.

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