BiP/Kar2p Serves as a Molecular Chaperone during Carboxypeptidase Y Folding in Yeast

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Abstract. Although transiently associated with numerous newly synthesized proteins, BiP has not been shown to be an essential component directly linked to the folding and oligomerization of newly synthesized proteins in the endoplasmic reticulum. To determine whether it is needed as a molecular chaperone, we analyzed the maturation of an endogenous yeast glycoprotein, carboxypeptidase Y (CPY) in several yeast strains with temperature-sensitive mutations in BiP. These *kar2* mutant strains have previously been found to be defective in translocation at the nonpermissive temperature (Vogel, J. P., L. M. Misra, and M. D. Rose. 1990. *J. Cell Biol.* 110:1885-1895). To circumvent the translocation block, we used DTT at permissive temperature to delay folding and intracellular transport. We then followed the maturation of the ER-retained CPY after shifting to the nonpermissive temperature and dilution of the DTT. Without the functional chaperone, CPY aggregated, failed to be oxidized, and remained in the ER. In contrast to wild-type cells, in which BiP binding was transient with no more than 10–15% of labeled CPY associated at any time, 30–100% of the CPY remained associated with BiP in the mutant strains. In a heterozygous diploid strain, CPY matured and exited the ER normally. Taken together, the results provide clear evidence that BiP plays a critical role as a molecular chaperone in CPY folding.

For most newly synthesized secretory proteins, plasma membrane proteins, and proteins of the vacuolar organelles, the site of folding and oligomerization is the ER. To assist in the translocation and maturation of these proteins, the ER lumen contains a high concentration of chaperones and folding enzymes which include BiP (also called GRP78), GRP94, peptidyl-prolyl *cis-trans* isomerase, protein disulfide isomerase, ERp72, and calnexin (Vogel et al., 1990; Gething and Sambrook, 1992; Bergeron et al., 1994). The ER also provides an oxidizing environment that facilitates the formation of disulfide bonds, a prerequisite for the proper folding of most membrane-bound and secretory proteins (Creighton, 1988; Jaenicke, 1991). Cotranslational addition of N-linked oligosaccharides is also important since the presence of glycans helps to prevent aggregation of folding intermediates and mediates the interaction of newly synthesized glycoproteins with calnexin, a lectin-like chaperone (Kern et al., 1992; Bergeron et al., 1994; Hammond et al., 1994).

Among the most abundant and best-characterized of the ER chaperones is BiP, a heat shock protein (hsp)[1] 70 analogue. It interacts transiently with numerous proteins during early stages of folding, and binds more persistently to misfolded proteins, incompletely assembled oligomers, and unassembled subunits (see Gething and Sambrook, 1992). Exposed hydrophobic peptide segments on the surface of such proteins are thought to serve as recognition sites (Flynn et al., 1991; Blond-Elguindi et al., 1993). Like other members of the hsp70 family, BiP is thought to repeatedly bind and release the substrate in a cycle involving ATP binding and hydrolysis (Kassenbrock and Kelly, 1989; Flynn et al., 1991; Knittler and Haas, 1992; Gaut and Hendershot, 1993). In this process, the release step is ATP dependent, but it is not known whether both ATP binding and hydrolysis are required.

In the yeast *Saccharomyces cerevisiae*, BiP is required for the translocation of proteins into the ER (Vogel et al., 1990; Sanders et al., 1992). This involves an interaction between BiP, encoded by the *KAR2* gene in yeast, and a DnaJ-like domain of Sec63p, an ER membrane protein associated with the translocation complex (Brodsky and Schekman, 1993; Scidmore et al., 1993). Hsp70 homologues in mitochondria, chloroplasts, and the cytosol are

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; hsp, heat shock protein; NEM, *N*-ethylmaleimide; TX-100, Triton X-100; wt, wild-type.

also known to assist protein translocation across membranes, suggesting a common function for this family of chaperones (Wild et al., 1992; Tsugeki and Nishimura, 1993; Stuart et al., 1994).

It has also been proposed that BiP plays a role in the retention of immature proteins in the ER (Bole et al., 1986; Gething et al., 1986; Pelham, 1986), in the regulation of ER degradation (Hurtley et al., 1989), and in the retrieval of incompletely folded proteins from the Golgi complex to the ER (Hammond and Helenius, 1994). Support for a role in the ER quality control system has been obtained in cultured mammalian cells that secrete von Willebrand factor, a mutant form of factor VIII, and plasminogen activator in a BiP-dependent fashion (Dorner et al., 1988, 1992). Moreover, yeast strains with defective BiP are unable to complete nuclear fusion during mating (Rose et al., 1989; Vogel et al., 1990) and show a defect in homotypic fusion of ER membranes (Latterich and Schekman, 1994).

Finally, it is widely believed that BiP actively supports proper folding and oligomeric assembly of polypeptides in the ER (Gething et al., 1986; Pelham, 1986). This is based on BiP's association with numerous newly synthesized proteins only as long as they are incompletely folded or assembled. It is also supported by known activities of other hsp70 analogues, such as mitochondrial and bacterial hsp70 proteins, that promote in vitro refolding of several proteins in an ATP-dependent manner (Kang et al., 1990; Skowyra et al., 1990; Schröder et al., 1993).

To determine whether BiP indeed has a chaperone function during folding in the ER, we have followed the maturation of newly synthesized carboxypeptidase Y (CPY) in temperature-sensitive *kar2* mutant yeast strains. CPY is a well-characterized vacuolar glycoprotein, synthesized in the ER, and transported as a monomer via the Golgi complex to the vacuole, where it is proteolytically activated (Hasilik and Tanner, 1978*a*; Hemmings et al., 1981; Stevens et al., 1982). It has four N-linked glycans (Hasilik and Tanner, 1978*b*), and recent x-ray crystallographic data have revealed five intrachain disulfide bonds (Endrizzi et al., 1994).

The requirement for BiP in translocation of newly synthesized proteins into the ER has precluded direct analysis of CPY folding at the nonpermissive temperature. To circumvent this problem, we used DTT, which has been shown to delay folding of disulfide-containing proteins in the ER of animal cells and yeast (Braakman et al., 1992*a*; Lodish and Kong, 1993; Tatu et al., 1993; Jämsä et al., 1994). This enabled us to accumulate radiolabeled unfolded CPY in the ER lumen at a permissive temperature, and to follow its posttranslational folding at the nonpermissive temperature after dilution of DTT. These studies provided evidence that BiP plays a direct role in the folding process.

Materials and Methods

Yeast Strains and Growth Conditions

The following *Saccharomyces cerevisiae* strains were used: SFNY26-6A (*his4-619*, MATα), MS1705 (*ade2-101*, *trp1Δ1*, *ura3-52*, *kar2-113*, MATα), MS177 (*ade2-101*, *ura3-52*, *kar2-159*, MATα), MS1351 (*leu2,3-112*, *ade2-101*, *trp1D1*, *ura3-52*, *kar2-203*, MATα), NY432 (*ura3-52*, *sec18-1*, MATα), and ANY123 (*his4-619*, *ura3-52*, *β1-1*, MATα).

Cells were grown at 25°C, unless otherwise stated, in either YP medium (1% yeast extract [Difco Laboratories, Inc., Detroit, MI], 2% Bacto-Peptone) containing 2% glucose, or before metabolic labeling experiments, overnight in yeast nitrogen base (Difco Laboratories, Inc.) that was supplemented with 2% glucose and the appropriate amino acids.

Antibodies

Anti-BiP/Kar2p antibody was obtained from the laboratory of Dr. Mark D. Rose and from Dr. Jeffrey Brodsky (University of California, Berkeley, CA). The CPY antibody was raised against a bacterially expressed fusion protein between β-galactosidase and the full-length open reading frame of CPY, and was obtained from Dr. Patrick Brennwald (Yale University School of Medicine, New Haven, CT). The anti-[H^+]ATPase antibody used was affinity purified as described (Chang and Slayman, 1991) and was a gift from Dr. Carolyn W. Slayman (Yale University School of Medicine).

Metabolic Labeling and Immunoprecipitations

For metabolic labeling, 2–5 OD_{599} units of cells in an early logarithmic growth phase were pelleted and resuspended in a volume of 100–400 μl of fresh yeast nitrogen base medium. For induction of the temperature-sensitive phenotype, the *bet1-1* and *sec18-1* strains were incubated for 30 min at the nonpermissive temperature (37°C) before metabolic labeling. Induction of the mutant phenotype in the *kar2-113*, *kar2-159*, and *kar2-203* mutants was done by shifting the cells for 20 min to 34°C. Samples that received DTT were preincubated for 10 min in the presence of 5 mM DTT, and the metabolic labeling was typically done by adding 100–200 μCi of a mix of [^{35}S]methionine and [^{35}S]cysteine (L-[^{35}S] In Vitro Cell Labeling Mix; Amersham Corp., Arlington Heights, IL). To terminate translation and incorporation of radiolabeled amino acids, 1 mM cycloheximide, and 10 mM each of cold methionine and cysteine were added.

All incubations were terminated by adding an equal volume of ice-cold PBS containing 40 mM *N*-ethylmaleimide (NEM), to prevent further rearrangement of disulfide bonds, and 20 mM sodium azide. Subsequently, cells were pelleted and resuspended in either 1% SDS in PBS, or 2% CHAPS (Pierce Chemical Co., Rockford, IL) in HBS buffer (200 mM NaCl, 50 mM Hepes, pH 7.6) for the coprecipitations. 1 mM PMSF (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin (Sigma Chemical Co., St. Louis, MO) were added to prevent postlysis protein degradation. Cells were disrupted by vortexing with glass beads, and samples in SDS were subsequently either boiled for 5 min, or in the case of [H^+]ATPase, incubated for 5 min at 37°C.

The extracellular α-factor was precipitated from the growth medium in 5% TCA for 30 min on ice, and the pellet was washed twice in ice-cold acetone. The dry pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 7.5, 6 M urea, 1% SDS, and 1 mM EDTA, and before the addition of anti-α-factor antibody the volume was increased 10-fold in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.5% Tween-20, 150 mM NaCl, and 0.1 mM EDTA.

Samples were immunoprecipitated overnight at 4°C in the presence of the appropriate antibodies and protein A-Sepharose (Sigma Chemical Co.). The immunoprecipitates from SDS-lysed cells were washed with a buffer containing 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 0.05% Triton X-100 (TX-100), and 0.1% SDS at room temperature. When coimmunoprecipitations using the anti-BiP antibody were performed, the immunoprecipitates were washed in HBS buffer containing 0.5% CHAPS at 4°C. The immunocomplexes were then disrupted by boiling in 1% SDS and the volume was increased 10-fold with 1% TX-100 in PBS before the addition of the anti-CPY antibody. The immunoprecipitates of the extracellular α-factor were washed in the Tween-containing immunoprecipitation buffer used during its precipitation, but which had been supplemented with 2 M urea.

Sucrose Gradient Centrifugation

Samples were prepared essentially as described above for CHAPS solubilization, except that 1% TX-100 in PBS was used to solubilize the cells. The cell lysates were loaded on top of 10–40% (wt/vol) linear sucrose gradients containing 0.1% TX-100 in PBS. Tubes were centrifuged for 7 h in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, CA) at 45,000 rpm, at 4°C and fractions were collected manually from the top of the gradients using a micropipette. CPY was immunoprecipitated directly from each fraction and analyzed by SDS-PAGE.

Gel Electrophoresis

Samples for gel electrophoresis were dissolved in Laemmli sample buffer (Laemmli, 1970) and either boiled for 5 min in the presence or absence of 25 mM DTT, or in the case of [H⁺]ATPase, incubated for 5 min at 37°C to avoid aggregation of the protein (Chang and Slayman, 1991).

CPY, [H⁺]ATPase, and the intracellular form of α-factor were analyzed by 7.5% SDS-PAGE (Laemmli, 1970). When analyzing [H⁺]ATPase, the gel was run until β-galactosidase of the prestained molecular weight marker (Bio-Rad Laboratories, Richmond, CA) had migrated through two-thirds of the gel (Chang and Slayman, 1991). The extracellular α-factor was analyzed on a tricine SDS-PAGE gel (Schrägger and von Jagow, 1987). Quantitation of radioactive signals was done by using a PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA).

To normalize the amount of radioactive material loaded in each lane of the SDS-PAGE, total incorporation of [^{35}S]methionine and [^{35}S]cysteine in labeling experiments was measured by TCA precipitation. The precipitated radiolabeled material was quantified by liquid scintillation counting in the presence of Optifluor (Packard Instrument Co., Inc., Meriden, CT).

Results

DTT Prevents Folding and Intracellular Transport of CPY

Three distinct intracellular forms of CPY can be identified on SDS-PAGE due to glycan modification and proteolytic cleavage: (*a*) a core glycosylated 67-kD ER form called p1CPY; (*b*) a Golgi form, p2CPY of 69 kD; and (*c*) the mature vacuolar mCPY of 61 kD, which is formed by proteolytic cleavage of p2CPY (Hasilik and Tanner, 1978*a*; Hemmings et al., 1981; Stevens et al., 1982). When wild-type cells were metabolically labeled for 30 min, labeled CPY of all three forms was recovered by immunoprecipitation (Fig. 1 *A*, lane *1*).

To determine whether DTT had an effect on CPY folding and transport, we first exposed yeast to various concentrations of DTT, and determined its effect on cell growth. With 0, 2.5, 5, 10, and 20 mM DTT added during the early logarithmic growth phase (0.1 ODU/ml), the doubling times were 2, 3, 4, 6, and >8 h, respectively (data not shown). Having confirmed that the cells were viable for several hours, we analyzed the folding of newly synthesized CPY in the presence of 5 or 10 mM DTT. We found that DTT caused a dramatic enrichment of p1CPY, but prevented the formation of the Golgi and the vacuolar forms. It was found that 5 mM DTT efficiently blocked the appearance of the post-ER forms if the pulse was preceded by a 10-min preincubation (Fig. 1 *A*, lane *2*).

To confirm that CPY was accumulating in the ER, we used a temperature-sensitive mutant strain, *bet1-1*, defective in ER to Golgi transport (Newman and Ferro-Novick, 1987). When these cells were metabolically labeled at a nonpermissive temperature, p1CPY was generated both in the presence and absence of DTT (Fig. 1 *A*, lanes *3* and *4*). This indicated that DTT, like the *bet1* mutation, interfered with the transport of CPY from the ER to the Golgi.

A small mobility difference was observed between p1CPY that accumulated in the presence or absence of DTT in the *bet1-1* strain, as well as in the wild-type strain (Fig. 1, compare lane *1* with lane *2*, and lane *3* with lane *4*). The reason for the shift is not known, but it was reversible upon dilution of the DTT (not shown).

When analyzed by nonreducing SDS-PAGE, the CPY from DTT-treated wild-type and *bet1-1* cells had the same mobility (Fig. 1 *A*, lanes *6* and *8*), and comigrated with in vitro reduced p1CPY (not shown). In contrast, the form that accumulated in the absence of DTT in the *bet1-1* strain comigrated with the middle band from the wild-type strain labeled under nonreducing conditions (Fig. 1 *A*, lanes *5* and *7*). The uppermost band in the wild-type sample probably represented the oxidized Golgi form and possibly partially oxidized p1CPY (Fig.1 *A*, lane *5*). We concluded that the CPY accumulating in the presence of DTT corresponded to reduced p1CPY, and that the faster moving form seen in the *bet1-1* mutant in the absence of DTT corresponded to oxidized p1CPY. Thus, DTT interfered directly with the oxidation of CPY.

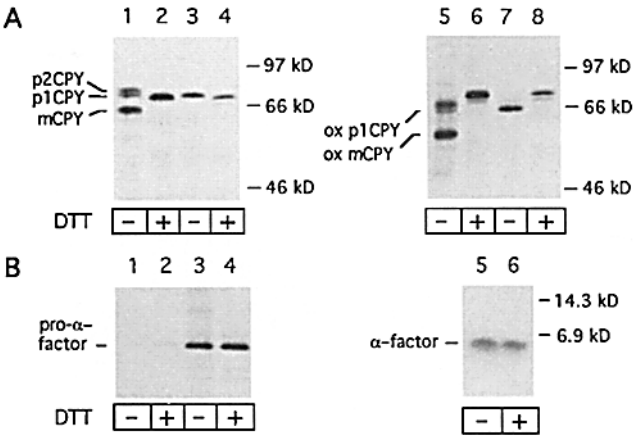

Figure 1. CPY but not pro-α-factor is retained in the ER in the presence of DTT. (*A*) Wild-type (lanes *1*, *2*, *5*, and *6*) and *bet1-1* (lanes *3*, *4*, *7*, and *8*) strains were incubated at 37°C for 30 min, preincubated another 10 min in the presence or absence of 5 mM DTT, and metabolically labeled with ^{35}S-labeled cysteine and methionine for 30 min. Subsequently, the cells were disrupted (as described in Materials and Methods) in the presence of 20 mM NEM, and CPY was recovered by immunoprecipitation. The immunoprecipitated samples were analyzed either by reducing (lanes *1–4*) or nonreducing (lanes *5–8*) SDS-PAGE. (*B*) Pro-α-factor was immunoprecipitated from the same lysates as in *A* and analyzed by SDS-PAGE. The immunoprecipitates from wt cells were analyzed in lanes *1* and *2*, and the *bet1-1*–derived immunoprecipitates in lanes *3* and *4*. Lanes *5* and *6* represent the mature secreted form of α-factor, that was TCA precipitated from the culture medium, followed by anti–α-factor antibody precipitation, and analyzed on a tricine SDS-PAGE gel.

We also assessed whether DTT, under the conditions described above, would have an effect on the transport of a disulfide-free protein, the mating pheromone α-factor. In the early secretory pathway, α-factor exists as a 26-kD protein which is proteolytically cleaved in the late Golgi compartment to generate the secreted 13–amino acid mature pheromone. We found that mature α-factor was efficiently secreted in the presence of 5 mM DTT (Fig. 1 *B*, lanes *5* and *6*), with only minor amounts of the intracellular form detected (lanes *1* and *2*). In contrast, in the *bet1-1* mutant labeled at the nonpermissive temperature, the 26-kD ER form accumulated (Fig. 1 *B*, lanes *3* and *4*).

These results confirmed previous observations that DTT blocks oxidation and ER to Golgi transport of proteins that require disulfide bonds, without interfering with the transport pathway per se (Braakman et al., 1992*a*; Lodish and Kong, 1993; Tatu et al., 1993; Jämsä et al., 1994).

DTT Effect Is Reversible and Reveals Folding Intermediates

Next, we determined whether the DTT block was reversible. Rather than washing the cells to remove the DTT (Braakman et al., 1992*a*; Jämsä et al., 1994), we used a protocol by which the DTT was diluted 10- or 20-fold. The cells were metabolically labeled for 10 min in the presence of 5 mM DTT as described, and the incorporation of label was terminated by the addition of cycloheximide and an excess of unlabeled methionine and cysteine. At the same time, the volume was increased 10-fold with fresh medium lacking DTT. Samples were taken at the end of the pulse and at timed intervals following the dilution, and immediately treated with NEM to prevent further formation of disulfide bonds.

As expected, when analyzed by reducing SDS-PAGE, only p1CPY was detected in a sample taken at the end of the pulse (Fig. 2 *A*, lane *8*). However, 5 min after dilution of the DTT, p2CPY as well as mCPY appeared, and by 30 min all the CPY had reached the vacuole (Fig. 2 *A*, lanes *9–14*). The transport block imposed by DTT was thus fully reversible.

When the CPY was analyzed on a nonreducing gel, a different pattern of bands was seen (Fig. 2 *A*, lanes *1–7*). In addition to the distinct vacuolar form, two additional bands were resolved. These could be identified as the reduced (*red p1CPY*) and the fully oxidized (*ox p1CPY*) ER forms of CPY based on their mobilities. Between them an additional difuse band was seen (Fig 2 *A*, lane *2*), corresponding either to p2CPY or partly oxidized p1CPY.

To confirm that oxidation of p1CPY was taking place after the DTT dilution, the experiment was repeated in a *sec18-1* mutant strain, which like the *bet1-1* strain is blocked in ER to Golgi transport at the nonpermissive temperature. The nonreducing gel (Fig. 2 *B*, lanes *1–4*), showed the disappearance of reduced p1CPY and the appearance of oxidized p1CPY after DTT dilution. Reduction of the samples before SDS-PAGE analysis confirmed that all CPY still resided in the ER (Fig. 2 *B*, lanes *5–8*).

In summary, our results demonstrated that DTT blocks efficiently the folding and subsequent transport of CPY, but has no effect on the transport pathway as such. Furthermore, the effect of DTT was reversible; the posttranslational folding process after DTT dilution resulted in quantitative formation of transport-competent CPY. Finally, as for influenza virus hemagglutinin and vesicular stomatitis virus G protein (Braakman et al., 1992*a*; Marquardt et al., 1993; Tatu et al., 1993), nonreducing SDS-PAGE proved to be a powerful means to follow the oxidation of CPY. Completely reduced and fully oxidized forms could be distinguished. These findings formed the basis for our experiments with the *kar2* mutant strains.

CPY Folding Is Defective in kar2-113, kar2-159, and kar2-203 Mutants

To examine BiP involvement in the posttranslational folding and intracellular transport of CPY, we followed its maturation in three *kar2* mutant strains, *kar2-113*, *kar2-159*, and *kar2-203*. All have mutations in the ATPase domain of BiP, and all show a severe translocation defect at the nonpermissive temperature (Vogel et al., 1990; Sanders et al., 1992; Rose, M. D., unpublished data). The mutant and wild-type cells were metabolically labeled for 10 min at permissive temperature (25°C) in the presence of 5 mM DTT. Translation was terminated by the addition of cycloheximide and unlabeled methionine and cysteine, after which BiP was inactivated by shifting the cells to the nonpermissive temperature (34°C) for 20 min. Cells were then diluted 20-fold in DTT-free prewarmed (34°C) growth medium and samples were taken at timed intervals, the first immediately before the dilution of the DTT.

In wild-type cells, the majority of CPY was found in the vacuole by 60 min of chase (Fig. 3 *A*, lane *4*). In the mutant cells, however, no CPY reached the vacuole. Instead, it remained in the ER, as evidenced by the accumulation of p1CPY (Fig. 3 *A*, lane *5–16*). Nonreducing SDS-PAGE demonstrated that no fully oxidized p1CPY was formed, and that a substantial part of the protein remained completely reduced throughout the chase (Fig. 3 *B*, lanes *5–16*). Some entered heterogeneous disulfide-linked complexes with slow SDS-PAGE mobility. As evident from *A*, these cross-linked species were recovered as monomers when the samples were reduced before SDS-PAGE. Since interchain disulfides are not part of CPY's structure, they rep-

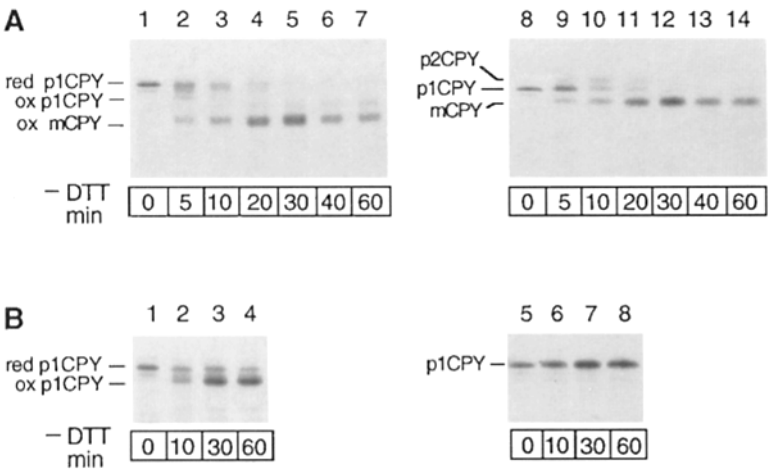

Figure 2. CPY folds posttranslationally and is transported out of the ER upon dilution of DTT. (*A*) The wt strain was preincubated for 10 min at 37°C in the presence of 10 mM DTT, followed by a 10-min radioactive pulse. The volume was then increased 10-fold with DTT-free medium and aliquots were transferred to PBS containing 20 mM NEM at the indicated time-points after the dilution. Cells were disrupted as described in Materials and Methods, and CPY was recovered by immunoprecipitation. The samples were analyzed by nonreducing (lanes *1–7*) or reducing (lanes *8–14*) SDS-PAGE. (*B*) The *sec18-1* strain was preincubated for 20 min at 37°C, DTT was added at 5 mM for 10 min, and the cells were metabolically labeled for 10 min. The cell suspension was diluted 20-fold with DTT-free medium and samples were collected at timed intervals and processed as in *A*. The samples were run on nonreducing (lanes *1–4*) or reducing (lanes *5–8*) SDS-polyacrylamide gels. *red*, reduced; *ox*, oxidized.

resented misfolded products (see upper part of gels in Fig. 3 *B*, lanes *5–16*). When a similar experiment was performed without shifting the mutant cells to the nonpermissive temperature, folding and transport of CPY was normal (not shown).

These experiments showed that CPY folding, as measured by the formation of disulfide bonds and transport out of the ER, was defective in the *kar2-113*, *kar2-159*, and *kar2-203* mutants. Intriguingly, a large part of CPY remained in a form devoid of intra- as well as intermolecular disulfide bonds. Apparently, it was trapped in a form that could not get oxidized. Although incompletely folded, the CPY was also protected against degradation during the chase period for at least 2 h (Fig. 3 *A*, lanes *5–16*, 2 h timepoint not shown).

Transport of a BiP-independent Protein is Unperturbed

Incubation of the *kar2-113*, *kar2-159*, and *kar2-203* mutants at the nonpermissive temperature results in an irreversible translocation block and eventual cell death (Vogel et al., 1990). The CPY folding phenotype was also irreversible; when cells were shifted from the nonpermissive back to the permissive temperature, the CPY remained unfolded and trapped in the ER (not shown).

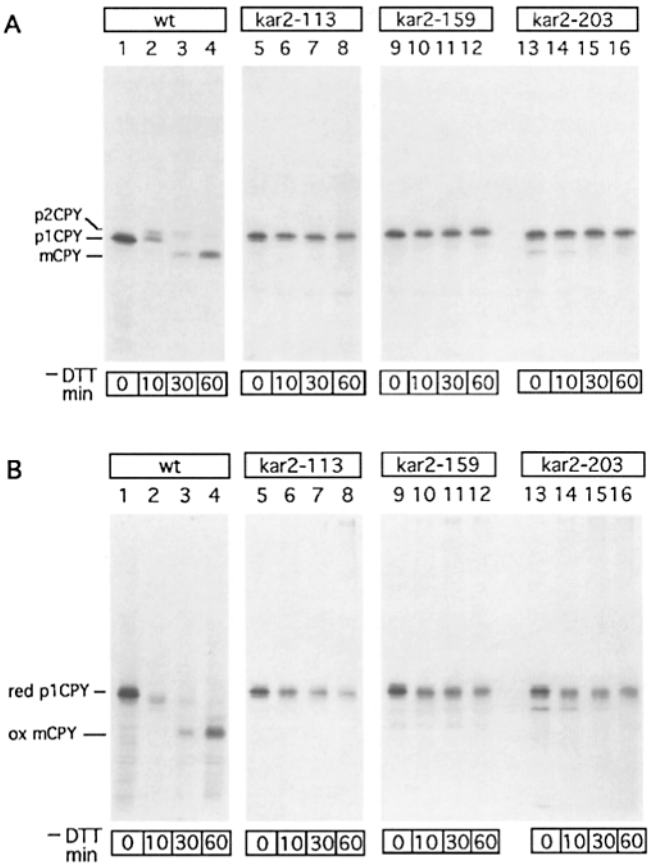

Figure 3. Folding and transport out of the ER is impaired in *kar2-113*, *kar2-159*, and *kar2-203* mutant strains. Wild-type or mutant strains were incubated for 10 min at 25°C in the presence of 5 mM DTT, pulse-labeled for 10 min, and subsequently shifted to 34°C for 20 min. The sample volume was then increased 20-fold with prewarmed (34°C) DTT-free medium and aliquots were removed at the indicated time-points and lysed as described in Fig. 1. The samples were analyzed by either reducing (*A*) or nonreducing (*B*) SDS-PAGE. The faster migrating band in lanes 13 and 14 represent nontranslocated CPY, demonstrating that under the labeling conditions used, the *kar2-203* mutant was partially defective in translocation.

To exclude the possibility that the observed ER retention of CPY was due to a general inability of the mutant cells to support any type of folding and transport, we studied the intracellular transport of cell surface [H⁺]ATPase. While this protein is transported along the secretory pathway (Brada and Schekman, 1988), it differs from CPY in that its translocation into the ER membrane is independent of BiP function (Chang et al., 1993). [H⁺]ATPase gets phosphorylated during transport and at the cell surface, resulting in a small mobility difference on SDS-PAGE between the newly synthesized protein and the plasma membrane form (Chang and Slayman, 1991).

We found, in agreement with Chang et al. (1993), that synthesis and transport of [H⁺]ATPase was normal in the *kar2* mutant cells under conditions mimicking those used for examining the maturation of CPY (Fig. *4*). Thus, the failure of CPY to fold and get transported was not due to a general block in the cells' metabolism and membrane traffic.

Aggregation of Misfolded CPY

In mammalian cells, ATP depletion leads to aggregation of newly synthesized proteins in the ER. These aggregates are frequently cross-linked by intermolecular disulfide bonds (Dorner et al., 1990; Braakman et al., 1992*b*; de Silva et al., 1993). To determine whether the mutant BiP in the *kar2* strains caused quantitative aggregation of CPY, wild-type and *kar2-159* cells were labeled at 25°C in the presence of DTT and shifted to 34°C for 20 min. The DTT was then diluted 20-fold, and samples were taken either immediately before the dilution or 1 h later, alkylated with NEM and solubilized with TX-100. Post-nuclear lysates were then subjected to velocity sedimentation centrifuga-

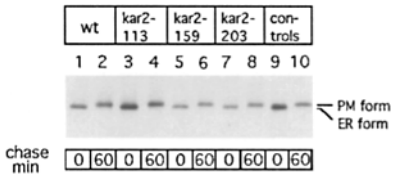

Figure 4. Synthesis, translocation and transport of cell surface [H⁺]ATPase is unaffected in *kar2-113*, *kar2-159* and *kar2-203* mutant strains. To mimic conditions used in Fig. 3, wild-type or *kar2* mutant strains were incubated for 20 min in 5 mM DTT at 25°C, then shifted to 34°C for 30 min, whereafter 10-fold excess of prewarmed (34°C) DTT-free medium was added. After another 30 min of incubation at 34°C, the cells were metabolically labeled for 5 min, and half of the cells were immediately disrupted, whereas the other half was chased for another 60 min at 34°C. The samples were prepared as described (Chang and Slayman, 1991), by omitting boiling, and analyzed by SDS-PAGE. The controls represent the ER form of [H⁺]ATPase synthesized at 37°C in the *bet1-1* strain (lane *9*), and the plasma membrane form obtained after a 1-h chase at 25°C in the same strain (lane *10*). *PM*, plasma membrane.

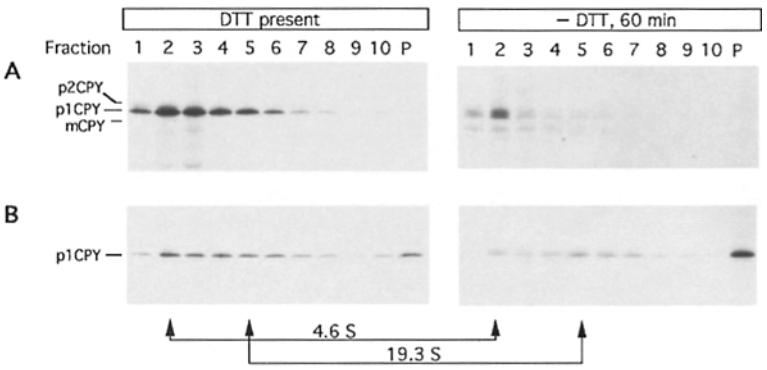

Figure 5. The ER bound CPY in the *kar2-159* strain is sequestered into aggregates after removal of DTT. Wild-type (*A*) and *kar2-159* (*B*) cells were metabolically labeled as described in Fig. 3. Samples were taken immediately before the dilution of the DTT and 60 min later. Cells were disrupted in 1% TX-100 in PBS as described in Materials and Methods and subjected to 10–40% sucrose gradient velocity sedimentation for 7 h at 45,000 rpm. Fractions were collected from the top of the gradient, and pelleted material was dissolved in 1% SDS and diluted 10-fold in 1% TX-100 in PBS. CPY was recovered by immunoprecipitation and analyzed by reducing SDS-PAGE. Parallel gradients were loaded with thyroglobulin (19.3 S, 660,000 kD) or BSA (4.6 S, 66,000 kD), and the positions of these proteins were determined by analyzing an aliquot of each fraction on SDS-PAGE, followed by Coomassie staining of the gels. *P*, pellet.

tion on sucrose gradients, and CPY was recovered from each fraction by immunoprecipitation.

In wild-type cells, the reduced CPY was found in the upper part of the gradient, with the bulk in fractions 1–5 (Fig. 5 *A*, *DTT present*), corresponding to S values between 2 and 20S. After DTT dilution, the CPY was converted to oxidized p1CPY (nonreducing gel not shown), p2CPY, and mCPY, most of which sedimented as monomeric CPY of 4 S in fraction 4 (Fig. 5 *A*, *−DTT, 60 min*). The loss of signal after 60 min of chase was mainly due to poor solubilization or antibody recognition of mCPY in TX-100–derived lysates.

In the *kar2-159* strain, the major part of CPY sedimented as in the wild-type strain at the nonpermissive temperature and in the presence of DTT. However, an appreciable amount was also found in the pellet (Fig. 5 *B*, *DTT present*). This suggested that functional BiP was needed to keep some of the reduced CPY from aggregating. After DTT dilution, a more dramatic departure from the distribution seen in the wild-type was observed; virtually all of the CPY pelleted in an aggregated form (Fig. 5 *B*, 60-min chase).

We concluded that the temperature-induced inactivation of BiP caused association of CPY, and possibly other newly synthesized proteins, into complexes which coalesced into large aggregates after DTT washout. This result suggested that one of BiP's functions is to prevent the aggregation of incompletely folded proteins in the ER, a function consistent with observations made with hsp70 proteins in cell-free refolding experiments (Langer et al., 1992).

Association of CPY and BiP

To determine whether BiP interacted with CPY before and after DTT dilution, coimmunoprecipitation experiments were performed using anti-BiP antibodies under mild detergent conditions. After BiP precipitation, samples were treated with SDS and reprecipitated with anti-CPY antibodies.

In wild-type cells, a variety of labeled proteins including p1CPY (10–15% of total), were precipitated with the anti-BiP antibody before dilution of DTT. After 60 min of chase, all CPY had exited the ER and no coprecipitation was observed (Fig. 6, *A* and *B*, anti-BiP precipitation only not shown). Only about one-eighth of the p1CPY molecules coprecipitated with BiP, suggesting either that BiP only bound to a fraction of the CPY residing in the ER at any given time, or that the interactions were partly disrupted during the precipitation procedure. The former explanation is consistent with a model in which substrates continuously bind to BiP and are released during the process of folding (see Gething and Sambrook, 1992; Hartl et al., 1994).

In the *kar2* mutants, the fraction of total CPY that coprecipitated with BiP at the nonpermissive temperature was much higher than in the wild-type cells (Fig. 6, *A* and *B*), depending on the strain 30–100% of CPY, as compared to the amount precipitated with the anti-CPY antibody, coprecipitated with BiP both before and after DTT dilution. In some experiments, more CPY was coprecipitated with BiP than with the anti-CPY antibody, probably due to missing or hidden epitopes in the aggregated CPY (e.g., Fig. 6, *A* and *B*, lanes *5* and *6*). Analysis of the samples by nonreducing SDS-PAGE revealed that both the covalently cross-linked as well as the non–cross-linked CPY was associated with BiP (Fig. 6 *C*, lanes *3* and *4*, only *kar2-159* shown).

These data demonstrate clearly that at the nonpermissive temperature the mutant BiP displayed enhanced association with CPY compared to the wild-type strain. These differences were also observed in the presence of DTT, suggesting that they were due to inherent properties of the mutant BiP molecules and not the folding state of CPY. Furthermore, Western blot analysis to determine the distribution of total BiP in the sucrose gradients in Fig. 5, showed that the bulk of it was in fractions 2 and 3, where monomeric and lower order oligomers would sediment. In fact, by this assay no BiP could be detected in the denser part of the gradient or in the pellet in either wild-type or *kar-159* cells under any conditions. This demonstrates that only a minor fraction of BiP was bound to the aggregated CPY, implying that the interaction was specific and not due to massive aggregation of mutant BiP.

Folding Defect of Kar2-159 Is Recessive

To shed more light on the mechanism of the folding defect caused by the BiP mutations, we analyzed CPY folding in a heterozygous diploid strain possessing a wild-type and a

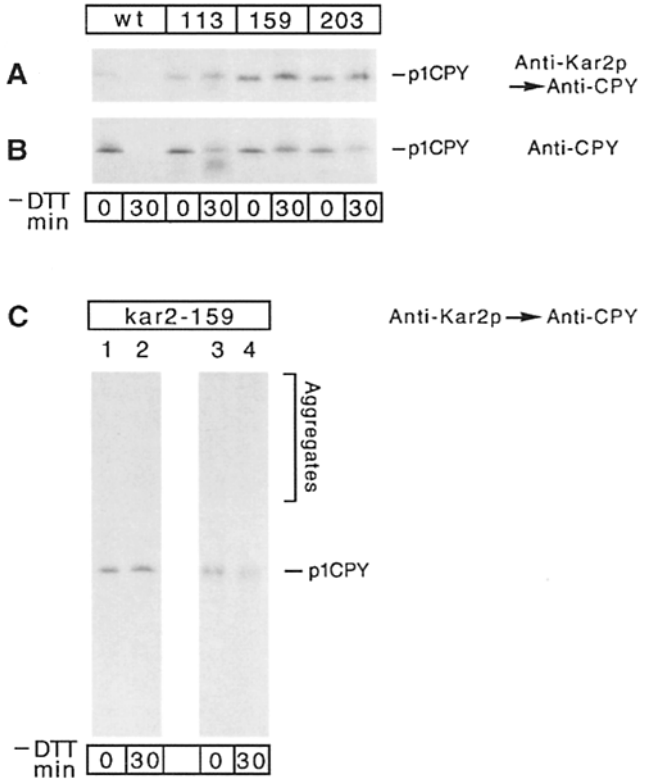

Figure 6. Binding of CPY to BiP is enhanced in the *kar2-113*, *kar2-159*, and *kar2-203* strains. Cells were radiolabeled as described in the legend of Fig. 3 and subsequently lysed in 2% CHAPS in the presence of 30 U/ml apyrase to preserve binding to BiP. Lysates were immunoprecipitated either with anti-CPY antibody (*B*), or first with anti-BiP antibody (*Anti-Kar2p*) followed by an anti-CPY antibody precipitation (*A*). The samples were analyzed by reducing SDS-PAGE. In *C* the samples were prepared as in *A* and *B*, but analyzed both by reducing (lanes *1* and *2*) and nonreducing (lanes *3* and *4*) SDS-PAGE.

kar2-159 allele of BiP. Previously, such strains have been shown to be viable and devoid of translocation defects at the nonpermissive temperature (Vogel et al., 1990), suggesting that the wild-type protein can neutralize any damage caused by the mutant BiP. We found that the CPY folding phenotype was recessive under a variety of conditions after DTT washout, or in the absence of any DTT treatment. Thus, the folding of CPY in the heterozygous diploid was indistinguishable from wild-type cells (Fig. 7).

This result suggests that the mutant BiP molecules do not bind irreversibly to CPY at the nonpermissive temperature; otherwise a fraction of CPY would have been retained in the ER as aggregated complexes. The same result would, however, also arise if the wild-type BiP, via its chaperone activity, would help to suppress the temperature-induced inactivation of the mutant BiP.

Discussion

We have investigated the involvement of BiP during the folding of CPY in the ER of yeast. In three mutant strains, *kar2-113*, *kar2-159*, and *kar2-203*, with mutations in the ATPase domain of BiP, folding and transport of CPY out of the ER was completely and irreversibly inhibited at the

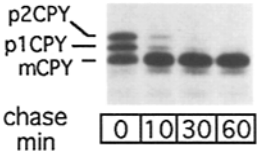

Figure 7. Folding of CPY occurs normally in a heterozygous diploid. Cells were incubated for 1 h at 37°C, then metabolically labeled for 5 min and chased in the presence of cycloheximide and excess unlabeled methionine and cysteine. Aliquots were removed at the indicated time-points after the radioactive pulse, lysed as described in the legend of Fig. 1, and analyzed by reducing SDS-PAGE.

nonpermissive temperature. Neither partially oxidized folding intermediates nor native, fully oxidized ER forms were generated. Instead, the CPY was sequestered in large, partially disulfide cross-linked, BiP-associated aggregates. The results showed that interference with the function of BiP affected the folding of CPY, and established that BiP is a molecular chaperone with an active role in the conformational maturation and transport of newly synthesized proteins.

All mutants displayed increased binding between BiP and CPY compared with the wild-type strain. This suggests that the ATP-induced release from substrates was affected consistent with observations that binding or hydrolysis of ATP is needed for the release of BiP and other hsp70 chaperones from a variety of substrates (Munro and Pelham, 1986; Hurtley et al., 1989; Kassenbrock and Kelly, 1989; Dorner et al., 1990; Gaut and Hendershot, 1993; Palleros et al., 1993; Schmid et al., 1994). The explanation for the failed folding could, therefore, be that BiP bound persistently to newly synthesized CPY, limiting the conformational freedom needed for its proper folding. BiP is thought to recognize transiently exposed hydrophobic peptides that get buried in the interior of the folded protein (Flynn et al., 1991; Blond-Elguindi et al., 1993). Persistent BiP binding to such peptides could prevent them from reaching their intended position in the interior of the protein. The restrictions in the peptide chain mobility, thus imposed, could also explain why native disulfide bonds failed to form or formed inefficiently in CPY.

While such a mechanism is consistent with known features of the hsp70 binding cycle and the role of the ATPase domain, it does not fully explain our observations. For example, it is not clear why the fraction of CPY that did not coprecipitate with BiP also failed to fold. Nor is it obvious why the *kar2-159* mutation was recessive; if binding to the mutant BiP molecules is irreversible, one would expect impaired folding of at least a fraction of the CPY molecules in the heterozygous diploid. We found, however, that CPY folding was entirely normal.

Studies (Vogel, J., and M. D. Rose, manuscript in preparation) indicate that the *kar2-159*, *kar2-113*, and *kar2-203* mutations do not map to the ATP binding site, but rather to other parts of the ATPase domain. Therefore, these mutations may affect the rate of ATP hydrolysis or the coupling between ATP hydrolysis and peptide release. Indeed, other genetic studies (Scidmore, M. A. and M. D. Rose, manuscript in preparation) suggest that these mutants do have some residual BiP function at the nonpermissive temperature. In contrast, mutations that map to the ATP-binding site, which would be expected to be defective in binding or hydrolyzing ATP and thereby unable to release from substrate, are dominant lethals (Vogel, J. P.,

K. Reynolds, J. Brodsky, and M. D. Rose, manuscript in preparation). In the *kar2-159*, *kar2-113*, and *kar2-203* mutants, it is thus conceivable that substrate release does occur, though perhaps more slowly than normal, and in such a way that incorrectly folded intermediates are formed. In the *KAR2/kar2-159* diploid, the wild-type BiP may rescue such misfolded molecules, explaining the recessive phenotype of the *kar2-159* mutation. If so, this would imply that the increased association between CPY and the mutant BiP molecules in the haploid strains is due to the accumulation of misfolded CPY in the ER as much as from the slower (or abolished) release from BiP.

That BiP is required for translocation in yeast is well known, but how essential is it for protein folding? That no CPY folded or was transported out of the ER in the *kar2-113*, *kar2-159*, and *kar2-203* mutants argues that it is crucial. Maturation of a modified glycan-free CPY has also recently been shown to depend on the presence of wild-type BiP (Te Heesen and Aebi, 1994). In addition, when we analyzed the maturation of CPY in another ATPase domain mutant, *kar2-127*, and in the two peptide domain mutants *kar2-1* and *kar2-133*, a partial folding and transport defect was observed. In these strains the ER-retained CPY did not remain in a reduced form, but was found in disulphide cross-linked aggregates (Simons, J. F., M. D. Rose, S. Ferro-Novick, and A. Helenius, unpublished observations). Together these observations support the importance of BiP during protein maturation, and indicate that a variety of mutations in BiP affect its ability to support efficient folding, and that the fidelity of its function becomes increasingly important as the tendency of the substrate to misfold increases.

An additional, although less likely explanation to the failure of CPY to fold in our experimental system includes a defect in the redox regulation of the ER lumen. Normally, the lumen is more oxidizing than the cytosol and allows the formation of disulfide bonds. The redox environment is probably modulated by the influx of oxidized glutathione from the cytosol (Hwang et al., 1992). It is conceivable that the system responsible for maintaining an oxidizing environment somehow depends on the presence of functional BiP. We also cannot exclude that the ATP content of the ER is dependent on functional BiP.

Aggregation is often seen when misfolding occurs in the ER (Leavitt et al., 1977; Doms et al., 1988; Hurtley et al., 1989; Valetti et al., 1991). It is thought to be caused by the hydrophobic properties of incompletely folded polypeptide chains, and prevented by molecular chaperones. Depletion of ATP from living cells causes aggregate formation in the ER (Doms et al., 1987; Dorner et al., 1990; Braakman et al., 1992*b*), and has been attributed to failed function of BiP or other ATP-dependent chaperones in the ER (Kassenbrock and Kelly, 1989; Clairmont et al., 1992). Consistent with this, we found the misfolded CPY in the *kar2* mutant cells in large aggregates. While a large part of it coprecipitated with BiP, there was no clear evidence that they associated directly with each other. The inactivation of BiP could, in fact, have resulted in massive precipitation of luminal ER proteins, with CPY and BiP being trapped nonspecifically in the same complexes. However, our observation that the majority of the BiP molecules did not aggregate suggested that the association of BiP with the aggregates was mediated by specific interactions supporting a model in which the aggregation was caused by inherent properties of the misfolded CPY.

We also observed that except for a minor loss during the first 10 min after DTT dilution, no degradation of CPY took place in any of the mutants up to 2 h. ER degradation has been reported to occur with highly variable half-times depending on the protein. For example, degradation of T cell receptor α chain proceeds with a 50-min $t_{1/2}$, whereas mutant α_1-antitrypsin in fibroblasts from certain PiZZ patients and misfolded influenza virus hemagglutinin disappear with $t_{1/2}$ of 2–2.5 and 6 h, respectively. It is thus possible that a longer chase would have revealed some degradation of CPY. However, since the temperature shift causes irreversible damage to the *kar2* mutants and eventually leads to cell death (Vogel et al., 1990), any degradation observed at such late time-points could be unrelated to normal degradation in the ER.

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