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Cooperation of ER-60 and BiP in the Oxidative Refolding of Denatured Proteins *In Vitro*

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ER-60 is a PDI family protein that has protein thiol-disulfide oxidoreductase activity. It has been shown to associate with BiP in the endoplasmic reticulum. Here, we analyzed the cooperation of ER-60 and BiP in the oxidative refolding of denatured proteins *in vitro*. ER-60 facilitated the refolding of 20 or 30% of denatured α-lactalbumin or ribonuclease B during incubation for 80 min, and these levels of nearly doubled on the addition of BiP to the reaction mixture. BiP alone could not refold denatured ribonuclease B, but could refold denatured α-lactalbumin a little. Enhancement of oxidative refolding of α-lactalbumin by ER-60 could be detected only when ER-60 was present from the start of refolding. On surface plasmon resonance analysis, ER-60 was shown to associate with BiP. The association was not influenced by ATP or ADP. Domains a and/or b′ of ER-60 were implicated in the association with BiP.

Key words: BiP, ER-60, molecular chaperone, protein folding, thiol-disulfide oxidoreductase.

Abbreviations: DTT, dithiothreitol; ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulfide; HEPES, [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; α-LA, α-lactalbumin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; PVDF, polyvinylidene difluoride; RNase B, ribonuclease B; SPR, surface plasmon resonance.

Many polypeptides synthesized in the endoplasmic reticulum (ER) become folded together with the formation of inner molecular disulfide bonds facilitated by protein disulfide isomerase (PDI) and related proteins (*1*, *2*). Mammalian PDI and related proteins have one to three thioredoxin-like motifs, which contain an active center Cys-Xaa-Yaa-Cys sequence for protein thiol-disulfide oxidoreductase activity (*3*). PDI is known to be a multifunctional folding catalyst: it functions not only as a protein thiol-disulfide oxidoreductase but also as a chaperone/anti-chaperone (*4*, *5*), transglutaminase (*6*), the non-catalytic subunit of microsome triacylglycerol transfer protein (*7*, *8*), and the non-catalytic subunit of prolyl 4-hydroxylase (*9*, *10*). ER-60 is known as a PDI family protein, ERp57, and also has protein thiol-disulfide oxidoreductase activity (*11*). ER-60 has been reported to possess multiple enzymatic activities such as endoproteinase (*12–14*) and transglutaminase (*15*) ones. The physiological roles of these enzyme activites other than that of thiol-disulfide oxidoreductase remain unclear.

PDI and ER-60 have been demonstrated to have different mechanisms for substrate recognition. PDI exhibited higher *in vitro* oxidative refolding activity toward denatured proteins than did ER-60 (*16*, *17*), since domain b' of PDI, but not that of ER-60, exhibited higher affinity for substrates (*18*). On the other hand, ER-60 was found to facilitate the oxidative folding of unfolded monoglucosylated glycoproteins *via* interaction with lectin-like molecular chaperones such as calnexin and calreticulin (*16*, *19–21*). Transient mixed-disulfide linkages between ER-60 and nascent glycoproteins were observed *in vivo*. The ER-60–enhanced oxidative refolding of reduced-denatured ribonuclease B (RNase B) was stimulated *in vitro* in the presence of calreticulin or the luminal domain of calnexin. The molecular regions for the association of ER-60 with calnexin or calreticulin were mapped to domains b and b′ and the C-terminal tail of ER-60 (*17*, *22–24*), and the arm domains of calnexin and calreticulin (*25*, *26*). Based on these findings, the idea that PDI is a general protein thiol-disulfide oxidoreductase and that ER-60 is a specialized one for glycoproteins has been accepted. Nevertheless, the actual situations of these proteins in the ER are presumed to be more complicated. Findings suggesting the cooperation of PDI or ER-60 with other ER-resident molecular chaperones for the formation of disulfide linkages in nascent polypeptides have been reported. For example, PDI has been shown to be a member of the complex for the folding of the immunoglobulin heavy chain that comprises BiP, GRP94, P5, ERdj3, cyclophilin B/SDF2-L1, ERp72, GRP170 and UDP-glucosyltransferase (*27*). In addition, PDI has been shown to cooperate with BiP in the oxidative folding of the Fab fragment of immunoglobulin *in vitro* (*28*, *29*). On the other hand, ER-60 associates with a receptor-associated protein, which is a specialized chaperone for a low density lipoprotein receptor–related protein (*30*). In this case, the receptor-associated protein was thought to recruit ER-60 to facilitate the oxidative folding of the low density lipoprotein receptor–related protein. In addition, ER-60 has been shown to associate with BiP in HepG2 cells on immunoprecipitation with anti-BiP or anti–ER-60 antibodies (*31*).

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In this study, we demonstrated that ER-60 was able to cooperate with BiP in the oxidative refolding of denatured RNase B and α-lactalbumin (α-LA) *in vitro*. ER-60 was suggested to be recruited by BiP for the folding of some kinds of proteins in the ER.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP was obtained from NEN Life Science Products Inc. A TSK gel G3000SW HPLC column was obtained from TOSOH. A NAP-5 column was purchased from Amersham. An ATP agarose column, RNase B and α-LA were from Sigma, Inc. A His-Bind quick cartridge and pET30Xa/LIC were obtained from Novagen. Polyvinylidene difluoride (PVDF) protein sequencing membranes and X-ray films, X-OMAT AR, were obtained from Bio-Rad and Eastman Kodak Co., respectively. CM5 sensor chips and an amino coupling kit containing *N*-ethyl-*N*′-(3-dimethyl aminopropyl)-carbodiimide hydrochloride, *N*-hydroxysuccinimide and ethanolamine-HCl were from Biacore AB. The Dye Termination Cycle Sequencing Kit was from Applied Biosystems. Tli DNA polymerase and goat HRP–anti–mouse Ig serum were purchased from Promega Co. Peptide pp52 (YVDRFIGW), which was synthesized using fluorenylmethoxycarbonyl-protected amino acids, was obtained from Sawady Technology Co., Tokyo. The cDNA clone of BiP (GeneStorm yORF Expression Vector containing an insert of H-X87949) from Human GeneStorm was purchased from Invitrogen Co. Mouse anti–78-kD glucose–regulated protein monoclonal antibodies (anti-BiP antibodies) were obtained from Stressgen Biotechnologies Co. Human recombinant ER-60 and the domain mutant fragments were prepared as described previously (*17*). All other chemicals were of reagent grade.

Construction of Expression Vectors for Human BiP—The coding region of human BiP, without a signal sequence, included in the pcDNA3.1/GS plasmid was amplified by polymerase chain reaction (PCR) with two primers: 5′-GGTATTGAGGGTCGCGAGGAGGAGGACAAGAAGGAGGACG-3′, corresponding to the recognition sequence for the LIC site of the vector, pET-30Xa/LIC, and the sequence coding the N-terminal amino acid sequence of mature human BiP, as a foward primer; and 5′-AGAGGAGAGTTAGAGCCCTACAACTCATCTTTTTCTGCTGTA-3′, corresponding to the LIC site and the sequence coding the C-terminal amino acid sequence of human BiP, as a reverse primer. The amplified PCR product was inserted into pET-30Xa/LIC. The nucleotide sequence of the construct was confirmed by the fluorescence dideoxy chain termination method.

Expression And Purification of Recombinant Human BiP—The resultant expression plasmid was transformed into the *E. coli* host strain, BL21 (DE3) (Novagen), grown as 2,000-ml cultures in the presence of 500 μg/ml ampicillin at 37°C, and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 30°C for 2 h. Recombinant BiP was produced as a soluble protein in *E. coli*. Cells were collected by centrifugation, disrupted by sonication in 80 ml of 20 mM Tris/HCl buffer, pH 7.9, containing 5 mM imidazole, 0.5 M NaCl and 1 mM $CaCl_2$ (binding buffer), and then centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was applied to a His-Bind quick cartridge. After washing of the cartridge with binding buffer, BiP was eluted with binding buffer containing 1 M imidazole. The eluted fraction was applied to an ATP-agarose column (1 ml) equilibrated with 20 mM [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer, pH 7.4, 0.5 M NaCl, 3 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride, and eluted with the same buffer containing 3 mM ATP. The eluted fraction was concentrated and subjected to gel filtration chromatography on a TSK gel G3000SW column equilibrated with 10 mM Tris/HCl buffer, pH 7.4, containing 0.15 M NaCl and 10% glycerol. BiP was eluted in both the void volume and the volume fractions corresponding to 80 kDa. The 80-kDa fractions were collected and used for the following assays. For surface plasmon resonance (SPR) analysis, the purified BiP was supplemented with a 600-fold molar excess of ADP, incubated at 4°C for 30 min, dialyzed against 10 mM HEPES, pH 7.0, 150 mM NaCl, 0.05% Tween 20 (HBS buffer) at 4°C for 16 h, and then used for experiments.

Oxidative Refolding of RNase B And α-LA—The preparation and refolding of reduced and denatured RNase B were carried out as described by Zapun *et al*. (*16*). Briefly, RNase B was reduced and denatured by incubation for 20 min at 25°C in 0.1 M Tris/HCl, pH 8, 6 M guanidine chloride, 20 mM dithiothreitol (DTT), and then desalted in 0.1% trifluoroacetic acid on a NAP-5 column and freeze-dried. Refolding was initiated by dissolving the freeze-dried reduced RNase B (73 μM) in 20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, 25 mM $MgCl_2$, 12.5 mM ATP, 0.5 mM glutathione disulfide (GSSG), 2 mM glutathione (GSH), 3.7 μM ER-60 with or without 11.1 μM BiP, and then the mixture was incubated at 25°C. Aliquots were withdrawn at various times, reacted for 5 min at 25°C with 0.25 volumes of 0.5 M iodoacetamide in 1.5 M Tris/HCl, pH 8.7, and then stored on ice until analysis by low pH nondenaturing polyacrylamide gel electrophoresis (PAGE) (*32*). Proteins were stained with Coomassie Brilliant Blue R-250.

The preparation of reduced and denatured α-LA was carried out as described by Ewbank and Creighton (*33*). Refolding was initiated by dissolving the freeze-dried reduced α-LA (74 μM) in 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 25 mM $MgCl_2$, 12.5 mM ATP, 0.5 mM GSSG, 2 mM GSH, 3.7 μM ER-60 with or without 148 μM BiP, and then the mixture was incubated at 25°C. In the case of preincubation experiments, the freeze-dried reduced α-LA (74 μM) was dissolved in 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 25 mM $MgCl_2$, 12.5 mM ATP, 0.5 mM GSSG, 2 mM GSH with or without 148 μM BiP and then preincubated at 25°C for 40 min. After the preincubation, ER-60 (3.7 μM) was supplemented and then the mixture was incubated at 25°C. Aliquots were withdrawn at various times, reacted for 5 min at 25°C with 0.25 volumes of 0.5 M iodoacetamide in 1.5 M Tris/HCl, pH 8.7, and then stored on ice until analysis by high pH nondenaturing PAGE (*34*). Proteins were stained with Coomassie Brilliant Blue R-250.

SPR Analyses of Protein–Protein Interactions with BIAcore—The BIAcore 2000 machine and analysis program were both from Biacore AB. A CM5 sensor chip was activated with a 1:1 mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride, following the instructions of the manufacturer. ER-60 (15 μg/ml in 35 μl of 10 mM acetate buffer,

pH 4.5), α-LA (1.1 mg/ml in 35 μl of 10 mM acetate buffer, pH 4), or BiP (4 μg/ml in 35 μl of 10 mM acetate buffer, pH 5.5) was injected over the activated surface. α-LA was reduced in 2 mM DTT for 30 min at 25°C before the injection. The remaining binding sites were blocked with 1 M ethanolamine, pH 8.5. The control channel on the sensor chip was activated and blocked using amine-coupling reagents, without immobilization of the protein. The binding of proteins to this control channel was subtracted from the specific binding. The experiments were performed at 20°C with HBS buffer containing 2 mM $MgCl_2$ (running buffer) with 1 mM ATP or ADP at the flow rate of 10 μl/min. The running buffer was also used for diluting the samples for injection. An equal volume of each protein solution was injected onto a control mock sensor chip to obtain a blank sensorgram.

Others—The ATPase activity of BiP was assayed by Seals's method (*35*). BiP (5 μM) was incubated in 20 ml of 20 mM HEPES, pH 7.0, containing 75 mM KCl, 5 mM $MgCl_2$ and 100 mM [γ-^{32}P]ATP (1 mCi) at 37°C. Twenty-five milliliters of 4 N H_2SO_4, containing 4% ammonium molybdate and 20 mM silicotungstic acid, and 100 ml of a xylene and isobutyl alcohol mixture (65:35) were added, followed by centrifugation at 12,000 rpm for 10 min. The radioactivity included in 50 ml of the upper solvent layer was counted.

The concentrations of proteins were measured with a BioRad protein assay kit with γ-immunoglobulin as a standard.

Western blotting was carried out with anti-BiP antibodies. Briefly, proteins separated by SDS-PAGE (*36*) were blotted onto PVDF membranes and then immunostained with goat horseradish peroxidase–conjugated mouse IgM serum as second antibodies, using Renaissance Chemiluminescence Reagent (NEN Life Science Products Inc.).

RESULTS

Effect of BiP on the Oxidative Refolding by ER60 of Denatured RNase B or α-LA—ER-60 is a PDI family protein that exhibits weaker protein thiol-disulfide oxidoreductase activity than that of PDI (*16*, *17*). ER-60 has been shown to associate with BiP in HepG2 cells by cross-linking experiments on cellular proteins followed by immunoprecipitation with anti–ER-60 or anti-BiP serum (*31*). Hence, we tried to determine the effect of BiP on the refolding activity of ER-60 toward denatured proteins. First, the human BiP recombinant protein was expressed in *E. coli* and purified (Fig. 1a). A His tag was inserted at the N-terminal of recombinant BiP to avoid contamination by *E. coli* DnaK on purification on a Ni^{2+}-column (His-Bind quick cartridge). During the purification procedures, the recombinant BiP bound to ATP agarose and was eluted with ATP. This suggested that the recombinant BiP molecules were properly folded. The expressed BiP was predominantly present as a monomer, which was eluted in volume fractions corresponding to 80 kDa on gel filtration column chromatography (Fig. 1b). A small proportion of oligomeric species was also obtained on the gel filtration column chromatography. The BiP monomer fractions were collected and used for experiments. No DnaK protein was detectable in the BiP preparation by Western blotting with anti-DnaK antibodies (data not shown). The purified BiP monomers showed ATPase activity: the specific activity was 7.2 pmol/min/μg protein (Fig. 1c). The activity was not affected by the addition of peptide pp52, which has been identified as a BiP-binding sequence and stimulates the ATPase activity of oligomeric BiP by dissociating it into monomers (*37*, *38*). This suggested that the purified BiP was stably present as monomers. Next, the effect of the recombinant BiP on the refolding of α-LA denatured by reduction with DTT was determined (Fig. 2, a and b).

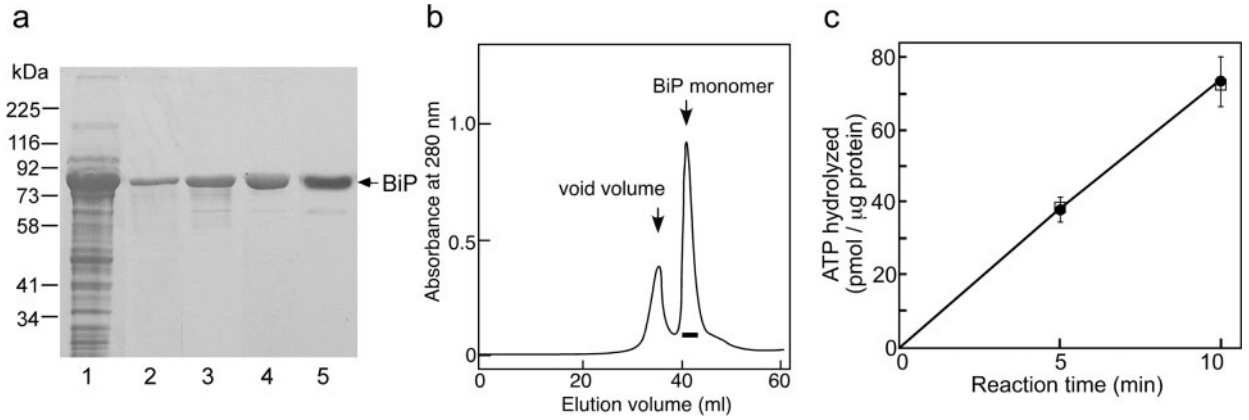


Fig. 1. **Expression and purification of the recombinant BiP.** (a) SDS-PAGE (10% gel) analysis of pooled fractions at various stages of purification of the recombinant BiP. Extract of *E. coli*, in which the recombinant BiP protein was expressed (lane 1); pooled fractions obtained from a His-Bind quick cartridge (lane 2); fractions obtained on ATP-agarose column chromatography (lane 3); fractions obtained on TSK gel G3000 column chromatography (lanes 4 and 5). Proteins were stained with Coomassie Brilliant Blue-R250 (lanes 1–4) or immunostained with anti-KDEL antibodies (lane 5) as described under "MATERIALS AND METHODS." (b) Gel filtration column chromatogram of the recombinant BiP expressed in *E. coli*. The fractions obtained on ATP-agarose column chromatography were subjected to TSK gel G3000SW gel filtration column chromatography. The elution of proteins was followed as the UV absorption at 280 nm. The fractions indicated by a bar were collected and used for experiments. (c) ATPase activity of the recombinant BiP. Activity was determined as described under "MATERIALS AND METHODS," The activity of the recombinant BiP assayed in the presence of 100 μM pp52 (squares) was the same as that assayed in the absence of pp52 (circles). Values are mean for three experiments.

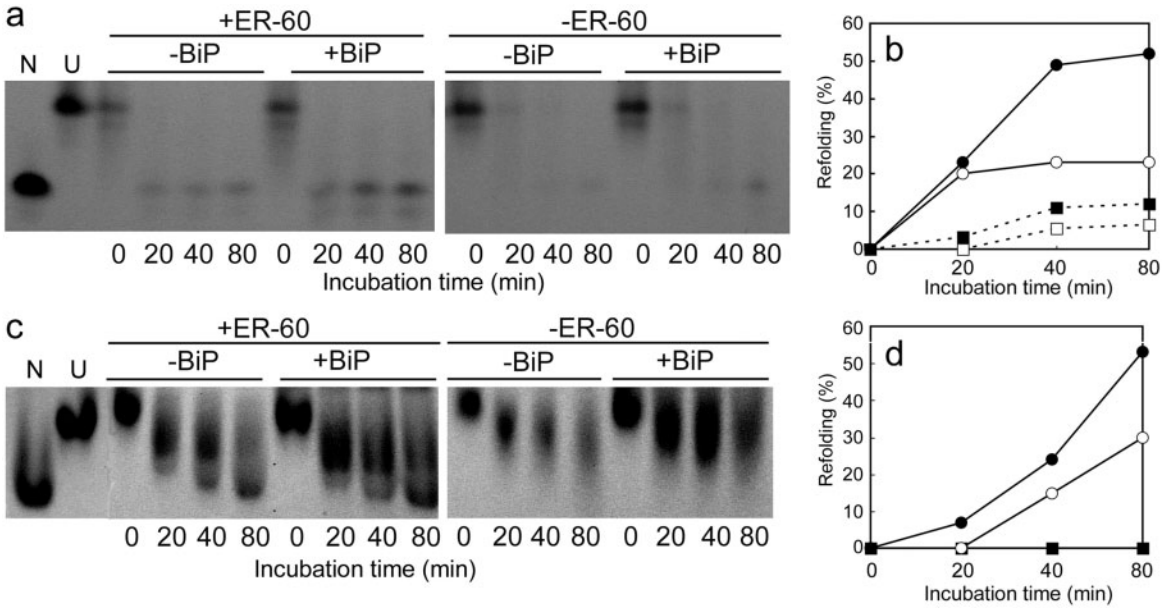


Fig. 2. **Refolding of α-LA or RNase B catalyzed by ER-60 with or without BiP.** Refolding of denatured α-LA (a) or RNase B (c) was carried out in the presence (+ER-60) or absence (–ER-60) of ER-60 with (+BiP) or without (–BiP) BiP. The reaction was terminated after the indicated times and then the conformation of α-LA or RNase B was examined by nondenaturing PAGE, as described under "MATERIALS AND METHODS." The native (N) and unfolded forms (U) of α-LA or RNase B were also electrophoresed on the same gel. Refolding % of α-LA (b) and RNase B (d) in the presence (circles) or absence (squares) of ER-60 with (closed symbols) or without (open symbols) BiP was quantified by densitometry of the gel shown in (a) or (c).

Seven percent of α-LA was spontaneously refolded in the refolding buffer. The addition of BiP to the refolding buffer increased the refolding to 12% of that in the case of the denatured α-LA. The refolding of the denatured α-LA was enhanced by the addition of ER-60 to threefold the spontaneous refolding observed in the absence of ER-60 and BiP. This oxidative refolding of the denatured α-LA enhanced by ER-60 increased in the presence of BiP to over twice as much as that in the absence of BiP. This value was higher than the sum of the yields obtained in the refolding reaction with BiP or ER-60 alone. Similar results were obtained for the refolding reaction of denatured RNase B (Fig. 2, c and d). No spontaneous refolding of RNase B was observed even in the presence of BiP. Refolding of denatured RNase B was clearly enhanced by ER-60. This oxidative refolding of the denatured RNase B enhanced by ER-60 increased in the presence of BiP to about twice as much as that in the absence of BiP. The positive effect of BiP on the oxidative refolding of the denatured protein may be caused by prevention of misfolding or aggregation of the denatured protein. To examine this, we performed refolding experiments after preincubation of reduced α-LA for 40 min in the absence or presence of BiP (Fig. 3). When unfolded α-LA was preincubated in the absence of BiP, the final refolding yield was 7%. This corresponded to the yield of spontaneous refolding in the absence of ER-60 and BiP (Fig. 2a). When unfolded α-LA was preincubated in the presence of BiP, the refolding yield was 15%. This is compatible with the refolding yield obtained in the presence of BiP alone (Fig. 2a).

BiP Associates with ER-60 in a Manner Different from Its Association with an Unfolded Polypeptide—Previously, ER-60 was revealed to associate with BiP in HepG2 cells (*31*). Thus, we examined the interaction of ER-60 and BiP

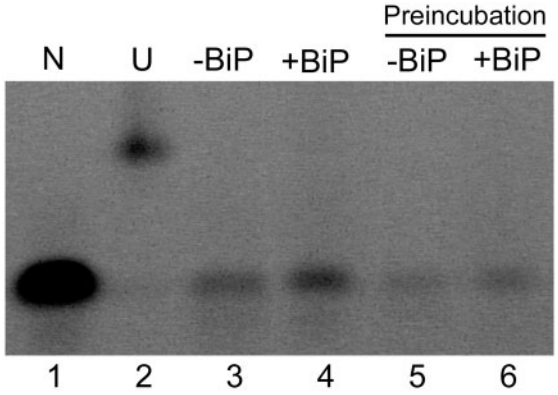


Fig. 3. **Influence of preincubation on the refolding of α-LA.** Denatured α-LA was preincubated at 25°C for 0 (lanes 3 and 4) or 40 (lanes 5 and 6) min in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of BiP, and then supplemented with ER-60. After incubation at 25°C for 80 min, the conformation of α-LA was examined by nondenaturing PAGE, as described under "MATERIALS AND METHODS." The native (lane 1) and unfolded (lane 2) forms of α-LA were electrophoresed on the same gel.

by SPR analysis to confirm whether or not ER-60 associated directly with BiP. BiP associated with ER-60 on the sensor chip (Fig. 4a). ATP or ADP did not influence this interaction. From the sensorgrams, dissociation constants, K_D, of 2.5 μM and 3.0 μM were obtained in the presence of ATP and ADP, respectively. On the other hand, the dissociation of BiP from the denatured α-LA was faster in the presence of ATP than ADP (Fig. 4b). The interaction of BiP with an unfolded protein has been demonstrated to be competitively inhibited by oligopeptides containing aromatic and hydrophobic amino acids,

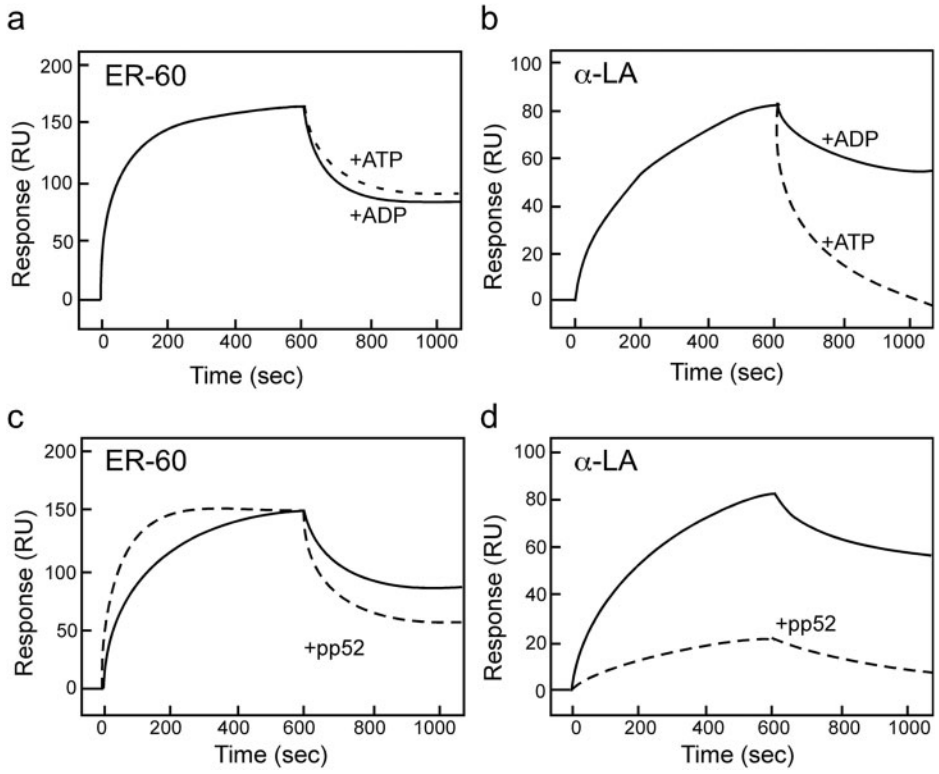


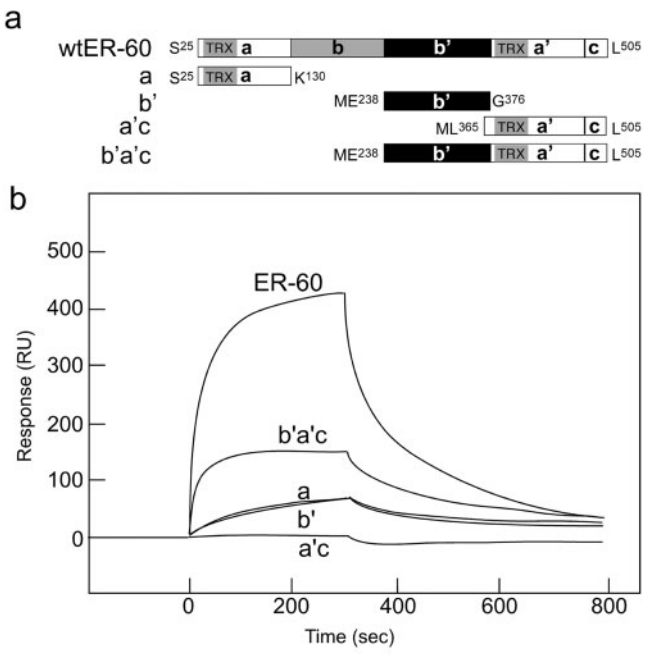
Fig. 4. **Examination of binding of BiP and ER-60 or reduced α-LA by SPR analysis.** Native ER-60 (38 fmol/mm^2) (a, c) or reduced α-LA (191 fmol/mm^2) (b, d) was immobilized on the surface of a SPR chip as described under "MATERIALS AND METHODS." In (a) and (b), at zero time, BiP (3.5 μM) was added to the running buffer containing 1 mM ADP passed continuously over the chip. At the time the curve began to fall, the BiP solution was replaced by running buffer containing 1 mM ADP (solid lines) or ATP (dashed lines). The non-specific response with a blank chip exposed in the same manner has been subtracted from the response units shown. In (c) and (d), 3.5 μM BiP mixed with (dashed lines) or without (solid lines) 100 μM pp52 in running buffer containing 1 mM ADP was passed over the chip with immobilized ER-60 (c) or reduced α-LA (d), and then the on and off rates for binding were determined.

like pp52 (*38*, *39*). As expected, the interaction of BiP with the denatured α-LA was inhibited by pp52 (Fig. 4d). On the other hand, the association of BiP with ER-60 was not inhibited by pp52 (Fig. 4c). Consequently, it is suggested that the interaction between BiP and ER-60 may differ from that between BiP and a denatured polypeptide such as reduced α-LA.

Domains a and b′ of ER-60 are able to Associate with BiP—To map the BiP binding site(s) on the ER-60 molecule, the association of recombinant domain proteins of ER-60 with BiP on a sensor chip was analyzed. In the previous study (*17*), recombinant domain proteins b and ab were demonstrated not to be properly folded in *E. coli*. Thus, recombinant domain proteins a, b′, a′c, and b′a′c (Fig. 5a), which were confirmed to be folded on urea gradient gel electrophoresis and CD analysis, were analyzed. Proteins b′a′c and b′, but not a′c, were able to associate with BiP (Fig. 5b). In addition, a was also able to associate with BiP. The dissociation constants of a, b′ and b′a′c from BiP were similar to that of ER-60 from BiP (Fig. 5c). Therefore, domain b′ and/or a are thought to be responsible for the association of ER-60 with BiP.

DISCUSSION

In this study, we investigated the effects of BiP on the ER-60–enhanced oxidative refolding of denatured RNase B and α-LA. Our results showed that the ER-60–enhanced oxidative refolding of denatured RNase B or α-LA was synergistically stimulated on the addition of BiP. When ER-60 alone was present from the initiation of refolding of α-LA or RNaseB, up to 20 or 30% of the molecules reached the native form. However, enhancement of oxidative refolding of α-LA by ER-60 could not be detected when ER-60 was added after preinitiation for 40 min. These results suggested that accessible unfolded substrates decreased in the mixture during the refolding reaction, in which misfolding or aggregation of the unfolded substrates occurred. Therefore, it was presumed that BiP may prevent misfolding or aggregation to keep the substrates available for ER-60. A similar phenomenon has been reported for the enhanced folding of a Fab fragment through cooperation of PDI with BiP (*28*). In addition, ER-60 was indicated to associate with BiP on SPR analysis. ER-60 may bind to a region other than the 18-kDa peptide binding domain of the BiP molecule (*40, 41*), since the binding of ER-60 with BiP was not affected by ATP, which accelerates the dissociation of BiP from a substrate polypeptide (*42*), or by pp52, which competitively inhibits the binding of substrate polypeptides to the substrate binding site of BiP (*37*). On the other hand, recombinant domain proteins of ER-60, a and b′, were shown to be able to bind to BiP. Previously, it was shown that fragments a and a′ of ER-60 had thiol-disulfide oxidoreductase



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Analyte	K_D (M)
ER-60	8.1×10^{-8}
a	2.4×10^{-8}
b'	1.5×10^{-7}
b'a'c	7.7×10^{-8}

Fig. 5. **Examination of binding of BiP and recombinant fragments of ER-60 by SPR analysis.** (a) Recombinant fragments of ER-60 used in this study. The nomenclature adopted is based upon the hypothesized domain structure of ER-60 (*17*). The N-terminal or C-terminal amino acid residue of each domain is given on the left or right side of the domain bar, respectively. M donates an initial methionine residue. TRX indicates thioredoxin-like motif. (b) BiP (13 fmol/mm^2) was immobilized on the surface of a SPR chip. ER-60 or recombinant fragments (0.55 μM) were added to running buffer passed continuously over the chip. The experiments were performed in the presence of 1 mM ADP as described under "MATERIALS AND METHODS." At the time the curve began to fall, the solution was replaced by running buffer containing ADP. The non-specific response with a blank chip exposed in the same manner has been subtracted from the response units shown. The analyses were performed with the same sensor chip. (c) Dissociation constants were calculated by nonlinear fitting of the sensorgram data in b using the BIA evaluation 2.1 software.

activity equivalent to almost half of that of wild-type ER-60 (*17*), suggesting that ER-60 was able to bind to the substrate protein through domains a and/or a′. From these results it is postulated that BiP binds to an unfolded protein by means of its substrate-binding region to slow down aggregation and misfolding of the substrate, and simultaneously binds to b′ of ER-60 by means of an unidentified ER-60–binding region. Thus, a possible mechanism for the synergistic effect of BiP on the enhancing activity of ER-60 toward oxidative refolding of denatured RNase B and α-LA is assumed to be that the oxidative refolding is stimulated by the formation of a complex of ER-60 and BiP on the unfolded protein. It is assumed that the apparent affinity of ER-60 for the denatured protein may increase as a result of the association of ER-60 with BiP, which is bound to the denatured protein, and this may increase the efficiency of the oxidative refolding of the denatured protein. However, it is unclear whether or not a complex of BiP and ER-60 is able to prevent aggregation or misfolding of unfolded proteins. Previously, ER-60 was shown to cooperate with calnexin and calreticulin, which bind to the monoglucosylated glycan moiety of the unfolded protein (*16*, *19–21*). Therefore, ER-60 is thought to be a thiol-disulfide oxidoreductase specialized for glycoproteins. This study suggested the possibility that ER-60 even acts on unfolded proteins other than monoglucosylated glycoproteins through cooperation with BiP.

Does ER-60 actually cooperate with BiP in the oxidative folding of nascent polypeptides in the ER? In rat thyroid cells and mouse lymphoma cells, BiP has been found to form a multiprotein complex with ERdj3, GRP94 and GRP170, irrespective of the presence of an unfolded protein (*27*, *43*). When the multiprotein complex associated with the unfolded immunoglobulin heavy chain in mouse lymphoma cells, the complex was shown to recruit cyclophilin B/SDF2-L1, UDP-glucosyltransferase and several protein thiol-disulfide oxidoreductases, and to form a large folding complex. In this folding complex, ER-60 was detected on mass spectrometry (*27*). In addition, the coimmunoprecipitation of BiP with ER-60 has been reported for Hep G2 cells (*31*). Recently, it was reported that ER-60 could bind to PDI, which acts exclusively in the BiP system (*44*). This finding also implies that ER-60 can function in the BiP complex. Possibly, BiP folding complexes may recruit different protein thiol-disulfide oxidoreductase(s), depending on the type of unfolded polypeptide chains associated.

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