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BiP and PDI Cooperate in the Oxidative Folding of Antibodies *in Vitro**

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Immunoglobulin heavy chain binding protein (BiP), a member of the Hsp70 chaperone family, and the oxidoreductase protein-disulfide isomerase (PDI) play an important role in the folding and oxidation of proteins in the endoplasmic reticulum. However, it was not clear whether both cooperate in this process. We show here that BiP and PDI act synergistically in the *in vitro* folding of the denatured and reduced Fab fragment. Several ATP-dependent cycles of binding, release, and rebinding of the unfolded antibody chains by BiP are required for efficient reactivation. Our data suggest that in the absence of BiP unfolded antibody chains collapse rapidly upon refolding, rendering cysteine side chains inaccessible for PDI. BiP binds the unfolded polypeptide chains and keeps them in a conformation in which the cysteine residues are accessible for PDI. These findings support the idea of a network of folding helper proteins in the endoplasmic reticulum, which makes this organelle a dedicated protein-processing compartment.

In the endoplasmic reticulum, several chaperones and folding catalysts are involved in the folding of secretory proteins (1–5). It is still poorly understood how these proteins cooperate to assist folding. Immunoglobulin heavy chain binding protein (BiP),[1] an ER-located member of the Hsp70 chaperone family, was originally found noncovalently associated with immunoglobulin heavy chains (6). It is known that BiP associates transiently with a large number of newly synthesized proteins in the ER (7–9). Like other Hsp70 proteins, BiP has a weak ATPase activity (10). Binding of ATP is necessary for the release of peptides bound to BiP (11). BiP has a binding site selective for linear sequences of seven amino acids containing hydrophobic residues (12). Based on this binding algorithm, BiP binding sequences were identified in the light chain and in the V_H, C_H1 and C_H3 domains of the heavy chain (13).

PDI is a homodimer of 55-kDa subunits, which catalyzes the formation of disulfide bonds (14). Because PDI also binds to different peptides that lack cysteines (15), it had been proposed that PDI acts not only as an enzyme but also as a chaperone (16–18). Both activities are required for reactivation of reduced and denatured acidic phospholipase A_2 (19). However, in the refolding of antibody fragments, PDI has no chaperone-like effect but acts as an enzyme that catalyzes disulfide bond formation and reshuffling (20).

The antibody Fab fragment is a good model system, because antibody domains contain a disulfide bond that is part of the hydrophobic core of the protein. We used the Fab fragment of the murine monoclonal antibody MAK33 (21) for examining the influence of BiP and PDI on the refolding and reoxidation of antibodies, because the effect of PDI on antibody reactivation had been investigated in detail before (20). PDI has to be present during the initial stages of folding to influence the reaction. This may be due to the kinetic competition between Fab structure formation and accessibility of the cysteines for PDI (20).

Here we addressed the question of whether the binding of unfolded antibody fragments to BiP may keep the cysteines accessible for PDI and whether these folding helpers cooperate in a synergistic way.

EXPERIMENTAL PROCEDURES

Reagents—Phosphocreatine, creatine phosphatase, apyrase, and BSA (RIA Grade) were from Sigma. MAK33 Fab was from Roche Diagnostics.

Purification of Recombinant Protein-disulfide Isomerase—Human PDI was expressed in *Escherichia coli* strain BL21(DE3). The pet 23a-based plasmid containing the coding sequence for PDI was obtained from R. Freedman and L. Ruddock (University of Kent, UK).

Cells were grown at 37 °C in LB medium supplemented with 100 μg ml^{-1} ampicillin at an A_{600} of 0.6. Gene expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. After 6–7 h, the cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C.

Cell pellets were washed and resuspended in 0.1 M Tris/HCl, pH 8, 2 mM EDTA containing a protease inhibitor mixture (Roche Diagnostics). The cells were lysed using a disrupter (Constant Systems). After centrifugation (40,000 × *g*, 40 min, 4 °C) to remove debris, PDI was enriched by heat treatment and ammonium sulfate precipitation. First the cell extract was heated up to 54 °C and maintained at this temperature for 15 min. After cooling, the extract was centrifuged (40,000 × *g*, 30 min, 4 °C), and the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 55% saturation. After stirring for 30 min at room temperature, the precipitate was centrifuged (40,000 × *g*, 40 min, 4 °C), and the pellet was discarded. Further $(NH_4)_2SO_4$ was added to a final saturation of 85%, and the material was centrifuged as before. The pellet was dissolved in 100 ml of 20 mM sodium-phosphate buffer, pH 6.3, and dialyzed against the same buffer.

The dialyzed material was loaded on to a resource Q column (Amersham Pharmacia Biotech) equilibrated with 20 mM sodium-phosphate buffer, pH 6.3. For elution, a linear NaCl gradient was applied. PDI was detected between 0.3 and 0.4 M NaCl by SDS-polyacrylamide gel electrophoresis.

Next, the PDI-containing fractions were bound to a hydroxylapathite column (Bio-Rad) equilibrated with 20 mM sodium phosphate buffer, pH

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[1] The abbreviations used are: BiP, immunoglobulin heavy chain binding protein; BSA, bovine serum albumin; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; Fab, proteolytically derived antigen-binding antibody fragment consisting of the entire light chain and the two NH_2-terminal domains of the heavy chain; Fab/red, Fab fragment with reduced cysteines; Fd, part of the immunoglobulin heavy chain consisting of the two amino-terminal domains (V_H and C_H1); MAK33, murine monoclonal antibody of subtype κ/IgG1 directed against human creatine kinase; PDI, protein-disulfide isomerase.

6.3, and eluted with a linear sodium phosphate gradient. The PDI-containing fractions were pooled and dialyzed overnight against 20 mM sodium phosphate buffer, 300 mM NaCl, pH 6.3.

The dialyzed fractions were applied to a Superdex 200-pg gel filtration column (Amersham Pharmacia Biotech) equilibrated with 20 mM sodium phosphate buffer, 300 mM NaCl, pH 6.3. The protein was concentrated by Amicon ultrafiltration, dialyzed against 50 mM NH_4HCO_3, and freeze-dried. The freeze-dried enzyme was stored at −20 °C.

The concentration of PDI was determined using the published extinction coefficient of $E^{1\,cm}_{280,\,0.1\%} = 0.795$. Here molar concentrations refer to the dimer.

Purification of Recombinant BiP—Murine BiP was expressed in the *E. coli* strain M15. The pASK-based plasmid contains the coding sequence for BiP with a His_6 tag at its COOH terminus (22).

Expression and purification was carried out basically as described previously (22). Cell pellets were washed and resuspended in 40 mM Hepes (pH 7.0) containing a protease inhibitor mixture (Roche Diagnostics) and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed using a disrupter (Constant Systems).

After centrifugation (40,000 × *g*, 30 min, 4 °C) to remove debris, the supernatant was loaded on a Ni^{2+}-nitrilotriacetic acid-Sepharose fast flow column. BiP was detected in a sharp peak at 150 mM imidazole.

The fractions containing BiP were pooled, dialyzed overnight against 40 mM Hepes, 0.5 M NaCl, 5% glycerol, pH 7.5, and concentrated by Amicon ultrafiltration.

The concentrated protein solution was applied to a Superdex 200-pg gel filtration column (Amersham Pharmacia Biotech) equilibrated with 40 mM Hepes, 0.5 M NaCl, 5% glycerol, pH 7.5.

The protein was dialyzed overnight against 40 mM Hepes, 5% glycerol, pH 7.5, and concentrated by Amicon ultrafiltration. The protein was frozen in liquid nitrogen and stored at −20 °C.

The concentration of BiP was determined using the published extinction coefficient of $E^{1\,cm}_{280,\,0.1\%} = 0.395$. Here molar concentrations refer to the monomer.

Unfolding and Refolding of the Fab Fragment—Denaturation and renaturation of the proteolytically derived Fab fragment were carried out as described previously (20). The Fab fragment was denatured in 6 M guanidinium chloride, 0.1 M Tris, pH 8.0, for 2 h at 25 °C. Reduction of the disulfide bonds was achieved by adding 0.3 M dithioerythritol to the denaturation solution. Renaturation was initiated by diluting the denatured protein 100-fold into the buffer preincubated at 15 °C containing 0.1 M Tris, pH 7.0, with vigorous stirring for 10 s. The renaturation buffer contained in addition 6 mM GSSG, and the final concentration of DTE was 3 mM. In the cases indicated, the renaturation buffer was supplemented with an ATP-regenerating system containing 0.5 mM ATP, 10 mM phosphocreatine, and 35 units/ml creatine phosphatase. Refolding was carried out at 15 °C. The final concentrations during renaturation were 0.2 μM for Fab and 60 mM for guanidinium chloride. PDI was reduced in the presence of 1 mM dithioerythritol for 10 min before it was added to the renaturation buffer.

Isolation and Solubilization of Light Chain and Fd Inclusion Bodies—Light chain and Fd fragment of MAK33 were expressed recombinantly in the *E. coli* strain BL21(DE3) harboring the plasmid for either the light chain or the Fd fragment (23).

Cells were grown at 37 °C in LB media supplemented with 100 μg ml^{-1} ampicillin at an A_{600} of 0.5. Gene expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. After 24 h, the cells were harvested by centrifugation at 5000 × *g* for 15 min at 4 °C.

Inclusion body isolation and solubilization were performed as described previously (23). The cell pellet was resolved in 0.1 M Tris, 1 mM EDTA, pH 7.0, and incubated with 1.5 mg of lysozyme/g of cells for 30 min at 4 °C. Cells were lysed with a disrupter (Constant Systems). The lysed cells were incubated with 10 μg of DNase I and 3 mM $MgCl_2$ for 30 min at 25 °C to digest the DNA. The inclusion bodies were isolated by adding 0.5 volumes of 60 mM EDTA, 6% Triton X-100, 1.5 M NaCl, pH 7.0, and incubation for 30 min at 4 °C. The inclusion body pellet was centrifuged (40,000 × *g*, 30 min, 4 °C) and washed in 0.1 M Tris, 20 mM EDTA, pH 7.0. The pellet was resolved in 0.1 M Tris, 8 M urea, 100 mM dithioerythritol, pH 8.0, and incubated for 2 h at 25 °C. After a pH shift to pH 4.0 and centrifugation (40,000 × *g*, 30 min, 4 °C) the solution was dialyzed overnight against 5 M urea, pH 4.0.

The purity and protein concentration of the preparations were checked by SDS-polyacrylamide gel electrophoresis and Bradford assay (24).

ELISA—The ELISA assay was carried out as described previously (23, 25). In short, dimeric muscle-specific human creatine kinase was attached to tubes using the biotin/streptavidin system. At the times indicated, aliquots of the refolding Fab fragment were diluted in ELISA buffer. To prevent further reactivation of the Fab fragment, the samples were kept on ice, and trypsin with a final concentration of 400 μg/ml was added. Binding of functionally refolded Fab fragments to dimeric muscle-specific human creatine kinase was detected by using peroxidase-coupled anti-mouse-IgG-antiserum. The amount of functionally reactivated protein was quantified by comparison with standard curves obtained with the authentic Fab fragment.

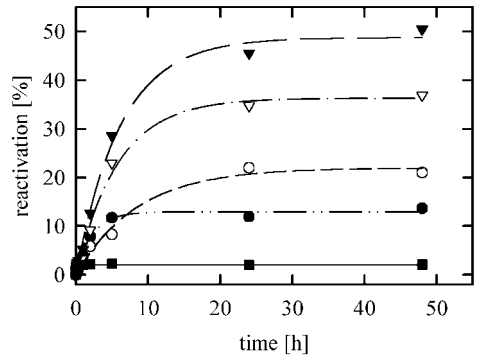

FIG. 1. **Influence of BiP and PDI on the refolding of Fab/red.** Reactivation of Fab/red (the final concentration of Fab was 0.2 μM) was initiated by a 100-fold dilution in 0.1 M Tris, pH 7.0, 6 mM GSSG in the presence of 3 μM BSA with 0.5 mM ATP and an ATP-regenerating system (■) (the same results were observed without ATP; data not shown), in the presence of 2 μM BiP with 0.5 mM ATP and an ATP-regenerating system (●) (the same results were observed without ATP; data not shown), in the presence of 0.5 μM PDI (○), in the presence of 2 μM BiP and 0.5 μM PDI (▽), and in the presence of 2 μM BiP, 0.5 μM PDI, 0.5 mM ATP, and an ATP-regenerating system (▲). Reactivation yields were determined by ELISA.

ATPase Measurements—ATPase activity of BiP was determined as described previously (13). The assay was carried out in the same buffer used for the refolding experiments (0.1 M Tris, pH 7.0, 50 mM KCl, and 2 mM $MgCl_2$). 5 μM BiP was incubated at 37 °C with 500 μM (final concentration) unlabeled ATP and 10 μCi of [α-^{32}P]ATP in a total volume of 30 μl. ATP hydrolysis was stopped after different times by adding EDTA (final concentration 24 mM). After thin layer chromatography on polyethyleneimine-cellulose (Merck), the ATP/ADP ratio was quantified with a Molecular Dynamics Storm 860 PhosphorImager. Hydrolysis rates were corrected for uncatalyzed, spontaneous ATP hydrolysis at the respective conditions.

RESULTS

PDI and BiP Affect the Refolding of Fab/red—It had been shown previously that PDI influences the reactivation of denatured and reduced Fab fragments *in vitro*. Whereas in the absence of PDI hardly any refolding could be achieved, the presence of PDI lead to reactivation yields of about 20% (Ref. 20 and Fig. 1). It is known that *in vivo* BiP associates transiently with immunoglobulin heavy and light chains (8, 9), and BiP binding sequences were determined in the Fab fragment of antibodies (13). It had therefore been proposed that BiP could act together with PDI in the folding of antibodies (20).

To test this hypothesis, we performed refolding experiments in the presence of either PDI, BiP, or both folding helpers. BSA was used to test whether nonspecific protein effects influence the reaction. As shown in Fig. 1, no reactivation could be achieved in the presence of BSA, whereas with PDI 20% reactivation was obtained, as described previously (20). In the presence of BiP alone, the reactivation yields were 15%. Surprisingly, when ATP was present in addition to BiP, no further increase in reactivation yields was observed. In the presence of PDI and BiP, 35% of the molecules reached the native state. This corresponds to the sum of the reactivation yields of BiP (15%) and PDI (20%). However, in the presence of BiP, PDI, and ATP, yields of 50% were observed (Fig. 1). This indicates that BiP and PDI act in a synergistic way and that this effect is ATP-dependent. Using radiolabeled [α-^{32}P]ATP, we demon-

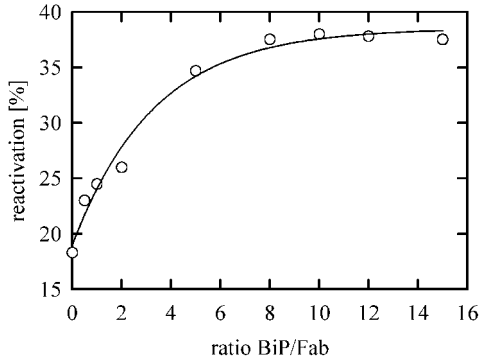

FIG. 2. **Influence of the BiP to Fab ratio on the refolding of Fab/red.** Reactivation of Fab/red (the final concentration of Fab was 0.2 μM) was initiated by a 100-fold dilution in 0.1 M Tris, pH 7.0, 6 mM GSSG, containing 0.5 mM ATP, an ATP-regenerating system, 0.5 μM PDI, and different amounts of BiP (0–3 μM). Reactivation was determined after 48 h of incubation.

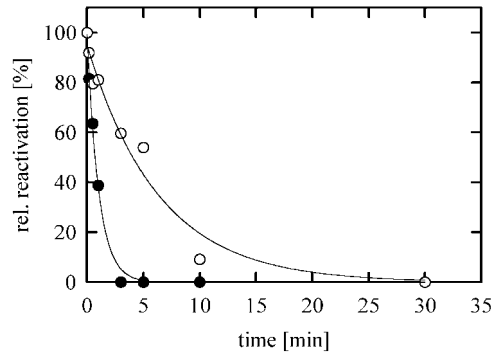

FIG. 3. **Timed addition of PDI to refolding Fab/red.** Reactivation of Fab/red (the final concentration of Fab was 0.2 μM) was initiated by a 100-fold dilution in 0.1 M Tris, pH 7.0, 6 mM GSSG, containing 0.5 mM ATP and an ATP-regenerating system. Reactivation yields were determined after a 48-h incubation in the absence of BiP (●) or in the presence of 2 μM BiP (○). At the times indicated, PDI was added to the refolding Fab fragment at a final concentration of 0.5 μM. The rate constants were 0.95 in the absence of BiP and 0.15 in the presence of BiP. Renaturation yields with PDI present from the start of renaturation were defined as 100%. The actual renaturation yields were 49% in the presence of BiP and 21% in the absence of BiP, respectively.

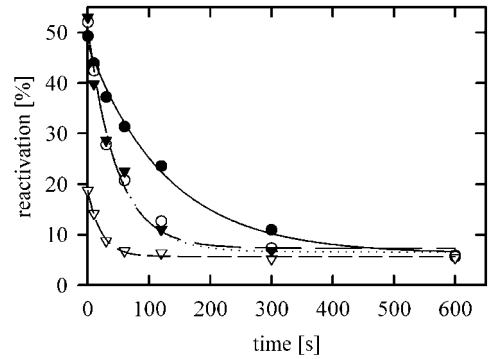

FIG. 4. **Refolding of Fab after separate preincubation of light chain and Fd fragment.** Light chain and Fd fragment were preincubated separately in 0.1 M Tris, pH 7.0, 6 mM GSSG in the presence and in the absence of 2 μM BiP. At the times indicated, light chain and Fd were combined, and PDI, ATP, and an ATP-regenerating system were added. The final concentrations for light chain and Fd were 0.2 μM each, 6 mM for GSSG, 2.0 μM for BiP, 0.5 μM for PDI, and 0.5 mM for ATP. After 48 h of reactivation, yields were determined by ELISA for light chain and Fd both incubated in the presence of BiP (●), light chain preincubated in the presence of BiP and Fd preincubated in the absence of BiP (▲), light chain preincubated in the absence of BiP and Fd preincubated in the presence of BiP (○), and light chain and Fd both preincubated in the absence of BiP (▽). The half-times were 91 s for both fragments incubated in the presence of BiP, 32 s for either light chain or Fd incubated in the presence of BiP, and 8 s for both fragments incubated in the absence of BiP. When the subunits were combined at the beginning of reactivation, the yields of active antibody were similar to those for the complete Fab fragment *i.e.* 50% in the presence of BiP and 20% in the absence of BiP.

strated that the ATPase of BiP did not lose activity during reactivation (48 h) and that the ATP-regenerating system stayed effective (data not shown).

Next, we asked how the influence of BiP on the refolding reaction depends on the BiP/Fab ratio. We performed experiments in which different amounts of BiP were present during the refolding reaction. As shown in Fig. 2, reaction yields increased with increasing BiP concentrations up to a BiP/Fab ratio of about 10:1. This indicates that there is a kinetic competition between binding to BiP and rapid structure formation or misfolding.

BiP Holds Cysteines Accessible for PDI during the Initial Phase of Refolding—It had been shown that the effect of PDI on refolding decreased when PDI was added after the initiation of refolding with a half-time of less than 10 s. After 30 s, the addition of PDI no longer influenced the reactivation process (20). This effect was not due to a change in the redox environment, since the number of sulfhydryl groups did not change during this period of time (20). We reasoned that the positive effect of BiP on the reactivation of the Fab fragment could be due to prevention of structure formation. If this way BiP kept the cysteines accessible for PDI for a longer period of time, we should be able to increase the time span during which the addition of PDI is effective.

To test this, we performed refolding experiments in the absence and presence of BiP, adding PDI at certain time points after the initiation of the refolding reaction. As shown in Fig. 3, reactivation yields decreased dramatically when PDI was added after the initiation of refolding in the absence of BiP. After 3 min, the addition of PDI had no detectable effect on Fab reactivation. In the presence of BiP, however, an influence on the reactivation was still observed, when PDI was added 20 min after starting reactivation. The decrease of reactivation yields was 6 times slower than in the absence of BiP.

BiP Binds Both Light Chain and Fd—From the previous experiments, it was clear that BiP influences the folding of the Fab fragment in an ATP-dependent way. However, it was not established whether this effect involves the interaction with one or both of the polypeptide chains of the Fab fragment. To answer this question, we used isolated Fd and light chain obtained as inclusion bodies after recombinant expression in *E. coli*. After denaturation and reduction of the respective inclusion body proteins, we performed folding reactions with either light chain or Fd in the presence or absence of BiP. In a second step, the individual folding reactants were combined and further incubated in the presence of PDI and ATP.

As shown in Fig. 4, nearly the same reactivation yields were achieved for the complete Fab fragment and for Fd and light chain combined immediately after initiating reactivation. However, if light chain and Fd were combined after starting refolding, reactivation yields decreased dramatically from 20 to 5% within the first minute. When either Fd or light chain was preincubated in the presence of BiP, reactivation yields of 50% were achieved if the antibody chains were combined immediately. The reactivation yields decreased as fast as in the absence of BiP if the chains were combined after starting reactivation. However, when light chain and Fd were both preincubated in the presence of BiP, this decrease could be decelerated effectively.

Since we did not observe any difference in the time course and yield of reactivation when either light chain or Fd were

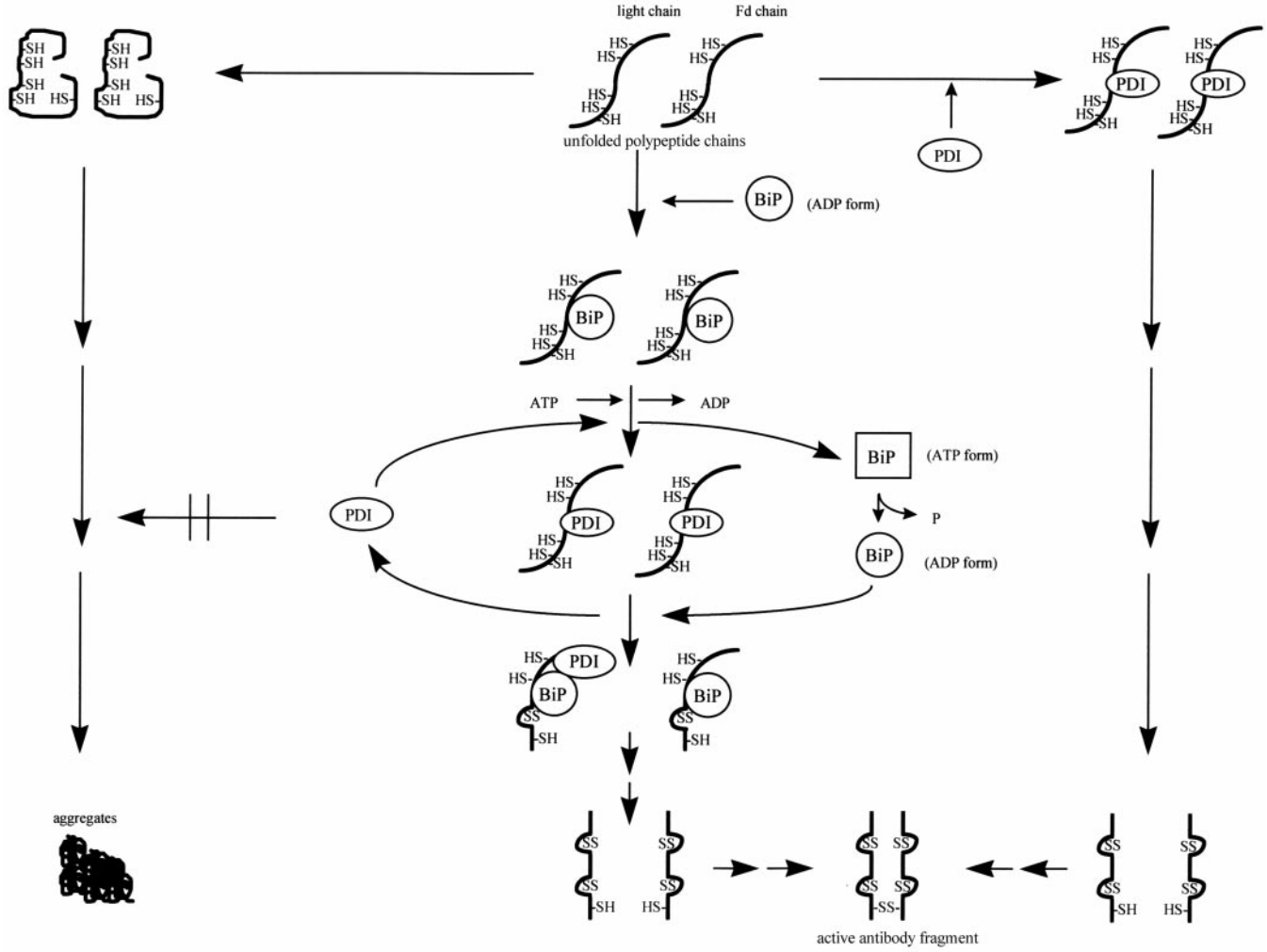

FIG. 5. **A schematic model for the role of BiP and PDI during the oxidative folding of antibodies *in vitro*.** Starting with the unfolded and reduced antibody chains, there are, dependent on the presence of folding factors, three different routes the polypeptide chains can take. In the absence of BiP and PDI, renaturation leads to completely inactive protein. In the presence of PDI, active antibodies are obtained with yields of about 20%. The chaperone BiP, when present during refolding, binds to both antibody chains. During the ATP-dependent cycles of binding, release, and rebinding, the cysteine residues of the antibody chains are kept accessible for PDI. This allows efficient disulfide bond formation and reshuffling. Further reaction steps lead to the fully oxidized and active antibody.

preincubated with BiP, it is obviously important for refolding that BiP binds to both antibody chains.

DISCUSSION

In vivo, the folding and formation of disulfide bonds in antibody chains seems to start cotranslationally, and complete oxidation is achieved posttranslationally (26). During these processes, the antibody chains interact with PDI, which catalyzes formation and reshuffling of disulfide bonds (14). Although PDI shows chaperone-like activity independent of its isomerase activity in the refolding of proteins containing no disulfide bonds (27, 28), the function of PDI in the folding of antibodies seems to be restricted to its isomerase activity (20). An influence of PDI on the folding of an antibody Fab fragment in vitro could be detected only when PDI was present during the initial phase of refolding. Based on these results, it was speculated that BiP may be able to keep the cysteine residues accessible for PDI (13, 20)

To test whether BiP and PDI cooperate in the folding of antibody chains, we performed *in vitro* folding experiments with the denatured and reduced Fab fragment of the monoclonal antibody MAK33 in the presence and absence of folding helpers. We were able to show that BiP and PDI act in a synergistic way, since the reactivation yields were not only the sum of the yields obtained in the presence of one of the folding factors.

An unexpected feature of the folding of antibodies was revealed by experiments in which the antibody chains were first preincubated separately and then combined during reactivation. The yields decreased dramatically even after short times of separation during refolding, suggesting that contacts between the two chains are important already during early stages of folding. This explanation is in agreement with results showing that specific domain interactions can occur in reduced antibody domains.[2]

The results presented here led to a model for the oxidative folding of antibodies *in vitro* shown in Fig. 5. Starting from the denatured and reduced polypeptide chains, folding of the Fab fragment can occur in three different reactions. Under the conditions used, spontaneous reactivation in the absence of folding factors is not possible. However, after optimization of buffer conditions, as shown previously, up to 40% of the reduced and denatured Fab could be reactivated (23). These experiments were performed in the presence of high concentrations of L-arginine. L-Arginine acts as a "labilizing" agent that

[2] M. Thies and J. Buchner, unpublished results.

preferentially destabilizes incorrectly folded or aggregation-prone species (29, 30). Because the correctly folded species is not affected by arginine, the folding reaction is shifted toward the correctly folded structure by an iterative mechanism. This strong dependence of the folding on reaction conditions suggests that misfolding is a major factor in structure formation of antibodies *in vitro*. In the presence of PDI and under otherwise nonpermissive folding conditions, up to 20% of the molecules reach the native state when PDI is present from the start of reactivation. The limited ability of PDI to influence antibody folding may be explained by a kinetic competition between Fab structure formation and accessibility of the cysteines for PDI. The cysteines seem to become rapidly buried in the core of the folding polypeptide chains, and the influence of PDI on disulfide bond formation is therefore very inefficient. In the presence of PDI and BiP, the antibody chains take a much more efficient folding route. Binding to BiP seems to prevent the unfolded antibody chains from forming conformations in which the cysteines are buried. Several ATP-dependent cycles of binding, release, and rebinding are necessary for maximum oxidation of the antibody chains. As previously observed for other chaperones, an excess of BiP over Fab is required in this assay. This is due to the kinetic competition between unproductive folding reactions and the binding to BiP. It seems that BiP has to be able to trap the unfolded polypeptide chains rapidly at the beginning of refolding. In addition, repeated ATP-dependent binding and release cycles of BiP and Fab are required during reactivation. The ATP dependence of the reaction is in agreement with previous results, demonstrating that the release of peptides bound to BiP is dependent on its ATPase activity (31–33). During these ATP-dependent cycles, PDI may bind to the antibody chains simultaneously with BiP or after BiP is released from the polypeptide chain.

In this context, it remains to be seen how co-chaperones and additional ER-specific folding helpers such as Grp94, PPI, calreticulin, and calnexin (2, 5) modulate the effects of BiP and PDI on antibody folding. Specifically, Hsp70 proteins are known to be regulated by J-domain-containing proteins (34). It has been demonstrated in *Saccharomyces cerevisiae* that BiP (Kar2p) interacts with at least three ER-located HSP70 proteins, Sec63p (35, 36), Scj1p (37), and Jem1p (38). Three DnaJ domain-containing proteins have been found in mammalian cells: Mtj1p, Sec63p (39, 40), and a Scj1p homolog (ERj3p) (41). Yeast Sec63p interacts with the ATP-bound form of BiP and stimulates hydrolysis, resulting in the rapid trapping of peptides, which are then only slowly released upon nucleotide exchange (36, 42).

Although BiP has a marked effect on antibody refolding, the overall folding rate remains very slow, as observed in previous studies (20). It is known that the oxidation of the cysteines and reshuffling of disulfide bonds take place during the whole time course of renaturation (20). Therefore, disulfide bond formation or a folding/association step which is required for correct disulfide bond formation seems to be the limiting step in antibody folding. It has been shown previously that formation of the intrachain disulfide bond of antibody domains is a slow process because the cysteine residues are buried in the core of the protein (43). Therefore, their accessibility is largely restricted for thiol reagents and even more for a large enzyme like PDI. Although we do not have direct experimental evidence, it is tempting to speculate that the inability of BiP and PDI to influence reactivation when added at later stages of folding may be due to a preceding structural collapse.

These results suggest that different classes of folding helper proteins including components of the protein translocation work together synergistically in a network-like manner in the ER of eukaryotic cells.

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BiP and PDI Cooperate in the Oxidative Folding of Antibodies*in Vitro*

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