Influence of Protein Disulfide Isomerase (PDI) on Antibody Folding in Vitro*

Hauke Lilié*, Stephen McLaughlin†, Robert Freedman‡, and Johannes Buchner‡‡

From the †Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, 93053 Regensburg, Germany and the ‡Biological Laboratory, University of Kent, Canterbury CT2 7NJ, Great Britain

The role of eucaryotic protein disulfide isomerase (PDI) in the folding and reoxidation of proteins in vitro was investigated using an antibody Fab fragment as a model substrate, since PDI is known to participate in the disulfide bond formation of immunoglobulins in vitro.

PDI has no effect on the folding of the Fab fragment with intact disulfide bonds, suggesting that, at least in this system, PDI is not able to influence the folding process in a chaperone-like manner. Instead, the role of PDI is limited to disulfide bond formation as demonstrated for the folding of uncleaved denatured and reduced Fab fragment. Here, PDI influences the yield of reactivation enormously with a maximum effect at about stoichiometric amounts of PDI and Fab. Furthermore, PDI changes the redox dependence of the reaction. In the presence of PDI, formation of the correct disulfide bonds is possible at higher oxidizing conditions compared to the spontaneous reaction.

The requirements both for stoichiometric amounts of PDI and for the presence of PDI during the first seconds of refolding suggest that there is a kinetic competition between rapid structure formation of the antibody domains and interaction of PDI with cysteine residues in the folding protein.

Protein folding in vitro is a spontaneous process leading to a uniquely folded structure depending only on the given amino acid sequence and the solvent conditions (Anfinsen, 1973; Jaenicke, 1987; Jaenicke and Buchner, 1993). However, both in vivo (Hurtley and Helenius, 1991) and in vitro (Buchner and Rudolph, 1991a), side reactions during folding may lead to inactive protein, especially in the case of disulfide-bonded proteins. In vitro, unproductive folding may be suppressed by careful composition of the folding conditions (Buchner and Rudolph, 1991a). In recent years, it has become increasingly clear that in vivo a set of helper proteins including folding catalysts and chaperones may guide polypeptides along the structure formation process (Freedman, 1992; Gething and Sambrook, 1992).

In this context, disulfide bond formation in the endoplasmic reticulum (ER) of eucaryotic cells seems to be influenced by the enzyme protein disulfide isomerase (PDI) (Freedman, 1991). Eucaryotic PDI, a resident ER protein, is a homodimer of 50-kDa polypeptides. The level of PDI is correlated to the amount of secreted disulfide-bonded protein (Brockway et al., 1980; Roth and Koshland, 1981; Myllyla et al., 1983). Furthermore, PDI-depleted microsomes have been shown to accumulate wrongly disulfide-bonded polypeptides upon in vitro translation and translocation of secretory proteins (Bulleid and Freedman, 1988). These findings suggest that the role of PDI is to facilitate disulfide bond formation in the cell. However, in addition to the homodimeric form, PDI is also found as a subunit in protein complexes whose functions are unrelated to disulfide bond formation, namely tetrameric prolyl 4-hydroxylase (Pihlajaniemi et al., 1987) or the triglyceride transfer protein complex (Wetterau et al., 1990).

In order to investigate further the role of PDI in folding and disulfide bond formation, we chose to use an antibody as substrate for PDI since the following evidence suggests that PDI is involved in the structure formation of these molecules in vivo. Covalent complexes of PDI with nascent immunoglobulin light chains that have been isolated from cells are direct evidence for the participation of PDI in the disulfide bond formation of antibody chains (Roth and Pierce, 1987). Furthermore, PDI catalyzes the assembly of IgM and IgA in vitro (Della Corte and Parkhouse, 1973; Delamette et al., 1975; Wilde and Koshland, 1978; Roth and Koshland, 1981). During lymphocyte differentiation into Ig-secreting plasma cells, there is a marked induction of PDI (Paver et al., 1989).

We used the Fab fragment of the murine monoclonal antibody MAK 33 (Buckel et al., 1987) as a model system because both the authentic murine Fab fragment and the respective recombinantly produced polypeptide chains have been used to study in detail the structure formation process after complete reduction and denaturation (Buchner and Rudolph, 1981b). It has been shown that under refolding conditions that allow the "reshuffling" of incorrect disulfide bonds in the presence of high concentrations of 1-arginine, about 40% of the antibody molecules reach their functional state. The kinetic of functional reactivation is very slow with a half-time of 15 h at 10°C. Aggregation of non-native polypeptide chains and formation of

vaccine serum albumin; DTE, dithioerythritol; Fab, antigen-binding antibody fragment consisting of the entire light chain and the two N-terminal domains of the heavy chain; Fab/ox, Fab fragment with intact disulfide bonds; Fab/red, Fab fragment with reduced cysteines; PDI, protein disulfide isomerase; MAK 33, murine monoclonal antibody of subtype κ/IgG1; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

1 The abbreviations used are: ER, endoplasmic reticulum; BSA, bovine serum albumin; DTE, dithioerythritol; Fab, antigen-binding antibody fragment consisting of the entire light chain and the two N-terminal domains of the heavy chain; Fab/ox, Fab fragment with intact disulfide bonds; Fab/red, Fab fragment with reduced cysteines; PDI, protein disulfide isomerase; MAK 33, murine monoclonal antibody of subtype κ/IgG1; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

*This work was supported by grants from the Fonds der chemischen Industrie, the Deutschen Forschungsgemeinschaft, and Boehringer Mannheim GmbH (to J. B.) and the ARC program (to J. B. and R. F.), a scholarship from the University of Regensburg (to H. L.), and grants from the Medical Research Council and the Science and Engineering Research Council (to R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany. Fax: 49-941-943-2813.

‡‡The abbreviations used are: ER, endoplasmic reticulum; BSA, bovine serum albumin; DTE, dithioerythritol; Fab, antigen-binding antibody fragment consisting of the entire light chain and the two N-terminal domains of the heavy chain; Fab/ox, Fab fragment with intact disulfide bonds; Fab/red, Fab fragment with reduced cysteines; PDI, protein disulfide isomerase; MAK 33, murine monoclonal antibody of subtype κ/IgG1; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

14290
wrong disulfide bonds are the major side reactions leading to inactive protein (Buchner and Rudolph, 1990). The presence of the intradomain disulfide bonds is a prerequisite for the formation of functional antibody molecules since renaturation under reducing conditions did not result in the formation of active protein (Buchner and Rudolph, 1991b). In contrast to reactivation of the denatured and reduced protein, renaturation of the denatured Fab fragment with intact disulfide bonds both in the individual domains and between the two constituent polypeptide chains is a fast but not completely reversible process (Schmidt and Buchner, 1992). The yield of functional refolding can be influenced by the chaperones GroE and Hsp90 (Schmidt and Buchner, 1992; Wiech et al., 1992). Furthermore, the extent of catalysts of the peptidyl prolyl isomerase family on antibody folding has been described (Lilie et al., 1993).

Here we addressed the questions of whether and how PDI interacts with refolding Fab in the oxidized and reduced state.

We demonstrate that PDI has no chaperone-like effect on the folding of the molecule with intact disulfide bonds (Fab/ox) at pH 7 under oxidizing conditions. However, PDI is able to effectively reduce refolding Fab/ox under redox conditions where the spontaneous refolding is dominated by the kinetics of the oxidized Fab. The refolding of denatured and reduced Fab (Fab/ red) is effectively influenced by PDI, which has been shown to be catalytically active in the scrambled RNase assay.

**EXPERIMENTAL PROCEDURES**

Reagents—PDI (EC 5.3.4.1) was purified from bovine liver as described (Lambert and Freedman, 1983; Mills et al., 1985). In the text, molar concentrations of PDI refer to the dimer.

MAK 33, a murine antibody subclass α/IgG1 directed against dimeric muscle-specific human creatine kinase (CK-MM, EC 2.7.3.2) (Buckel et al., 1987), and the respective Fab fragment were obtained from the Freiburg University Mannheim GmbH. The Fab fragment was produced by proteolytic digestion of the intact murine monoclonal antibody as described (Johnstone and Thorpe, 1987). Since proteolysis is performed in the presence of reducing agent, interchain disulfide bonds within the Fab fragment will be partially reduced. The contents of non-covalently linked antibody polypeptide chains that can be separated by SDS-PAGE varies between different preparations. The yield of spontaneous reactivation seems to vary with the amount of non-covalently linked Fab fragments. In previous studies using the MAK 33 Fab fragment, spontaneous reactivation was found to be about 30% (Schmidt and Buchner, 1992; Lilie et al., 1993). In this study, spontaneous reactivation of a different preparation of the Fab fragment yielded about 70% of native molecules possibly due to a lower content of reduced material. Despite the difference in yield, the two preparations behave indistinguishably concerning their interaction with folding catalysts and chaperones.

For some experiments, the recombinantly produced antibody Fab fragment was used (see below). The concentration of the Fab fragment was determined spectrophotometrically using an extinction coefficient of 1.6 for a 0.1% solution (Schmidt and Buchner, 1992; Lilie et al., 1993). In this study, spontaneous reactivation of a different preparation of the Fab fragment yielded about 70% of native molecules possibly due to a lower content of reduced material. Despite the difference in yield, the two preparations behave indistinguishably concerning their interaction with folding catalysts and chaperones.

For some experiments, the recombinantly produced antibody Fab fragment was used (see below). The concentration of the Fab fragment was determined spectrophotometrically using an extinction coefficient of 1.6 for a 0.1% solution (Schmidt and Buchner, 1992; Lilie et al., 1993). In this study, spontaneous reactivation of a different preparation of the Fab fragment yielded about 70% of native molecules possibly due to a lower content of reduced material. Despite the difference in yield, the two preparations behave indistinguishably concerning their interaction with folding catalysts and chaperones.

ELISA reagents including biotinylated dimeric muscle-specific human creatine kinase were obtained from Boehringer Mannheim GmbH. Insulin Assay—Porcine insulin was dissolved as described by Holdgren (1979). The aggregation reaction of insulin (65 μM) was carried out in 0.1 M Tris, pH 6.5, 2 mM EDTA, 5 mM DTE at 20 °C. The turbidity of the solution was monitored photometrically at 650 nm in 1-mm cuvettes in the presence or absence of 0.25 μM PDI.

SDS-PAGE—Western—SDS-PAGE was performed essentially according to Laemmli (1970). Subsequently, proteins were transferred to nitrocellulose using standard procedures (Khyse-Anderson, 1984). Detection of MAK 33 Fab was achieved using a goat peroxidase-conjugated anti-mouse antibody and the ECL detection system (Amer sham Corp.). Prior to separation on SDS-PAGE, the samples were precipitated in the presence of sodium deoxycholate according to Bensadoun and Wein stein (1987), with the modification that phosphoric acid was used instead of trichloroacetic acid. The pellet was subsequently dissolved in Laemmli buffer.

**RESULTS**

**PDI Does Not Influence the Folding of the Fab/ox Fragment**—Since PDI is found as a subunit of several enzyme complexes unrelated to disulfide bond formation (cf. Freedman

---

2 Since proteolytic digestion of the antibody by papain is performed in the presence of a low concentration of reducing agent (Johnstone and Thorpe, 1987), a number of molecules will be present as non-covalently linked dimers. The Fab/ox preparations used for the experiments described here contained about 5% of these species, as judged by scanning of non-reducing Coomassie-stained SDS gels.

3 In most experiments, the redox system was established by adding 3 mM DTE to 6 mM GSSG in the refolding buffer. DTE reduces the oxidized glutathione, thus creating a redox couple with a redox potential almost identical to a system of 6 mM GSH and 3 mM GSSG (Buchner and Rudolph, 1991b). According to its redox potential, the effect of DTE/ox on the GSH/GSSG system is negligible.

4 M. Schmidt, U. Bücheler, B. Kaluzna, and J. Buchner, manuscript in preparation.
Fab was diluted 100-fold in 0.1 M Tris/HC1, 2 mM EDTA, 8 mM GSSG, pH 7.0, at 15 °C. The final protein concentration was 0.2 μM. PDI was preincubated in the renaturation buffer for 20 min. At the time point indicated, aliquots were withdrawn and the regain of activity was monitored by ELISA after adding trypsin to prevent further reactivation. Renaturation was performed in the absence of PDI, in the presence of 0.5 μM PDI, in the presence of 0.5 μM alkylated PDI, and in the presence of 0.76 μM BSA.

(1992) and Noiva and Lennarz (1992) and since it has been shown to bind to several different peptides, some of which even lack cysteines (Noiva et al., 1991), it has been proposed that PDI may also work in a chaperone-like manner (Noiva and Lennarz, 1992). To test whether PDI could act chaperone-like in the refolding of Fab/ox comparable to GroE (Schmidt and Buchner, 1992), we performed renaturation assays in the presence and absence of excess PDI (Fig. 1). In addition to active PDI, PDI inactivated by alkylation (Hawkins and Freedman, 1991) was used. Alkylated PDI was inactive in the insulin reduction assay, even though the structural integrity of the enzyme was maintained, as demonstrated by size exclusion chromatography and fluorescence spectroscopy (data not shown).

Both catalytically active and alkylated PDI had no effect on the reactivation of Fab/ox, even if present at a 2.5-fold molar excess. However, under the same experimental conditions, the GroE chaperones influenced the folding process significantly (Schmidt and Buchner, 1992). Therefore we conclude that, at least in our assay system, the function of PDI is clearly limited to disulfide bond formation (see below).

PDI Is Able to Reduce Fab/ox During Folding—Next, we asked whether PDI is able to influence the folding of Fab/ox in the presence of a dithiol/disulfide redox system consisting of 3 mM GSSG and 6 mM GSH. We performed refolding experiments of Fab/ox under conditions used for the refolding of denatured and reduced Fab (see below). The yields of spontaneous refolding of Fab/ox under these conditions were about 2-fold lower (maximum 35%) compared to refolding without redox equivalents (70%) (Fig. 2A; cf. Fig. 1). Thus, disulfide bonds of non-native molecules may have been reduced during reactivation. However, in the presence of PDI the kinetics and yield of refolding were completely different. PDI seemed to strongly interfere with the refolding of Fab (Fig. 2A). To demonstrate unambiguously that the effects observed were really due to the reduction of disulfide bonds of the Fab fragment by PDI, aliquots were withdrawn at different time points during refolding in the presence and absence of PDI and analyzed by immunoblotting following SDS-PAGE (Fig. 2B). While in the absence of PDI the amount of the covalently linked dimer did not change significantly during the time course of refolding, in the presence of PDI, a drastic reduction of the Fab species corresponding to the dimer was observed. This effect was not due to a proteolytic activity in the PDI preparation (data not shown).

The signal of the monomeric chain (κ) was much weaker in the immunoblotting since the antiserum reacted much stronger.
Influence of PDI on Antibody Folding in Vitro

with Fab than κ. Fab was not detectable at all. These results show clearly that PDI is able to reduce disulfide bonds of Fab/ox during refolding.

To further characterize the ability of PDI to reduce refolding Fab/ox, the enzyme was added either immediately or at certain time points after initiation of refolding. As shown in Fig. 2C, PDI was able to reduce Fab molecules even if added 15 min after diluting the denatured protein.

Correct Disulfide Bonds Are Formed during the Whole Time Range of Reactivation of Fab/Red—It has been shown previously that the refolding of Fab fragments starting from the completely unfolded and reduced protein is a very slow process. Regain of activity is correlated to dimer formation as monitored by ELISA, size exclusion chromatography, and non-reducing SDS-PAGE (Buchner and Rudolph, 1991b; Buchner and Rudolph, 1990). However, from these results it was not clear whether redox reactions that influence the yield of functional reactivation take place over the whole time range of the reaction. To address this question, we refolded denatured and reduced Fab (cf. Buchner and Rudolph (1991a)) and alkylated all accessible SH groups of the Fab/Red fragment at different time points during refolding with iodoacetamide. The samples representing the disulfide bond pattern at the given time point were then allowed to renature further. After 140 h, all samples were tested for antigen binding activity. If folding and association reactions alone were rate-determining, freezing of disulfide bond formation and reshuffling should not affect the yield at later stages of reactivation. However, blocking of cysteines completely suppresses further renaturation at all stages during the kinetics of reactivation (Fig. 3). We conclude that disulfide bond formation and reshuffling processes take place during the whole range of the renaturation process of the denatured and reduced Fab. Whether the rate-limiting step is disulfide bond formation itself or whether a slow folding step preceding disulfide bond formation cannot be distinguished by this experiment.

Active PDI Influences the Reactivation of Fab/Red—Previously, we have established an optimized redox system for the reactivation of antibodies from the completely unfolded and reduced state (Buchner and Rudolph, 1991b). We used a mixture of the oxidized and reduced form of the low molecular weight thiol reagent glutathione (GSSG and GSH, respectively) at alkaline pH in combination with additives like urea or L-arginine, which allows both correct disulfide bond formation and suppression of side reactions such as aggregation (Rudolph, 1990; Buchner and Rudolph, 1991b; Buchner et al., 1992). In order to be able to test the influence of PDI on disulfide bond formation of the refolding Fab fragment, we had to change the optimum buffer conditions since PDI became inactivated at high L-arginine concentrations (data not shown) and at alkaline pH (Hawkins and Freedman, 1991). Renaturations in the absence of additives resulted in significantly lower yields of active molecules.

Using this system, we next asked whether PDI influences disulfide bond formation during refolding. As shown in Fig. 4A, PDI clearly increases the number of correctly folded molecules. Parallel experiments, in which alkylated PDI or BSA (not shown) were used, showed that the catalytically active form of PDI is required to achieve this effect (Fig. 4A).

In the Fab fragment, there are two different types of disulfide bonds, the intrachain disulfide bonds within each of the four domains and the disulfide bond connecting the two

![Fig. 3. Influence of disulfide bond formation on the kinetics of refolding.](image)

![Fig. 4. Influence of PDI on the refolding of Fab/Red.](image)
polypeptide chains. To further analyze the effect of PDI on Fab folding and disulfide bond formation, we used a recombinantly produced Fab variant lacking the respective cysteine residues forming the interchain disulfide bond. The resulting Fab fragment is stable and functionally associated without the interchain disulfide bond. Spontaneous refolding and reoxidation gave yields and kinetics similar to those of the proteolytically derived Fab molecule (Fig. 4B). Again, PDI influenced the renaturation of the variant increasing the yield of functional refolding. Alkylation of PDI was inactive in influencing the refolding and reoxidation of the Fab fragment (Fig. 4B). Together, these findings suggest that PDI participates in the formation of the intradomain and interchain disulfide bonds.

**PDI Renders Fab/red Renaturation Concentration-independent**—As shown in Fig. 5A, there was little influence of PDI on the renaturation of Fab at subequimolar ratios. The yield of active molecules increased with increasing PDI concentrations up to about equimolar concentrations of PDI compared to refolding antibody polypeptide chains (i.e., light chain and Fd part of heavy chain). An additional increase in PDI did not result in an additional increase in yield.

Next, we asked how PDI influences the concentration dependence of Fab refolding. Previously, we have shown that the refolding process is strongly dependent on the concentration of denatured protein allowed to renature (Buchner and Rudolph, 1991b; Kießhaber et al., 1991). To address this question, we performed renaturation experiments, in which we varied the concentration of the denatured Fab from 5 to 100 μg/ml corresponding to 0.1–2 μg antibody chains. Renaturation at each concentration was carried out: (i) in the absence of PDI, (ii) in the presence of a fixed amount of PDI (0.5 μg), and (iii) in the presence of a 2.5-fold molar excess of PDI over Fab at the given concentration (Fig. 6A).

**PDI Changes the Redox Dependence of Fab/red Refolding**—Reactivation of Fab/red is critically dependent on the redox conditions of the refolding system. Maximum reactivation was achieved at GSH2/GSSG ratios between 10 and 20 m (Buchner and Rudolph, 1991). To further characterize the effect of PDI on the disulfide bond formation of Fab/red, we determined the redox dependence of Fab/red refolding in the presence and absence of PDI. For the spontaneous reaction, the redox dependence was found to be similar to previously published data (Fig. 6B), although the reaction conditions were slightly different (cf. Buchner and Rudolph, 1991). However, in the presence of PDI a completely different redox dependence was obtained (Fig. 6B). While in the absence of PDI no reactivation was detected below a GSH2/GSSG of 3 mM, with PDI highest reactivation yields (about 50%) were obtained at GSH2/GSSG ratios between 0.15 and 6 mM. This means that in the presence of PDI the optimal conditions of reactivation can be shifted to strongly oxidizing conditions under which no spontaneous reactivation at all is observed. Under more reducing conditions (i.e., GSH2/GSSG > 10 mM), the redox dependence of both the spontaneous and the assisted reactivation are similar.

**PDI Must Be Present during the Initial Phase of Refolding of Fab/red**—To determine at which stages of the refolding process PDI affects the formation of native molecules, experiments
**Influence of PDI on Antibody Folding in Vitro**

**FIG. 7. Timed addition of PDI to refolding Fab/red.** Refolding was performed and monitored as described in the legend to Fig. 4. Reactivation yields were determined after a 50-h incubation in the absence of PDI (○) or in the presence of PDI (●). At times indicated, PDI was added to the refolding Fab (0.2 μM) fragment at a final concentration of 0.5 μM.

were performed in which PDI was added at different time points during refolding of Fab varying from 0 to 8 h after the initiation of reactivation. The number of sulfhydryl groups of the redox system did not change during this period of time (not shown). Therefore, differences observed in the ability of PDI to influence Fab reactivation are not due to a changing redox environment. PDI influenced refolding when present from t = 0 s in the refolding solution into which the denatured and reduced Fab was diluted. However, when PDI was added after initiation of refolding, its effect decreased with a half-time below 10 s and after 30 s, the addition of PDI no longer influenced the yield of reactivation (Fig. 7).

**DISCUSSION**

We have investigated here the role of eucaryotic PDI in the folding and disulfide bond formation of denatured antibody Fab fragments in the oxidized and reduced state.

Several recent findings have led to the speculative suggestion that PDI may function as a chaperone or (polypeptide)-binding protein, in addition to being an isomerase (Geetha-Habib et al., 1988); eucaryotic PDI has been found as a subunit of a number of enzyme complexes unrelated to disulfide bond formation but involved in the modification of (nascent) polypeptide chains (cf. Freedman (1992)). In the case of prolyl 4-hydroxylase, it has been demonstrated that PDI, the β-subunit of the tetrameric enzyme, prevents the aggregation of the α-subunits during synthesis (John et al., 1993). Furthermore, it has been shown that PDI has some peptide binding properties (Noiva et al., 1991).

Thus, refolding of Fab/ox seems to be a good model system to examine chaperone-like properties of PDI, in addition to its role in disulfide bond formation. However, refolding experiments of Fab/ox performed under oxidizing conditions showed that both the kinetics and yield of the reaction were not affected by PDI. In contrast, we have previously shown that members of the “protein binding family” of chaperones do influence the refolding process of Fab/ox (Schmidt and Buchner, 1992; Wiech et al., 1992). The procaryotic GroE system, the role model chaperone system, increases the yield of functionally refolded Fab molecules about 2-fold in an ATP-dependent reaction. GroEL alone slows down the refolding process, most likely by binding and slow release reactions (Schmidt and Buchner, 1992). Similar effects on the yield were achieved with Hsp90 (Wiech et al., 1992). Therefore, we conclude that at least in this system, PDI does not act as a chaperone independent of disulfide bond formation.

Although PDI is not able to influence the folding process of Fab/ox as a chaperone, PDI is able to function as an isomerase breaking disulfide bonds of refolding Fab/ox molecules provided that appropriate redox conditions (6 mM GSH, 3 mM GSSG) are used. This demonstrates that the interaction of PDI with the folding Fab/ox fragment is limited to disulfide shuffling. Under the folding conditions described, PDI predominantly breaks the interchain disulfide bond of the Fab fragment but also to a certain extent intradomain disulfides. The observed slow refolding kinetics and lowered reactivation yields compared to Fab/ox folding are possibly due to subsequent reassociation and reoxidation processes similar to those occurring in the refolding of Fab/red.

Reactivation of the Fab/red fragment is dominated by the formation of the intradomain disulfide bonds. PDI does influence this process resulting in significantly higher yields of reactivation depending on the redox conditions used. As one control for effects of PDI not related to disulfide bond formation we used alkylated PDI, which is unable to participate in redox reactions due to the modification of the active site cysteines. In contrast to native PDI, alkylated PDI had no effect on the refolding of Fab/red strongly suggesting that the effect of PDI observed is due to its disulfide isomerase activity. This suggestion is further corroborated by the influence of PDI on the redox dependence of Fab/red reactivation. It is possible to achieve yields of functional refolding of up to 50% in the presence of PDI while under the same set of conditions the spontaneous reaction is negligible. In order to be able to compare PDI-assisted reactivation with the spontaneous reaction, we performed the experiments to characterize the function of PDI under optimum conditions for spontaneous reactivation under which the effect of PDI is still significant.

Interestingly, no significant change in the kinetics of reactivation of Fab/red was observed in the presence of PDI compared to spontaneous refolding. Furthermore, experiments in which PDI was either present from the beginning of renaturation or added after initiation of refolding showed that PDI is effective only if present during the initial phase of folding.

During this time period antibody domains seem to fold into a compact intermediate. Subsequent slower reactions led to the formation of the native structure (Kawata and Hamaguchi, 1991). Fluorescence spectra of the refolding Fab fragment suggest that here also formation of a collapsed state occurs in the time range of seconds (data not shown). Previously, Goto and Hamaguchi (1981) showed elegantly 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. Thus, their accessibility even for low molecular thiol reagents is largely restricted and the kinetics of oxidation is dependent on the equilibrium between the native and the denatured state under the conditions used (Kikuchi et al., 1986).

In light of these results, it seems likely that the effectiveness of PDI is limited by accessibility of the intradomain cysteines for PDI. The shielding of the cysteine residues from solvent may explain the rapid drop in the ability of PDI to influence the refolding if added after starting the folding process. Furthermore, this interpretation would also explain why the kinetics of refolding are independent of the presence or absence of PDI. The prerequisite for any redox process involving intradomain cysteines is accessibility of the cysteines by spontaneous unfolding (Kikuchi et al., 1986). Under the solvent conditions used, unfolding of the intermediate structure with buried cysteines is not a very likely process. Therefore, once the cysteines are buried in the interior of the protein, spontaneous unfolding...
of structured intermediates will become the rate-limiting step for disulfide bond formation. Similarly, it has been shown in the case of peptidyl prolyl isomerase that accessibility of the respective Xaa-Pro peptide bond during the folding process limits the catalysis of isomerization (Kiefhaber et al., 1990).

Taken together our results clearly demonstrate that the effect of PDI in the reactivation of Fab\'ed is due to its specific redox properties, suggesting that PDI acts as an isomerase predominantly during the early phases of structure formation, while at later stages the effect of PDI may be veiled by a rate-limiting unfolding step.

In vivo, antibody polypeptide chains begin to fold and form disulfide bonds contrationally (Bergman and Kuehl, 1979). During the structure formation process, they seem to interact with several ER proteins in addition to PDI. Two of these proteins have been identified as members of the chaperone protein family, namely BiP (Haas and Wabl, 1983; Knittler and Haas, 1992; Melnick et al., 1992) and Grp94 (Melnick et al., 1992). Chaperones have been shown to interact with folding or unfolding proteins preventing premature folding and irreversible side reactions (cf. Jaenicke and Buchner (1993)). In this context, one may speculate that in vivo the accessibility of the intradomain disulfide bonds of antibody polypeptide chains for PDI may be modulated by the binding of a set of helper proteins present in the ER.

Acknowledgments—We thank Marion Schmidt for experimental help, Helmut Lenz for support, and Jim Bardwell, Hilary Hawkins, Rainer Jaenicke, Rainer Rudolph, and Franz Schmid for stimulating discussions and critically reading the manuscript.

REFERENCES
Anfinsen, C. B. (1973) Science 181, 223-230
Bensadoun, A., and Weinstein, W. (1976) Anal. Biochem. 70, 241-250
Bergman, L. W., and Kuehl, M. W. (1979) J. Biol. Chem. 254, 6690-6694
Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
Brockway, B. E., Forster, S. J., and Freedman, R. B. (1980) Biochem. J. 191, 873-878
Buchner, J., and Rudolph, R. (1990) in Dechema Biotechnology Conferences (Behrens, D., and Driesel, A. J., eds) Vol. 3, pp. 1035-1039, Verlag Chemie, Weinheim
Buchner, J., and Rudolph, R. (1991a) Curr. Opin. Biotechnol. 2, 522-528
Buchner, J., and Rudolph, R. (1991b) Bio/Technology 9, 157-162
Buchner, J., Pestan, I., and Bruckmann, U. (1992) Anal. Biochem. 205, 263-270
Buckel, P., Hulzer-Parajaz, C., Mattes, R., Lenz, H., Haug, H., and Beaucamp, K. (1987) Gene (Amst.) 51, 13-19
Bulleid, N. J., and Freedman, R. B. (1989) Nature 335, 649-651
Delamette, F., Marty, M. C., and Panjuel, J. (1976) Cell. Immunol. 19, 262-275
DellaCorte, E., and Parkhouse, R. M. E. (1973) Biochem. J. 136, 597-606
Ellman, G. L. (1955) Arch. Biochem. Biophys. 82, 70-78
Flynne, G. C., Chappell, G., and Rothman, J. E. (1989) Science 245, 385-390
Flynne, G. C., Poh, J., Flaco, M. T., and Rothman, J. E. (1991) Nature 353, 726-730
Freedman, R. B. (1991) in Conformation and Forces in Protein Folding (Nall, B. T., and Ill, K., eds) pp. 204-214, American Association for the Advancement of Science, Washington, DC
Freedman, R. B. (1989) in Protein Folding (Creighton, T. E., ed) pp. 455-568, W. H. Freeman and Co., New York
Geetha-Habs, M. M., Noiva, R., Kaplan, H. A., and Lennarz, W. J. (1988) Cell 54, 1053-1060
Gething, M. J., and Sumbrook, J. (1992) Nature 355, 33-45
Goto, Y., and Hamaguchi, K. (1981) J. Mol. Biol. 146, 321-340
Haas, I. G., and Wabl, M. (1983) Nature 306, 387-389
Hawkins, C. H., and Freedman, R. B. (1991) Biochem. J. 275, 335-343
Holmgren, A. (1979) J. Biol. Chem. 254, 9627-9632
Hurtley, S. M., and Helensia, A. (1989) Annu. Rev. Cell Biol. 5, 277-307
Jaenicke, R. (1987) Prog. Biophys. Mol. Biol. 49, 117-157
Jaenicke, R., and Buchner, J. (1984) Current Topics in Membrane Research, Vol. 4, I-30
John, D. C. A., Grant, M. E., and Balleid, N. J. (1993) EMBO J. 12, 1587-1595
Johnstone, A., and Thorpe, R. (1987) Immunocytochemistry in Protein, pp. 55-59, Blackwell Scientific Publications, Oxford
Kawata, Y., and Hamaguchi, K. (1991) Biochemistry 30, 4367-4373
Khyse-Anderson, J. (1984) J. Biochem. Biophys. Methods 16, 203-209
Kiefhaber, T., Kohler, H. H., Rudolph, R., and Buchner, J. (1991) Bio/Technology 9, 825-829
Kiefhaber, T., Quass, R., Hahn, U., and Schmid, F. C. (1990) Biochemistry 29, 3061-3070
Klich, H., Goto, Y., and Hamaguchi, K. (1986) Biochemistry 25, 2009-2013
Knittler, M. R., and Haas, I. G. (1992) EMBO J. 11, 1573-1581
Laemmli, U. K. (1970) Nature 227, 580-583
Lambert, N., and Freedman, R. B. (1983) Biochem. J. 213, 225-234
Lilie, H., Lang, K., Rudolph, R., and Buchner, J. (1993) Protein Sci. 1490-1496
Melnick, J., Aviel, S., and Argon, Y. (1992) J. Biol. Chem. 267, 21303-21306
Milks, E. N. C., Lambert, N., and Freedman, R. B. (1993) Biochem. J. 218, 245-248
Miyazaki, K., Koiva, Pihlajaniemi, T., Kivirikko, K. I. (1988) Eur. J. Biochem. 134, 7-11
Noiva, R., and Lennarz, W. J. (1982) J. Biol. Chem. 267, 5553-5556
Noiva, R., Kimura, H., Roos, J., and Lennarz, W. J. (1991) J. Biol. Chem. 266, 19645-19649
Pauer, J. L., Freedman, R. B., and Parkhouse, R. M. E. (1986) FEBS Lett. 242, 352-362
Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllyla, H., Huihala, M., Koiva, J., and Kivirikko, K. I. (1987) EMBO J. 6, 443-449
Roth, R. A., and Kashland, M. E. (1981) Biochemistry 20, 6594-6599
Roth, R. A., and Pierce, S. B. (1987) Biochemistry 26, 4179-4182
Rudolph, R. (1990) in Modern Methods in Protein and Nucleic Acid Research (Toccheas, H., ed) pp. 149-171, Walter DeGruyter, New York
Schmidt, M., and Buchner, J. (1992) J. Biol. Chem. 267, 16829-16833
Wetterau, J. R., Combs, K. A., Spinelli, S. N., and Joiner, B. J. (1990) J. Biol. Chem. 265, 8806-8807
Wiese, H., Buchner, J., Zimmermann, R., and Jakob, U. (1992) Nature 358, 169-170
Wilde, C. E., and Koshland, M. E. (1978) Biochemistry 17, 3209-3214