Catalytic Activity and Chaperone Function of Human Protein-disulfide Isomerase Are Required for the Efficient Refolding of Proinsulin

Received for publication, August 15, 2001, and in revised form, October 22, 2001
Published, JBC Papers in Press, November 1, 2001, DOI 10.1074/jbc.M107532200

Jeannette Winter‡§, Peter Klappa¶, Robert B. Freedman¶, Hauke Lilie‡, and Rainer Rudolph‡¶
From the ‡Martin-Luther Universität Halle-Wittenberg, Institut für Biotechnologie, Kurt-Mothes-Str. 3, 06120 Halle, Germany and the ¶Department of Biosciences, University of Kent, Canterbury CT2 7NJ, United Kingdom

Protein-disulfide isomerase (PDI) catalyzes the formation, rearrangement, and breakage of disulfide bonds and is capable of binding peptides and unfolded proteins in a chaperone-like manner. In this study we examined which of these functions are required to facilitate efficient refolding of denatured and reduced proinsulin. In our model system, PDI and also a PDI mutant having only one active site increased the rate of oxidative folding when present in catalytic amounts. PDI variants that are completely devoid of isomerase activity are not able to accelerate proinsulin folding, but can increase the yield of refolding, indicating that they act as a chaperone. Maximum refolding yields, however, are only achieved with wild-type PDI. Using genistein, an inhibitor for the peptide-binding site, the ability of PDI to prevent aggregation of folding proinsulin was significantly suppressed. The present results suggest that PDI is acting both as an isomerase and as a chaperone during folding and disulfide bond formation of proinsulin.

Protein-disulfide isomerase (PDI) is a protein of the endoplasmic reticulum involved in the oxidative folding of many disulfide-bonded proteins (1–3). PDI consists of four domains arranged in the order a-b-b’-a’ with an acidic extension at the C terminus (c domain) that contains the KDEL retention sequence. The first two domains show significant homology to thioredoxin (4, 5). Although there are no structural data available for the b’ and a’ domains, it can be inferred from their sequence similarities to the b and a domain, respectively, that they also contain the thioredoxin fold. Both, the a and a’ domains of PDI, contain the local sequence -WCCHCK- that is essential for the catalytic activity of PDI. Both isolated domains can operate as thio:disulfide oxidoreductases, but all other PDI domains are required to assist protein refolding and formation of native disulfide bonds with maximum catalytic activity (6). No function has been assigned to the b domain so far, however, the b’ domain provides the principle peptide-binding site (7). This domain alone is sufficient to bind peptides of 10–15 residues but binding of larger peptides or non-native proteins requires the contribution of either the a and b domains or the a’ domain (7).

In vitro, PDI can assist folding of proteins that contain no disulfide bonds, demonstrating its function as a chaperone (8, 9). PDI can influence folding of proteins with multiple disulfide bonds as a chaperone and also as an isomerase (10–17). Yao et al. (11) proposed that PDI can fulfil both activities on the refolding of acidic phospholipase A2. When using mitochondrial malate dehydrogenase (10) or rhodanese (8) as a substrate PDI influences refolding in a chaperone-like manner. PDI and also alkylated PDI displaying no isomerase activity can chaperone refolding of D-glyceraldehyde-3-phosphate dehydrogenase indicating that the active sites are not required for the chaperone activity of PDI (9, 18). The isomerase activity is involved in refolding of lysozyme (19), an antibody fragment (13), insulin (16), and proinsulin (17). Furthermore, under certain redox conditions PDI can reduce protein substrates with native disulfide bonds (13, 20). In addition, PDI can catalyze disulfide bond formation and rearrangements within kinetically trapped, structured folding intermediates (21). This demonstrates that the substrate specificity of PDI is not restricted to misfolded proteins containing no or non-native “scrambled” disulfide bonds.

It is possible to discriminate between isomerase and chaperone activity of PDI by using mutants or variants displaying only one of both activities. Active site mutants or alkylated PDI have been used to generate species that act as a chaperone only (11, 13). On the other hand mutants lacking chaperone activity do not exist as it is not clear which amino acid residues in the peptide-binding domain are involved and essential for substrate binding. Tsibris et al. (22) could show that isomerization of scrambled RNase and reduction of insulin are inhibited by estrogens which might be due to the similarity of PDI parts with estrogen receptor segments. Additionally, compounds with estrogenic activity can inhibit peptide binding to PDlp, a pancreas-specific member of the protein-disulfide isomerase family (23).

To dissect the different activities of PDI we used human proinsulin as model substrate. Human proinsulin consists of a single polypeptide which, after trypsin and carboxypeptidase B cleavage, can be converted to the biologically active insulin and the c-peptide in vitro (24). Proinsulin contains three disulfide bonds (1) Cys7-Cys72, (2) Cys19-Cys85, and (3) Cys71-Cys76 which are essential for its native structure. In addition to the efficient spontaneous oxidative folding of reduced proinsulin at...
Chaperone and Isomerase Activity of PDI

pH 10.5, native proinsulin can also be generated at neutral pH starting from the scrambled molecule in the presence of PDI in a molar ratio PDI/substrate of 0.1 (17).

Here we investigated the mechanism of PDI function in proinsulin refolding. We show that PDI influences the refolding of denatured and reduced proinsulin both as an isomerase and as a chaperone. PDI in catalytic amounts is able to increase the refolding rate and the refolding yield while PDI variants devoid of isomerase activity influence the refolding yield only when present in stoichiometric amounts. The chaperone function is essential during the first seconds of refolding because aggregation of folding proinsulin is a major side reaction. Inhibition of the peptide-binding site of PDI leads to a suppression of the aggregation preventing role of PDI. Besides the chaperone function the isomerase activity is also required at the beginning of proinsulin folding, but the late refolding process does only depend on the isomerase activity.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human proinsulin was provided by BIOBRAS, Montes Claros, Brazil. ELISA antibodies were obtained from Biochemicals, Penzberg, Germany. The vectors pET23-PDI and pET23-PDI-b were purchased from Dr. L. W. Rudbeck, University of Kent, Canterbury, UK. PDI and PDI variants were purified from Escherichia coli lysates as described below. Genistein and the homobifunctional cross-linking reagent disuccinimidyl glutarate were purchased from Sigma, iodoacetamide was obtained from ICN. X-ray films and Bolton-Hunter 125I labeling reagent were purchased from Amersham Bioscience, Inc.

Unfolding and Refolding of Proinsulin—The human proinsulin, containing a N-terminal His6-Arg-tag was subject to reductive unfolding by adding 20 mg of the native proinsulin to 1 ml of 6 M guanidinium chloride, 10 mM Tris/HCl, pH 8.5, 1 mM EDTA, 1.1 M dithiothreitol. The sample was incubated for 8 h at 37 °C and then extensively dialyzed at 4 °C against 8 M guanidinium chloride, 5 mM EDTA, pH 3. For cross-linking, the denatured and reduced proinsulin was desalted to a final concentration of 2 M guanidinium chloride using a NAP-5 column (Amersham Bioscience, Inc.).

Refolding of proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C by diluting the unfolded and reduced proinsulin to a final concentration of 100 μM if not otherwise indicated. The sample was immediately mixed. At distinct time points aliquots were removed and acetonitrile (final concentration 20% (v/v)) and trifluoroacetic acid (final concentration 0.1% (v/v)) were added, if not otherwise indicated.

Refolding of proinsulin in the presence of genistein was performed with proinsulin at a final concentration of 27 μM. Genistein (10 mM solution in MeSO) was added to a final concentration of 20 μM. Aliquots were removed and digested with trypsin and carboxypeptidase B (see below).

Quantification of Proinsulin Folding—Refolding of denatured and reduced proinsulin was monitored by reversed phase HPLC (RP-HPLC) using a C18 column (Macherey-Nagel) equilibrated with 100% solvent A (solvent A: 20% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid; solvent B: 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid). The protein was eluted at 20 °C with a flow rate of 0.5 ml/min of a linear gradient from 20 to 40% solvent B within 20 min. For samples containing genistein and for samples after digestion, the column was equipped with 10% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. The protein was eluted with a linear gradient from 10 to 58% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid with a flow rate of 0.5 ml/min within 40 min. Peaks were detected by absorbance at 214 nm and quantified according to a calibration curve.

Tryptic Digestion and ELISA—Refolding samples containing genistein were digested with trypsin and carboxypeptidase B (final concentration 1.35 μg/ml each) for 20 min at ambient temperature. Afterward acetonitrile and trifluoroacetic acid were added to a final concentration of 10 and 0.1% (v/v), respectively, and the samples were analyzed by RP-HPLC as described above.

To verify the native structure of the refolded proinsulin, a digestion was performed with native and refolded proinsulin at refolding conditions (100 μg/ml proinsulin). After incubation for 20 min at ambient temperature the digestion was stopped by addition of soybean trypsin inhibitor and EDTA. The final concentrations were 5 μg/ml trypsin, 5 μg/ml carboxypeptidase B, 25 μg/ml trypsin inhibitor, and 125 mM EDTA. Samples were analyzed by RP-HPLC as described above and by an insulin-ELISA as described previously (25).

Light Scattering—Light scattering due to aggregation was measured at 500 nm in a stirrable 2-ml cuvette using a fluorescence spectrometer (FLUOROMAX, Spec Instrument, CA). The concentration 20–100 μM both excitation and emission slits were adjusted to 1 nm band width, for lower concentrations to 3 nm. The temperature of the refolding buffer (see above) was adjusted to 25 °C. The protein to be measured was added to the stirred refolding buffer and aggregation was recorded for 120 to 600 s. At the same conditions a calibration curve was generated for denatured and reduced proinsulin (from 0.2 to 10 μM).

According to this calibration curve, the light scattering signal caused by the aggregation of proinsulin could be calculated to micrograms/ml aggregating proinsulin. Related to the amount of proinsulin used one can calculate the corresponding amount of proinsulin protected from aggregation.

Generation of PDI Variants—Point mutations to replace the codons for cysteine in the active sites of PDI by serine were generated using the vector pET23-PDI for the quick change procedure (Qiagen). The wild-type sequences 5′-TGTTGCAACTGC-3′ for the a domain (corresponding to -379CGHC382-) and 5′-TGTTGCACTGC-3′ for the a’ domain (corresponding to -379CGCH382-) were changed to 5′-TCCGTCACCTCT-3′ encoding the amino acid sequence -SGHS-. The active site in either the a domain (PDIAC1) or a’ domain (PDICA2) was mutated to generate a PDI-single mutant. The PDI-double mutant (PDICA1,2) was produced by replacing the cysteine codons of both active sites by serine. The correct sequence was verified by DNA sequencing.

Expression and Purification of PDI and PDI Variants—For the production of wild-type PDI, PDI-b, c, and the mutants PDICA1, PDICA2, E. coli BL21(DE3)-plSyS was transformed with the corresponding plasmid-DNA. The cells were incubated at 37 or 30 °C on LB medium containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Three hours after induction with 1 mM isopropyl-thio-β-D-galactopyranosid, the cells were harvested by centrifugation. The cell pellet was suspended in buffer A (20 mM Na-phosphate, pH 7.3) and DNase was added to a final concentration of 10 μg/ml. After freezing and thawing the suspension twice, the lysate was centrifuged. After ultrafiltration (0.2 μm), the supernatant was loaded onto a Ni-NTA column (volume 12 ml, Qiagen), which, after activation with NiSO4 and washing with double-diluted water, was equilibrated with buffer A. Proteins bound nonspecifically onto the matrix were removed by washing the column with buffer A containing 500 mM NaCl and 50 mM imidazole, followed by buffer A. Recombinant protein was eluted with buffer A containing 10 mM EDTA. The elution fraction was loaded onto a Resource Q column (6 ml, Amersham Bioscience, Inc.) equilibrated with buffer A and eluted with a linear gradient from 0 to 0.5 M NaCl in buffer A. Fractions containing homogenous PDI or PDI variants were pooled and dialyzed against 10 mM Tris, 1 mM glycine, pH 7.5, 0.1 mM EDTA. The concentration of purified protein was determined by UV spectrophotometry (molar absorbance coefficient 45,380 M⁻¹ cm⁻¹ (280 nm)).

The molecular mass of the purified proteins was determined by mass spectrometry. PDICA1,2 was centrifuged (70,000 × g, 4 °C, 30 min) prior to use and afterward the correct concentration was determined by UV spectrophotometry.

Alkylation of Cysteines—PDI was incubated with 5 mM dithiothreitol. Alkylation was performed at 25 °C, iodoacetamide was added to a final concentration of 50 mM and the sample was incubated for 45 min at 25 °C. Excess dithiothreitol and iodoacetamide was removed by dialysis against 1 mM Tris, 1 mM glycine, pH 7.5, 0.1 mM EDTA and the alkylated PDI lyophilized and stored at −20 °C. For refolding experiments, lyophilized alkylated PDI was dissolved in double distilled water to a concentration of ~5 mg/ml. After centrifugation (70,000 × g, 4 °C, 30 min), the correct concentration was determined by UV spectrophotometry.

125I-Bolton-Hunter—Bolton-Hunter-labeled carboxamidated and reduced proinsulin (5 mg/ml in 2 M guanidinium chloride, pH 3.0) was carried out as recommended by the manufacturer. Radiolabeled proinsulin was incubated with the E. coli cell extract that expressed PDI (10 μg/ml) on ice. After 10 min 0.1 volume of disuccinimidyl glutarate (5 mM in MeSO) was added and the sample incubated for additional 60 min. The reaction was stopped by the addition of SDS sample buffer (26). Proteins were separated on 12.5 or 15% polyacrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane and subsequently analyzed by autoradiography.

2 J. Winter, unpublished results.
Reactivation of denatured and reduced proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C. The final concentration was 10 μM for proinsulin and 70 mM for guanidinium chloride. At the times indicated an aliquot was withdrawn and acetonitrile (final concentration 20% (v/v)) were added. The yield of refolded proinsulin was analyzed by RP-HPLC. A, influence of different concentrations of PDI on the kinetics. Refolding was performed in the absence of PDI (○), in the presence of PDI in molar ratio (PDI/proinsulin) of 0.01 (▲), 0.033 (●), 0.1 (◆), 0.33 (△), or 1 (□). The renaturation kinetic of spontaneous proinsulin folding (●) was fitted single exponentially with a rate constant of $k_{app} = 0.0018$ s$^{-1}$. B, influence of PDI variants on the kinetic of refolding. Refolding was carried out in the absence (○) or presence (●) of PDI, or in the presence of the PDI mutants PDIΔC1 (▲) and PDIΔC1,2 (◆). The final concentration of PDI and the PDI variants was 2 μM; C, influence of PDI and PDIΔC1,2 on the refolding yield. PDI (○) or PDIΔC1,2 (□) were added to the refolding proinsulin at the molar ratios indicated. Shown are the amounts of native proinsulin analyzed after the renaturation was completed (the samples were measured at least as duplicates).

**Fig. 1** Influence of PDI on the kinetics and yield of proinsulin refolding. Reactivation of denatured and reduced proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG at 25 °C. The final concentration was 10 μM for proinsulin and 70 mM for guanidinium chloride. At the times indicated an aliquot was withdrawn and acetonitrile (final concentration 20% (v/v)) were added. The yield of refolded proinsulin was analyzed by RP-HPLC. A, influence of different concentrations of PDI on the kinetics. Refolding was performed in the absence of PDI (○), in the presence of PDI in molar ratio (PDI/proinsulin) of 0.01 (▲), 0.033 (●), 0.1 (◆), 0.33 (△), or 1 (□). The renaturation kinetic of spontaneous proinsulin folding (●) was fitted single exponentially with a rate constant of $k_{app} = 0.0018$ s$^{-1}$. B, influence of PDI variants on the kinetic of refolding. Refolding was carried out in the absence (○) or presence (●) of PDI, or in the presence of the PDI mutants PDIΔC1 (▲) and PDIΔC1,2 (◆). The final concentration of PDI and the PDI variants was 2 μM; C, influence of PDI and PDIΔC1,2 on the refolding yield. PDI (○) or PDIΔC1,2 (□) were added to the refolding proinsulin at the molar ratios indicated. Shown are the amounts of native proinsulin analyzed after the renaturation was completed (the samples were measured at least as duplicates).
beginning of the process, no increase in yield was observed if PDLΔC1,2 was added to refolding proinsulin after 7 s of refolding. PDI inactivated by alkylation (13) was tested under the same conditions (data not shown) and its activity on refolding was identical to that of PDLΔC1,2 thus excluding unspecific effects of possible structural changes caused by the point mutations in PDLΔC1,2. Both, PDLΔC1,2 and alkylated PDI, showed identical results in the timed addition experiment.

In contrast, wild-type PDI, even when added 1 min after initiation of folding could improve renaturation. This improvement, however, was not as efficient as when present from the beginning. This indicates that the significant decrease in the refolding yield due to the absence of the chaperone function in the first seconds of refolding can be partly compensated by the isomerase activity of PDI. The observed decrease in the yield of refolding of proinsulin when PDI was added to the refolding sample at different time points exhibits a biphasic kinetic. A very fast first phase with an apparent rate constant $k_{app} = 0.075 \text{ min}^{-1}$ was followed by a slow phase with a $k_{app} = 0.0043 \text{ min}^{-1}$. Compared with the rate constant of formation of native proinsulin ($k_{app} = 0.0018 \text{ s}^{-1}$, Fig. 1A) the second phase occurred in a similar time range but the first phase was much faster. From this we conclude that upon renaturation of proinsulin there is a kinetic competition between an overall slow folding process and an ~20 times faster off-pathway reaction. This off-pathway seems to depend on redox reactions subsequently leading to aggregation.

**Chaperone Function of PDI Prevents Aggregation of Proinsulin**—Furthermore, PDI and PDLΔC1,2 were tested with respect to peptide binding. Chemical cross-linking with an amino-specific cross-linking reagent is a useful method to detect interaction partners for PDI (7, 26). Both variants of PDI were capable of binding radiolabeled denatured and reduced proinsulin (Fig. 3). Furthermore, the binding was reversible, as it could be competed with other substrate peptides and proteins (data not shown). This clearly demonstrates that PDI and PDLΔC1,2 were indistinguishable with respect to their interactions with substrates. PDLΔC1,2 shows two bands in the cross-linking (Fig. 3). The signal at lower molecular weight corresponded to a degraded PDLΔC1,2. Both, the full-length and the degraded PDLΔC1,2 were able to bind denatured and reduced proinsulin. This degradation was probably due to proteases present in the *E. coli* cell extract used for the cross-linking assay. No degradation of PDLΔC1,2 was observed with purified protein under refolding conditions as analyzed by SDS gels (data not shown). To demonstrate that the observed effects of the PDLΔC1,2 were not due to misfolding of one or more of its domains its stability was compared with that of wild-type PDI. Incubation of PDLΔC1,2 or the wild-type PDI with various concentrations of proteinase K showed that there was no significant difference in the protease sensitivity indicating that PDLΔC1,2 can adopt as compact a structure as the wild-type and that there was no difference in the structural stability of both proteins (data not shown).

Aggregation of folding proinsulin as detected by light scattering was proportional to the proinsulin concentration in the refolding sample (Fig. 4B, inset). It occurred in the first seconds of refolding, reaching a maximum after 20–30 s (Fig. 4A). This indicates that aggregation of proinsulin during renaturation occurred in the same time range as the fast phase of the unproductive reaction observed in the timed addition experiment of PDI (Fig. 2). PDI suppressed aggregation of refolding proinsulin substantially and was already effective in catalytic amounts (Fig. 4B) which is in agreement with the refolding data shown in Fig. 1A and the timed addition data from Fig. 2. PDI at a molar ratio of PDI/proinsulin of 0.05 could prevent about 20% of the folding proinsulin from aggregation. In contrast, alkylated PDI in the same molar ratio could suppress only 10% of the aggregation (data not shown). Only when present in stoichiometric amounts could alkylated PDI prevent aggregation of folding proinsulin to the same extent as PDI. Under identical conditions bovine serum albumin did not suppress aggregation (data not shown).

This clearly indicates that PDI, present in catalytic amounts, possesses chaperone activity which alone is not sufficient to protect folding proinsulin from aggregation. Early intermediates in the proinsulin folding pathway, probably containing wrong disulfide bonds, seem to be highly susceptible to aggregation. In the presence of wild-type PDI these aggregation-prone intermediates can isomerize to folding intermediates that are less susceptible to aggregation. This results in reduced aggregation and, as a consequence, in an enhanced refolding yield. When present in very high concentrations alkylated PDI was similarly effective, indicating that a noncatalyzed isomer-
excess of genistein over proinsulin was required to completely suppress binding of denatured and reduced proinsulin to PDI as well as to PDI\(_{C1,2}\).

Genistein-inhibited PDI or alkylated PDI were significantly affected in their ability to prevent proinsulin from aggregation (Fig. 5B). In refolding samples analyzed by light scattering genistein-inhibited PDI and genistein-inhibited alkylated PDI could protect about 11% of the folding proinsulin from aggregation. This indicates that both PDIs still had some residual chaperone activity under the experimental conditions. Genistein inhibited 66% of the chaperone function of PDI. For comparison, the inhibitory effect of genistein on alkylated PDI was less pronounced (about 50% inhibition). The lower inhibition effect for alkylated PDI was due to its two times higher concentration in the refolding sample and therefore a two-times less excess of genistein over alkylated PDI compared with PDI.

In agreement with the results shown above, the inhibition of the chaperone function of PDI drastically changed the PDI-assisted refolding behavior of proinsulin (data not shown). In PDI-catalyzed refolding a 10-fold molar excess of genistein over proinsulin decreased the refolding rate four times and the yield of proinsulin refolding was only 37% compared with 50% in the presence of non-inhibited PDI. For refolding in the presence of genistein-inhibited PDI\(_{C1,2}\) or alkylated PDI the stimulating effect mediated by the variants with no isomerase activity decreased by 60% (corresponding to about 25% refolded proinsulin). The rate constant did not change compared with refolding without genistein. This shows that the inhibited PDIs still had some residual chaperone activity and that in the case of wild-type PDI the isomerase activity can facilitate a higher refolding yield. Similar results were obtained for refolding of proinsulin with a four times and a 100 times molar excess of genistein (data not shown). This is in agreement with the cross-linking data shown above. As a control we confirmed that the aggregation and refolding properties of proinsulin were not changed in the presence of genistein alone (see above). Additionally, Me\(_2\)SO used to solubilize the genistein did not influence proinsulin refolding, neither in the absence nor the presence of PDI or PDI variants (data not shown). This proves that by binding of genistein to the peptide-binding domain of PDI binding of folding proinsulin is suppressed. This inhibition of the chaperone function of PDI leads to enhanced aggregation of folding intermediates and to a reduction of the yield of refolding.

The Chaperone and Isomerase Activity of PDI Are Not Required during the Entire Refolding Process—To analyze whether both the chaperone and isomerase function of PDI were only required during the first seconds of refolding or whether they were essential during the entire refolding process, the refolding of proinsulin was monitored in the presence of PDI and PDI\(_{C1,2}\), and genistein was added at different times after initiation of refolding (Fig. 6A). Similar to these experiments we performed proinsulin refolding and added genistein-inhibited PDI at different time points after refolding started (Fig. 6B).

As described above, refolding of proinsulin in the presence of genistein-inhibited PDI or PDI\(_{C1,2}\) resulted in a significant decrease of the yield of refolding. In contrast, genistein added 25 s after initiation of refolding of proinsulin in the presence of PDI or PDI\(_{C1,2}\) did not influence the refolding yield. For both, refolding of proinsulin with PDI or PDI\(_{C1,2}\), the obtained refolding yield was similar to the yield of refolding without genistein. This indicates that at this time, 25 s after refolding started, the chaperone function was not longer required (Fig. 6A).

These results are in agreement with the light scattering data (Fig. 4A) showing that aggregation occurred in the first few seconds of refolding. Timed addition of genistein-inhibited

---

Fig. 4. Influence of PDI on proinsulin aggregation. Refolding of proinsulin was performed in 10 mM Tris, 10 mM glycine, pH 7.5, 1 mM EDTA, 1 mM GSH, 2 mM GSSG, with different concentrations of PDI at 25 °C in a stirred cuvette. The final concentration was 10 μM for denatured and reduced proinsulin and 40 μM for guanidinium chloride. Aggregation was monitored by light scattering at 500 nm. A, kinetics of the aggregation of proinsulin in the absence of PDI (○), and in the presence of 2 μM PDI (△), or 10 μM PDI (□); B, dependence of the aggregation of proinsulin on the concentration of PDI. The amount of proinsulin protected from aggregation was calculated according to the calibration curve shown in the inset. Inset, calibration curve of proinsulin aggregation from 10 to 100 μg/ml proinsulin.

Proinsulin Binding to PDI Is Suppressed by the Inhibitor Genistein—To analyze whether the interaction of PDI and PDI\(_{C1,2}\) with denatured and reduced proinsulin can be suppressed by an inhibitor of the chaperone activity we used the small molecular weight substance genistein. It has been shown previously that genistein, a substance with estrogenic activity, can suppress the binding of α-somatostatin to PD1p, a member of the protein-disulfide isomerase family (23), and also PDI. 3 Both, PDI and PDI\(_{C1,2}\) showed the same genistein binding properties (Fig. 5A) indicating that the peptide-binding domain of PDI\(_{C1,2}\) was not affected by the mutations in both active sites. By titration of genistein we found that a 3-fold molar

3 L. W. Ruddock and P. Klappa, unpublished observations.
PDI to refolding proinsulin exhibited a decrease in the refolding yield with a biphasic characteristic and an apparent rate constant for the fast, first phase of \( k_{\text{app}} = 0.029 \) s\(^{-1}\). PDI with isomerase activity could increase the refolding yield significantly even when added 1 or 5 min after starting refolding. This catalytic effect was independent of the chaperone activity of PDI. The results of the above experiments clearly show that the isomerase activity could compensate for an impaired chaperone activity of PDI. Maximum rate and yield of proinsulin refolding can only be achieved if both, the chaperone and the isomerase activity of PDI were present.

Although PDI exhibits isomerase and chaperone activity PDI seems to be more efficient as an isomerase than as a chaperone (13, 27). PDI acts only as an isomerase on refolding of an antibody fragment (13). This effect is limited to the first seconds of refolding. However, if the chaperone BiP is simultaneously present BiP can efficiently bind and re-bind folding intermediates. Thus it keeps the cysteines of the folding antibody fragment accessible to PDI over a much longer time scale and PDI can sequentially act as an isomerase (27). The active site sequence -WCGHC- of PDI enables very efficient disulfide isomerization as compared with wild-type thioredoxin. A Pro to His mutation (-CGPC- to -CGHC-) in the active site of thioredoxin increases the isomerization activity (28). For thioredoxin a flat and hydrophobic molecular surface area on one side of the redox active disulfide bond has been described that is suggested to be the binding area for redox interactions with other proteins (29, 30). On the basis of sequence homologies to thioredoxin, it was suggested that PDI also has protein binding capacity. The principle peptide-binding site is located in the b’ domain of PDI, however, for efficient binding of proteins the contribution of the a’ domain (b’a’c) or the a and b domains

**DISCUSSION**

Previous work on refolding activities of PDI in vitro showed that PDI has catalytic activity as a thiol:disulfide isomerase and can act as chaperone. In most cases PDI affects only one or the other of these functions (8, 9, 13). Yao et al. (11) proposed that for the reactivation of acidic phospholipase \( A_2 \) both functions are required. They stated that in their model system only a small part of PDI acts catalytically as an isomerase and the residual part functions as a molecular chaperone, which can be replaced by alkylated PDI. In our model system refolding of proinsulin is dependent on the redox conditions and influenced by aggregation and disulfide bond formation. A PDI variant with no isomerase activity can substantially suppress aggregation of refolding proinsulin. However, this PDI variant was not as efficient as wild-type PDI with respect to refolding yield and refolding rate, indicating that disulfide isomerization also plays an important role in the refolding of proinsulin. Maximum rate and yield of proinsulin refolding can only be achieved if both, the chaperone and the isomerase activity of PDI were present.
with respect to proinsulin refolding they can significantly increase the refolding rate and yield. We demonstrated that (i) the b’c domain construct of PDI can catalyze the refolding of proinsulin, (ii) the presence of one active site was sufficient to accelerate proinsulin refolding, however, (iii) full-length PDI was needed for maximum catalytic activity. This is in agreement with the data of Darby et al. (6) who demonstrated that the b’ domain of PDI has an especially important role in catalysis, and that maximum catalytic activity in disulfide bond rearrangements requires the involvement of all PDI domains. It was proposed that all PDI domains participate in substrate binding and especially the binding of non-native proteins might require all domains of PDI (7, 39).

Using isomerase-inactive PDI variants and PDI with inhibited chaperone function, we now can clearly distinguish between both functions PDI provides. The mutation in the active site did change the enzyme characteristic but did not effect the chaperone activity. This was concluded as both, alkylated PDI and PDIΔC1,2, were equally active regarding peptide binding as shown by cross-linking and refolding of denatured and reduced proinsulin. Genistein, which can suppress Δ-somatostatin binding to the pancreatic protein-disulfide isomerase PDP (23), is also able to bind to the peptide-binding site of PDI. Thus genistein can efficiently suppress binding of other substrates to PDI as shown by cross-linking experiments. By inhibition of the principal peptide-binding site the refolding yield of proinsulin was significantly decreased although not completely abolished indicating that other domains can contribute to the chaperone activity of PDI. However, in this case the interactions might be too weak to be detected in the cross-linking experiments. Genistein-inhibited PDI variants lacking isomerase activity that should not influence refolding were still able to increase proinsulin refolding, but not as efficient as the non-inhibited variant. This might indicate that proinsulin binding occurs not only at the b’ domain but extends through all PDI domains or that PDI contains more than one peptide-binding site with only one site as a target for genistein. Furthermore, neither for genistein nor for proinsulin the binding constant to PDI is known. Hence we cannot exclude that the binding of refolding proinsulin to PDI is possible with different affinities for different folding intermediates, even in the presence of an excess of genistein.

In spontaneous refolding of proinsulin about 20% of the denatured and reduced proinsulin was folding competent. The remaining part was excluded from productive refolding by very fast aggregation of completely reduced proinsulin or folding intermediates. Providing optimum conditions (e.g. refolding at pH 10.5) aggregation of folding proinsulin is reduced and about 60% native proinsulin can be formed. Similarly, IGF-I that belongs to the insulin superfamily and contains three motif-specific disulfide bonds yields under optimized conditions about 60% native protein within 5 h (40). Proinsulin and also porcine insulin precursor (41) form no stable intermediate as shown by cross-linking and refolding of denatured and reduced proinsulin. Genistein-inhibited PDI variants lacking isomerase activity of PDI. However, in this case the interactions with respect to proinsulin refolding they can significantly increase the refolding rate and yield. We demonstrated that (i) the b’c domain construct of PDI can catalyze the refolding of proinsulin, (ii) the presence of one active site was sufficient to accelerate proinsulin refolding, however, (iii) full-length PDI was needed for maximum catalytic activity. This is in agreement with the data of Darby et al. (6) who demonstrated that the b’ domain of PDI has an especially important role in catalysis, and that maximum catalytic activity in disulfide bond rearrangements requires the involvement of all PDI domains. It was proposed that all PDI domains participate in substrate binding and especially the binding of non-native proteins might require all domains of PDI (7, 39).

Using isomerase-inactive PDI variants and PDI with inhibited chaperone function, we now can clearly distinguish between both functions PDI provides. The mutation in the active site did change the enzyme characteristic but did not effect the chaperone activity. This was concluded as both, alkylated PDI and PDIΔC1,2, were equally active regarding peptide binding as shown by cross-linking and refolding of denatured and reduced proinsulin. Genistein, which can suppress Δ-somatostatin binding to the pancreatic protein-disulfide isomerase PDP (23), is also able to bind to the peptide-binding site of PDI. Thus genistein can efficiently suppress binding of other substrates to PDI as shown by cross-linking experiments. By inhibition of the principal peptide-binding site the refolding yield of proinsulin was significantly decreased although not completely abolished indicating that other domains can contribute to the chaperone activity of PDI. However, in this case the interactions might be too weak to be detected in the cross-linking experiments. Genistein-inhibited PDI variants lacking isomerase activity that should not influence refolding were still able to increase proinsulin refolding, but not as efficient as the non-inhibited variant. This might indicate that proinsulin binding occurs not only at the b’ domain but extends through all PDI domains or that PDI contains more than one peptide-binding site with only one site as a target for genistein. Furthermore, neither for genistein nor for proinsulin the binding constant to PDI is known. Hence we cannot exclude that the binding of refolding proinsulin to PDI is possible with different affinities for different folding intermediates, even in the presence of an excess of genistein.

In spontaneous refolding of proinsulin about 20% of the denatured and reduced proinsulin was folding competent. The remaining part was excluded from productive refolding by very fast aggregation of completely reduced proinsulin or folding intermediates. Providing optimum conditions (e.g. refolding at pH 10.5) aggregation of folding proinsulin is reduced and about 60% native proinsulin can be formed. Similarly, IGF-I that belongs to the insulin superfamily and contains three motif-specific disulfide bonds yields under optimized conditions about 60% native protein within 5 h (40). Proinsulin and also porcine insulin precursor (41) form no stable intermediate as possible intermediates seem to be either highly susceptible to aggregation or can fold to the native protein (41). In contrast, IGF-I yields two isoforms that are stable under refolding conditions (42–44). The first one is native IGF-I with the disulfide bonds: 1) Cys6-Cys48, 2) Cys35-Cys61, and 3) Cys52-Cys57 which correspond to Cys5-Cys72, Cys19-Cys85, and Cys33-Cys76 in proinsulin. The second isoform is non-native IGF-I with the disulfide bonds 1 and 3 mismatched. These species occur in a ratio of 60% native to 40% mismatched.

The apparent rate constant determined in timed addition experiments with PDI showed that in the first seconds of proinsulin refolding very fast off-pathway reactions play an important role. These unproductive reactions are reduced by both...
the chaperone and isomerase activity. The chaperone function of PDI was important during the first seconds but not at later stages of refolding. In the absence of the chaperone activity, however, the isomerase function of PDI became essential to ensure an increased refolding yield. The isomerase function of PDI is acting during the whole refolding process although 10 min after initiation of refolding its effect was less apparent. From the kinetics of spontaneous proinsulin refolding we know that refolding was completed after about 30–60 min. This shows that folding and possibly isomerization occurred even 10–30 min after refolding started when aggregation was already completed. These late intermediates were not particularly susceptible to aggregation and PDI could catalyze these reactions but did not enable a significant increase in the yield of refolding of proinsulin. In the presence of PDI, aggregation as the major side reaction was suppressed although not completely even in stoichiometric concentrations. Under these conditions the proinsulin species protected from aggregation (about 40%) became folding competent and refolded completely as the refolding yield during catalyzed refolding was increased to about 50–55%. This indicates that if side reactions can be efficiently suppressed, either by accelerated isomerization or reduced aggregation, denatured and reduced proinsulin can form the native molecule. However, off-pathway reactions occurred very fast and obviously they cannot be reversed by PDI. This might be the major reason why we cannot force the proinsulin to refold quantitatively.

Acknowledgments—We are indebted to BIOBRA–S, Brazil, for the generous gift of the recombinant proinsulin and Roche, for the gift of the ELISA antibodies. Stefan Gleiter is acknowledged for stimulating discussions, Peter Neubauer for support, and Frank Hoffmann for critically reading the manuscript. We thank Lloyd W. Ruddock for providing PDI constructs, and Kevin Howland and Angelika Schierhorn for performing mass analysis.

REFERENCES
1. Freedman, R. B., and Klappa, P. (1999) in Molecular Chaperones and Protein Folding (Bukau, B., ed) pp. 437–459, Harwood Academic Press, London
2. Naiva, R. (1999) Semin. Cell Dev. Biol. 10, 481–493
3. Wang, C. C. (1999) Biochemistry (Mosc.) 63, 497–412
4. Kemmink, J., Darby, N. J., Dijkstra, K., Nilges, M., and Creighton, T. E. (1997) Curr. Biol. 7, 239–245
5. Kemmink, J., Darby, N. J., Dijkstra, K., Nilges, M., and Creighton, T. E. (1996) Biochemistry 35, 7684–7691
6. Darby, N. J., Penka, E., and Vincentelli, R. (1998) J. Mol. Biol. 276, 239–247
7. Klappa, P., Ruddock, L. W., Darby, N. J., and Freedman, R. B. (1998) EMBO J. 17, 927–935
8. Song, J. L., and Wang, C. C. (1995) Eur. J. Biochem. 231, 312–316
9. Cai, H., Wang, C. C., and Tsou, C. L. (1994) J. Biol. Chem. 269, 24550–24552
10. van den Berg, B., Chung, E. W., Robinson, C. V., Mateo, P. L., and Dobson, C. M. (1999) EMBO J. 18, 4794–4803
11. Yao, Y., Zhou, Y., and Wang, C. C. (1997) EMBO J. 16, 651–658
12. McClelland, D. A., McLaughlin, S. H., Freedman, R. B., and Price, N. C. (1995) Biochem. J. 311, 133–137
13. Lilie, H., McLaughlin, S., Freedman, R., and Buchner, J. (1994) J. Biol. Chem. 269, 14290–14296
14. Puig, A., and Gilbert, H. F. (1994) J. Biol. Chem. 269, 7764–7771
15. Tang, B., Zhang, S., and Yang, K. (1994) Biochem. J. 301, 17–20
16. Wang, C. C., and Tsou, C. L. (1991) Trends Biochem. Sci. 16, 279–281
17. Tang, J. G., Wang, C. C., and Tsou, C. L. (1988) Biochem. J. 255, 451–455
18. Quan, H., Fan, G., and Wang, C. C. (1995) J. Biol. Chem. 270, 17078–17080
19. Katayama, S., Tilt, E. A., and Lennarz, W. J. (2001) Biochim. Biophys. Acts 1548, 47–56
20. Zheng, J., and Gilbert, H. F. (2001) J. Biol. Chem. 276, 15747–15752
21. Weissman, J. S., and Kim, P. S. (1993) Nature 365, 185–188
22. Tsibris, J. C., Hunt, L. T., Ballejo, G., Barker, W. C., Toney, L. J., and Spellacy, W. N. (1989) J. Biol. Chem. 264, 13967–13970
23. Klappa, P., Freedman, R. B., Langenbuch, M., Lan, M. S., Robinson, G. K., and Ruddock, L. W. (2001) Biochem. J. 354, 553–559
24. Kemmler, W., Peterson, J. D., and Steiner, D. F. (1971) J. Biol. Chem. 246, 6786–6791
25. Winter, J., Neubauer, P., Glockshuber, R., and Rudolph, R. (2000) J. Biotechnol. 84, 175–185
26. Klappa, P., Hawkins, H. C., and Freedman, R. B. (1997) Eur. J. Biochem. 248, 37–42
27. Mayer, M., Kies, U., Kamermeier, R., and Buchner, J. (2000) J. Biol. Chem. 275, 29421–29425
28. Lundstrom, J., Krause, G., and Holmgren, A. (1992) J. Biol. Chem. 267, 9047–9052
29. Jeng, M. F., Campbell, A. P., Begley, T., Holmgren, A., Case, D. A., Wright, P. E., and Dyson, H. J. (1994) Structure 2, 853–868
30. Eklund, H., Cambillau, C., Sjoberg, B. M., Holmgren, A., Jornvall, H., Hoog, J. O., and Branden, C. I. (1984) EMBO J. 3, 1443–1449
31. Kiefhaber, T., Rudolph, R., Kohler, H., and Buchner, J. (1991) Bio/Technology 9, 825–829
32. Ganesh, C., Zaidi, N. P., Udgaonkar, J. B., and Varadarajan, R. (2001) Protein Sci. 10, 1635–1644
33. Zhang, N., Li, J., and Wang, C. (2000) J. Protein Chem. 19, 569–574
34. Zelikowsky, M. (1999) J. Biol. Chem. 274, 28083–28086
35. Li, W., and Churchich, J. E. (1997) Eur. J. Biochem. 246, 127–132
36. Diamant, S., Ben Zvi, A. P., Bukau, B., and Goloubinoff, P. (2000) J. Biol. Chem. 275, 21107–21113
37. Goloubinoff, P., Mogk, A., Zvi, A. P., Tomoyasu, T., and Bukau, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13732–13737
38. Veinger, L., Diamanti, S., Buchner, J., and Goloubinoff, P. (1998) J. Biol. Chem. 273, 11032–11037
39. Klappa, P., Koivunen, P., Pihlajaniemi, P., and Creighton, T. E. (1999) Biochemistry 38, 6007–6012
40. Hejnaes, K. R., Bayne, S., Norskov, L., Sorensen, H. H., Thomsen, J., Schaffer, L., Wallner, A., and Skriver, L. (1999) Protein Eng. 12, 797–806
41. Giss, Z. S., Gao, Z. Y., and Feng, Y. M. (2001) Biochemistry 40, 2662–2668
42. Yang, Y., Wu, J., and Watson, J. T. (1999) J. Biol. Chem. 274, 37598–37604
43. Milner, S. J., Carver, J. A., Ballard, F. J., and Francis, G. L. (1999) Biotechnol. Bioeng. 62, 693–703
44. Rosenfeld, R. D., Miller, J. A., Narhi, L. O., Hawkins, N., Katta, V., Lauren, S., Weiss, M. A., and Arakawa, T. (1997) Arch. Biochem. Biophys. 342, 298–305
Catalytic Activity and Chaperone Function of Human Protein-disulfide Isomerase Are Required for the Efficient Refolding of Proinsulin
Jeannette Winter, Peter Klappa, Robert B. Freedman, Hauke Lilie and Rainer Rudolph

J. Biol. Chem. 2002, 277:310-317.
doi: 10.1074/jbc.M107832200 originally published online November 1, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107832200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 21 of which can be accessed free at http://www.jbc.org/content/277/1/310.full.html#ref-list-1