Kinetic Analysis of the Mechanism and Specificity of Protein-disulfide Isomerase Using Fluorescence-quenched Peptides*

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Protein-disulfide isomerase (PDI) is an abundant folding catalyst in the endoplasmic reticulum of eukaryotic cells. PDI introduces disulfide bonds into newly synthesized proteins and catalyzes disulfide bond isomerizations. We have synthesized a library of disulfide-linked fluorescence-quenched peptides, individually linked to resin beads, for two purposes: 1) to probe PDI specificity, and 2) to identify simple, sensitive peptide substrates of PDI. Using this library, beads that became rapidly fluorescent by reduction by human PDI were selected. Amino acid sequencing of the bead-linked peptides revealed substantial similarities. Several of the peptides were synthesized in solution, and a quantitative characterization of pre-steady state kinetics was carried out. Interestingly, a greater than 10-fold difference in affinity toward PDI was seen for various substrates of identical length. As opposed to conventional PDI assays involving larger polypeptides, the starting material for this assay is homogenous. It is furthermore simple and highly sensitive (requires less than 0.5 μg of PDI/assay) and thus opens the possibility for quantitative determination of PDI activity and specificity.

One of the reactions associated with folding of secretory proteins is the formation and isomerization of disulfide bonds. This is a slow process when uncatalyzed, but takes place rapidly in vivo, where the enzyme protein-disulfide isomerase (PDI) facilitates the formation and rearrangement of disulfide bonds. PDI is a 57-kDa protein present in the endoplasmic reticulum (ER) of eukaryotes. The protein is organized in an abba'c domain structure where the a and b domains are structurally related to thioredoxin and the c domain is rich in acidic amino acid residues (1–4). The catalytic activity of PDI resides in the a and a' domains, where the catalytically active cysteine residues are found in a WCGHK motif in each domain. In each active site the two cysteines cycle between a reduced and an oxidized state, enabling the enzyme to exchange reducing equivalents with substrate sulfhydryls. PDI is capable of catalyzing several kinds of disulfide reactions: 1) oxidation reactions, in which the intramolecular disulfide bond of the CGHC motif is transferred to a pair of sulfhydryls in a substrate, 2) isomerization reactions in which disulfides are rearranged via the formation of a transient, mixed disulfide between the first cysteine residue of the CGHC motif and the substrate (5), and 3) reduction of mixed disulfides, in which PDI catalyzes the reductive cleavage of a disulfide bond (6–8).

PDI has been studied in detail in vitro with a variety of polypeptide substrates such as bovine pancreatic trypsin inhibitor, insulin, lysozyme, and ribonuclease A (RNase) (recently reviewed by Ruddon and Bedows (45) and Creighton (46)). The reactivation of “scrambled” RNase (sRNase) is the classical assay for measuring PDI activity (9). Oxidation of reduced RNase in vitro results in non-native pairing of the sulfhydryl groups, giving the inactive RNase derivative, sRNase. The conversion to the native protein requires the presence of a thiol reagent, and the process is catalyzed by PDI (9–11).

In most in vitro studies of PDI, activity has been investigated using a redox-buffer containing reduced and oxidized glutathione; GSH and GSSG, respectively. In the ER, the active site cysteine residue of PDI presumably reacts with endogenous glutathione, which is present in mM amounts with a [GSH]/[GSSG] ratio in the range 1–3 (12). Similarly, the redox state of PDI is also determined by the relative levels of GSH and GSSG in most in vitro assays. The likely mechanism of the catalysis of disulfide bond formation involves the transfer of an active site disulfide bond from PDI to the substrate via a PDI-substrate disulfide intermediate (2). PDI cycles between reduced and oxidized states and the re-oxidation of the reduced PDI by GSSG limits the overall reaction rate in vitro (2, 13). This suggests that GSSG is a poor substrate of PDI. New evidence suggests that the yeast ERO1 gene product is required for oxidation of protein thiols in the ER. Loss of Ero1p function results in the accumulation of proteins whose folding is dependent upon disulfide bond formation. Together, these observations argue that Ero1p in vivo may be directly responsible of reoxidation of PDI (14, 15).

Even though the nature of PDI and its substrate binding has been investigated in various ways (16–20), little is known about the specificity of PDI in catalysis of disulfide rearrangement and redox reactions. This, and the lack of small, well defined substrates with high affinity for PDI prompted us to carry out the present study. In analogy with substrates of proteolytic enzymes (21), a fluorescent and internally quenched disulfide-linked library of peptides (22) was used to identify a series of heptameric peptides that are extremely sensitive sub-

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§§ The abbreviations used are: PDI, protein-disulfide isomerase; hPDI, human PDI; PDIsh, reduced PDI; PDIi, oxidized PDI; ER, endoplasmic reticulum; Abz, a-amino benzoyle; Tyr(No2), 3-nitrotyrosine; sRNase, scrambled ribonuclease; DTT, dithiothreitol; PEGA, polyethylene glycol-poly- N,N-dimethyl acrylamide copolymer; VV0, the "α" after a sub-

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states of PDI. A surprisingly high degree of similarity was found in the amino acid sequences identified by screening the library of peptides on resin beads. Human PDI (hPDI) favored substrates containing the following features: small/helix breaker-cysteine-X-hydrophobic/basic-hydrophobic. A quantitative kinetic characterization was carried out by measuring the time-dependent change in fluorescence due to reduction of soluble substrates by hPDI. This analysis suggests a higher degree of specificity of hPDI than previously anticipated. The main difference between the substrates was seen for the dissociation constant, which showed a 10-fold difference. This indicates that the specificity of hPDI is primarily determined by the association between enzyme and substrate rather than catalytic effectiveness.

EXPERIMENTAL PROCEDURES

Purification of hPDI—The gene encoding hPDI was a cDNA clone isolated by K. Kivirikko (University of Oulu, Oulu, Finland). hPDI was purified from the Escherichia coli strain BL21(DE3) carrying the plasmid pET12a-PDI2 (23). Five hours after induction with isopropyl-1-thio-β-D-galactopyranoside at 30 °C, the cells were harvested and suspended in 50 mM Tris-HCl (pH 8), 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1 mM dithiothreitol (DTT). The cell suspension was sonicated 3 × 30 s/0.5 liter water. After centrifugation, ammonium sulfate was added to the supernatant to 25% saturation and centrifuged at 10000 g for 20 min. Additional ammonium sulfate was added to the supernatant to 100% saturation, and the precipitated protein was recovered by centrifugation. The pellet was dissolved in 50 mM Tris-HCl (pH 8), 1 mM EDTA, and the cleared extract was dialyzed against 50 mM Tris-HCl (pH 8), 1 mM EDTA. After dialysis, NaCl was added to the sample to a final concentration of 1 M and the sample was applied to an Octyl-Sepharose column equilibrated with 1 M NaCl in 50 mM Tris-HCl (pH 8), 1 mM EDTA. After a wash with 1 M NaCl in 50 mM Tris-HCl (pH 8), 1 mM EDTA, the protein was eluted in one fraction with 50 mM Tris-HCl (pH 8), 1 mM EDTA. This resulted in more than 80% pure hPDI estimated by SDS-polyacrylamide gel electrophoresis. The hPDI was further purified by applying the dialyzed sample to a Mono Q ion exchange column equilibrated with 50 mM Tris-HCl (pH 8), 1 mM EDTA and was subsequently eluted with a linear gradient from 0.1 to 0.5 M NaCl in the same buffer. The hPDI eluted at ~0.3 M NaCl and was at least 95% pure, estimated by SDS-polyacrylamide gel electrophoresis. The pooled fractions were dialyzed against 50 mM Tris-HCl (pH 8), 1 mM EDTA and stored frozen at ~80 °C until use. The concentration of hPDI was determined using an absorbance coefficient of 56399 cm−1 M−1 s−1 (23). The yield was approximately 5 mg/0.5 liter water. The hPDI used in the reduction assays was reduced by adding 10-fold molar excess of DTT. The sample was incubated in an argon atmosphere for at least 20 min at room temperature after the removal of the DTT on a NAP-5 column (Pharmacia Biotech Inc.) equilibrated with 50 mM Tris-HCl (pH 8.0) 1 mM EDTA. The total sulfhydryl content of hPDI was determined by Ellman’s reaction (24, 25). Between 3.3 and 3.9 free cysteines were found per molecule of hPDI.

Screening a Library of Internally Fluorescence-quenched Peptides for hPDI Substrates—Two peptides linked by an interchain disulfide bond, a fluorescent probe, and a quencher group were synthesized as described (22) and purified by semi-preparative reverse phase high performance liquid chromatography to more than 98% purity as determined by amino acid analysis. The substrate concentrations were determined by amino acid analysis, and confirmed by titration with reduced hPDI as follows: Reduced hPDI with known active site concentration, ε280 (approximately 6 mM), was added to a surplus of substrate (100–300 nM) and the increase in fluorescence was measured. When all the reducing equivalents from hPDI were transferred to the substrate, the change in intensity of fluorescence corresponding to the amount of reduced substrate was determined (ΔFred). Total reduction of the substrate was obtained by addition of DTT (10 mM final concentration) (ΔFtotal). The substrate concentration [S]0 is obtained from: [S]0 = ε280 ∆Fmax/ε280 ∆Ftotal. The amount of N,N-dimethylformamide and further diluted to 10 μM in water. The amount of N,N-dimethylformamide in the assay (<0.5%) did not affect the activity of hPDI (data not shown).

Kinetics of the hPDI-catalyzed Reduction of the Soluble Fluorescent Peptide Substrates—The substrate concentrations were in the range 10–500 nM, and the concentration of reduced hPDI was 3 nM. The buffer and substrate were mixed in the cuvette before the addition of reduced hPDI. The rate of reduction was determined by the increase in fluorescence as a function of time. In each experiment the reduction of the substrate was followed for at least 600 s. The background fluorescence signal caused by the buffer and the non-reduced substrate was small and constant during the time course of the assay. In all cases, Equation 1, described as “Results,” described a satisfying fit to the measured data. The concentration dependence of the kinetic parameters, kobs and Fmax, obtained from fits of Equation 1, using the program Kaleidagraph (Synergy Software Ltd.), were further analyzed. As expected, all Fmax values were similar (data not shown), whereas kobs showed a non-linear dependence on the concentrations.

RESULTS

Experimental Approach—To develop a simple quantitative assay for investigating hPDI activity and substrate preference, we tested the following simple notions: (a) Tyr(NO2)3 would quench the fluorescent probe Abz when these groups were placed in individual peptides linked by a disulfide bond, and (b) reduction of the disulfide bond would result in a significant

FIG. 1. The test substrate VW0. During reduction of the disulfide bond, the Tyr(NO2)3-containing peptide chain is separated from the Abz-containing peptide, resulting in an increase in fluorescence.

GSH, some beads particularly susceptible to uncatalyzed reduction became fluorescent and were removed and defined as false positive. The rest of the beads were incubated under the same conditions with catalytic amounts of hPDI (0.2 μM), and the beads that turned fluorescent within 1 h were collected. These beads were washed with DTT in order to completely reduce the substrate so only the random peptide chain remained attached to the bead. This peptide was re-coupled with the Tyr(NO2)3-containing peptide via a disulfide bond (22), and the procedure was repeated. Beads in the original screen that were not fluorescent after 3 days were also collected in order to identify poor substrates for hPDI. The amino acid sequence of peptides on individual beads was determined directly using a protein sequencer (model 470A) from Applied Biosystems. As a control, the peptides from two pools of 2000 beads were sequenced collectively. This showed, as expected, that all amino acids except cysteine were present at about equal amounts at each position in the library (data not shown), confirming that the library was random. Soluble peptide substrates were synthesized as described previously (22).

Fluorescence Measurements—the reduction of internally quenched disulfide-linked substrates was measured as a function of time using a Perkin-Elmer Luminescence Spectrometer LS50B with excitation at 320 nm and emission at 420 nm. The excitation and emission slit widths were 15 nm each. The assay was performed at 25 °C in a 3-ml 1 × 1 cm quartz cuvette from Hellma using a thermostated, stirred single-cell holder. Complete mixing of the sample was achieved in approximately 1 s. The buffer (50 mM Tris-HCl (pH 8), 1 mM EDTA) was filtered and purged with argon, and the assays were performed in an argon atmosphere in order to avoid uncontrolled re-oxidation.

Characterization of the Soluble Fluorescent Peptide Substrates—Soluble substrates were synthesized as described (22) and purified by semi-preparative reverse phase high performance liquid chromatography to more than 98% purity as determined by amino acid analysis. The substrate concentrations were determined by amino acid analysis, and confirmed by titration with reduced hPDI as follows: Reduced hPDI with known active site concentration, ε280 (approximately 6 mM), was added to a surplus of substrate (100–300 nM) and the increase in fluorescence was measured. When all the reducing equivalents from hPDI were transferred to the substrate, the change in intensity of fluorescence corresponding to the amount of reduced substrate was determined (ΔFred). Total reduction of the substrate was obtained by addition of DTT (10 mM final concentration) (ΔFtotal). The substrate concentration [S]0 is obtained from: [S]0 = ε280 ∆Fmax/ε280 ∆Ftotal. The amount of N,N-dimethylformamide and further diluted to 10 μM in water. The amount of N,N-dimethylformamide in the assay (<0.5%) did not affect the activity of hPDI (data not shown).

Kinetics of the hPDI-catalyzed Reduction of the Soluble Fluorescent Peptide Substrates—The substrate concentrations were in the range 10–500 nM, and the concentration of reduced hPDI was 3 nM. The buffer and substrate were mixed in the cuvette before the addition of reduced hPDI. The rate of reduction was determined by the increase in fluorescence as a function of time. In each experiment the reduction of the substrate was followed for at least 600 s. The background fluorescence signal caused by the buffer and the non-reduced substrate was small and constant during the time course of the assay. In all cases, Equation 1, described as “Results,” described a satisfying fit to the measured data. The concentration dependence of the kinetic parameters, kobs and Fmax, obtained from fits of Equation 1, using the program Kaleidagraph (Synergy Software Ltd.), were further analyzed. As expected, all Fmax values were similar (data not shown), whereas kobs showed a non-linear dependence on the concentrations.
increase in the fluorescence yield. A model substrate (VW0°), composed of two peptide chains linked by a disulfide bond, was synthesized as described in Ref. 22 (Fig. 1). We found that VW0° was readily reduced by DTT, giving a 20-fold increase in fluorescence. This indicated that the system could in principle be used as a sensitive tool to study the reduction of disulfide bonds. However, the model substrate was not easily reduced by hPDI and therefore not a sensitive substrate of hPDI.

Characterization of the Fluorescence-quenched Peptide Library—In order to identify a better substrate of hPDI, an immobilized fluorescence-quenched peptide library was generated by the split synthesis method to obtain one peptide compound per bead (28). Like the compound VW0°, each consisted of two peptides linked together by a disulfide bond: one, with the sequence Abz-Gly-Xaa-Cys-Xaa-Met, synthesized on a methionine residue linked to the resin bead. Xaa indicates an amino acid residue except cysteine, varying from bead to bead. The other peptide contained the sequence Ala-Tyr(NO2)-Cys-Ala-NH2 (22) (Fig. 2A). This relatively small peptide chain was chosen as the non-random quenching peptide chain because VW0°, containing a similar sequence, was an extremely poor substrate. This ensured that the hPDI specificity would be mainly dependent on the random amino acid chain.

Screening the Library for Suitable hPDI Substrates—Beads containing suitable substrates quickly showed fluorescence when treated with reduced hPDI (Fig. 2B). These beads were picked up under a stereo microscope, completely reduced, washed, and the amino acid sequence determined (Table I). Thirteen peptides were picked up as fluorescent beads within 1 h, 2 were collected the next day, and finally 2 peptides were picked up as being non-fluorescent after 3 days of incubation with reduced hPDI (Table I).

When we inspected the first 13 sequences in Table I, certain amino acid residues seemed to be over-represented at certain positions. The first substrates to fluoresce had an over-representation of helix-breakers (such as proline and glycine) and small amino acid residues at the C1 position.2 There is an even distribution between non-polar and polar residues, but only one basic amino acid residue, an Arg in VW20. The hydrophy index (30) is varying widely around zero, suggesting that it does not matter whether the amino acid residue is hydrophobic or not. Although there does not seem to be a preference for any particular amino acid at the C1 position, a hydrophobic residue is found in more than 70% of the sequences. This tendency is reflected in an average hydrophathy index of 1.3. At the C2 position, on the contrary, there is a preference for basic amino acid residues. Seven sequences, among the first group of substrates, contain an amino acid residue with a basic side chain.

2 To facilitate the discussion of substrate preference we wish to introduce a nomenclature similar to that of protease substrates; C1 and C1′ indicate the residues directly NH2- and COOH-proximal, respectively, to the cysteine residue engaged in disulfide bond formation. The subscripts indicate the distances of the residues from the cysteine residue.

Abz-containing peptide. B, the beads are non-fluorescent at the beginning of the screen due to the direct linkage of the two peptides. After addition of a reducing reagent, GSH, the easily reduced substrates become fluorescent because the disulfide bond is cleaved and the quencher and fluorescent chromophore are separated. Using a fluorescence microscope, the fluorescent beads were removed and hPDI was added to the remaining beads. Beads that became fluorescent were picked up. To ensure that these indeed were substrates for hPDI, the disulfide bonds were re-generated (22) and the screen repeated. Only beads positive in the second screen were collected and fully reduced by treatment with DTT, which left only the random part of the substrate coupled to the bead. Sequence was determined on a protein sequencer, and results are listed in Table I.

FIG. 2. The fluorescence-quenched peptide library containing an interchain disulfide bond. A, the library consists of a cysteine residue flanked by randomized amino acid residues at both sides and a fluorescent Abz group at the amino terminus. A peptide containing the quenching chromophore, Tyr(NO2), is coupled via a disulfide bond to the
whereas the remaining five all have a hydrophobic amino acid residue. The average hydropathy index is −1.4, indicating a tendency to favor polar side chains. There are five lysine residues present at the C$_2$ position, and the probability that this is a coincidence is less than 1%. The C$_3$ position is the only one in which amino acids containing acidic side chains are found, but the position is dominated by hydrophobic amino acid residues. However, because of the strongly hydrophilic amino acid residues, aspartate and glutamate, the average hydropathy index is only 1.2. Even though aromatic amino acid residues are present in some of the preferred substrates, it is striking that the two substrates that were not reduced after 3 days of incubation with reduced hPDI, VW30 and VW31, contain a large number of aromatic residues. These two substrates were, however, easily reduced and were fluorescent after treatment with DTT. The most plausible reason for the lack of hPDI-mediated reduction of these substrates is that the amino acid residues, containing fairly bulky side chains, cause steric hindrance, making the disulfide bond between the two peptides inaccessible to hPDI.

Kinetic Characterization of the Reduction of Substrates by hPDI—Screening of the library allowed the identification of good substrates, but it gave only a qualitative impression of substrate specificity. To obtain a more quantitative measure for the activity of hPDI, we synthesized selected substrates in soluble form as described previously (22). These disulfide-linked peptide substrates turned out to be extremely sensitive to reduction by reduced hPDI. The reaction of the substrates with DTT was investigated under pseudofirst-order conditions using various concentrations of DTT (100 - 500 μM) and a fixed concentration of substrate (40 nM). All substrates showed the same second order rate constant in the DTT reduction ($k_{DTT} = 5.3 ± 0.6$ s$^{-1}$ μM$^{-1}$) (data not shown).

Figs. 3 and 4 show the reduction of substrate by reduced hPDI in an increase in fluorescence plotted against time. The time course for reduction of substrate VW6 by 2.5–3.0 nM hPDI at three different substrate concentrations, 15, 75, and 430 nM (Fig. 3, panels A, B, and C, respectively), is shown. When looking at three different substrates, VW1, VW17, and VW19, all at 100 nM, it is clear that the reduction of the substrates by hPDI (3 nM) occurs at different rates (Fig. 4). Since the substrate concentration is in large excess as compared with enzyme concentration, we make the approximation that the substrate concentration is constant throughout the reaction. The reaction is therefore approximated to a pseudo-first-order. The solid lines shown in Figs. 3 and 4 represent fits to Equation 1.

$$F(t) = F_s (1 - \exp(-k_{obs} \cdot t))$$  (Eq. 1)

Here, $k_{obs}$ is the observed first-order rate constant, and $F(t)$ is the intensity of fluorescence at time $t$. $F_s(t)$ attains the value $F_s$ at the end of the reaction.

Since the reaction is approximated as pseudofirst-order, Equation 1 holds only for substrate concentrations much higher than enzyme concentrations. Nevertheless, employing Equation 1 to the data set resulted in a reasonable fit even at substrate concentrations only 5 times larger than enzyme concentrations (Fig. 3A).

Kinetic Analysis of the hPDI-mediated Reduction of Substrate—The usual kinetic parameters of a Michaelis-Menten equation do not apply to this system since hPDI is added in the reduced form and is oxidized upon reaction with substrate. The system is therefore not a classic enzyme-substrate system, but can be described by presteady-state kinetics.

The dependence of the first-order rate constant, $k_{obs}$, on the concentration of the substrate [S]$_0$ obtained by fitting the data to Equation 1 is shown in Fig. 5. The hyperbolic pattern of $k_{obs}$ with [S]$_0$ for most of the substrates is not in agreement with a simple one-step mechanism (Reaction 1), which would have implied a linear dependence of $k_{obs}$ on the substrate concentration, i.e. $k_{obs} = k_1 [S]_0 + k_0 − 1$ (31).

$$PDH_{SH} + A^{SS}B \rightleftharpoons PDH_{SH}^A + B^{SS}$$

**REACTION 1**

In the reaction schemes, the reduced hPDI is written as PDH$_{SH}$ and the substrate composed of two peptides linked via a disulfide bond (Abz-peptide$^{SS}$-peptide-Tyr(NO$_2$)) (Fig. 1) is symbolized by A$^{SS}$-B. The SH group in superscript of hPDI (PDH$_{SH}$) indicates the NH$_2$-proximal cysteine residue in one of the two CXXX motifs, whereas the PDH$_{SH}$ indicates the COOH-proximal cysteine residue of the active site. These two cysteine residues are involved in the formation of the internal disulfide bond of oxidized hPDI, whereas only the NH$_2$-proximal cysteine residue is.

### Table I

| Abz C$_1$ | Gly C$_2$ | Xaa C$_1'$ | Cys | Xaa C$_2'$ | Xaa C$_1''$ | Met C$_1''$ |
|-----------|-----------|------------|-----|-----------|-------------|------------|
| VW1$^a$  | Abz       | Gly        | Ser | Cys       | Thr         | Phe        | Ile        | Met        |
| VW3$^a$  | Abz       | Gly        | Ala | Cys       | Met         | Lys        | Val        | Met        |
| VW4$^a$  | Abz       | Gly        | Ile | Cys       | Ala         | Tyr        | Met        | Met        |
| VW6$^a$  | Abz       | Gly        | Ala | Cys       | Thr         | Met        | ?          | Met        |
| VW8$^a$  | Abz       | Gly        | Ser | Cys       | ?           | Lys        | ?          | Met        |
| VW10$^a$| Abz       | Gly        | Cys | Cys       | Met         | Ala        | Leu        | Met        |
| VW11$^a$| Abz       | Gly        | Pro | Cys       | Asn         | ?          | Asp        | Met        |
| VW18$^a$| Abz       | Gly        | Gly | Cys       | Leu         | Lys        | Met        | Met        |
| VW19$^a$| Abz       | Gly        | Pro | Cys       | Ile         | Lys        | Met        | Met        |
| VW20$^a$| Abz       | Gly        | Arg | Cys       | Met         | Met        | Glu        | Met        |
| VW21$^a$| Abz       | Gly        | Pro | Cys       | Ala         | Arg        | Ser        | Met        |
| VW23$^b$| Abz       | Gly        | Gly | Cys       | Phe         | Lys        | Pro        | Met        |
| VW24$^a$| Abz       | Gly        | Leu | Cys       | Pro         | His        | Leu        | Met        |
| VW17$^b$| Abz       | Gly        | Tyr | Cys       | Ser         | Gly        | His        | Met        |
| VW22$^b$| Abz       | Gly        | Ile | Cys       | Asn         | Ile        | Thr        | Met        |
| VW23$^b$| Abz       | Gly        | Leu | Cys       | Trp         | Trp        | Trp        | Met        |
| VW31$^b$| Abz       | Gly        | Tyr | Cys       | Phe         | Trp        | Met        | Met        |

$^a$The first 13 sequences were coupled to beads that were picked up within the first hour of incubation with reduced hPDI.

$^b$VW17 and VW22 were picked up the following day as fluorescent beads.

$^c$Beads containing VW30 and VW31 did not react upon treatment with reduced hPDI.

$^d$A question mark in the sequence indicates that the residue could not be identified during the sequencing.
teine residue is involved in the formation of mixed intermediates between hPDI and a substrate (5, 32).

Reaction 1 was rejected due to the hyperbolic pattern of \( k_{\text{obs}} \) with \([S]_0\) (Fig. 5). This pattern is, however, consistent with the two-step mechanism presented in Reaction 2.

\[
PDI_{\text{SH}}^{\text{SH}} + AS-B \xrightleftharpoons[k_{-1}]{k_1} PDISH_{\text{SH}}^{\text{SH}}A^{\text{SH}}B \xrightleftharpoons[k_{-2}]{k_2} PDISH_{\text{SH}}^{\text{SH}}A + B^{\text{SH}}
\]

**REACTION 2**

This involves an association of enzyme, PDISH \( ^{\text{SH}} \), and substrate, AS–SB, followed by a change in the first association complex when hPDI reduces the substrate, giving rise to an increase in fluorescence. The association step is characterized by the dissociation constant, \( K_1 \) (Equation 2), whereas the \( k_2 \) value represents the turnover number in this reaction.

\[
K_1 = \frac{k_{-1} [PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}B]}{k_1 [PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}B]} \quad (\text{Eq. 2})
\]

It is assumed that the first step in Reaction 2 is much faster than the second step; thus, only the second step is limiting and \( K_1 \) rapidly reaches equilibrium. Detection can occur only when the two peptides of the substrate are separated from each other. This occurs in the slower process when the mixed intermediate, PDISH \( ^{\text{SH}} \), is formed.

\[
\frac{d[PDISH_{\text{SH}}^{\text{SH}}]}{dt} = k_2 [PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}B] - k_2 [PDISH_{\text{SH}}^{\text{SH}}][B^{\text{SH}}] \quad (\text{Eq. 3})
\]

The concentration of the enzyme can be written as follows.

\[
[PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}B] + [PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}A] + [PDISH_{\text{SH}}^{\text{SH}}][B^{\text{SH}}] = [PDISH_{\text{SH}}^{\text{SH}}]_{t_0} - [PDISH_{\text{SH}}^{\text{SH}}]_0 \quad (\text{Eq. 4})
\]

Here, \([PDISH_{\text{SH}}^{\text{SH}}]_{t_0}\) is \([PDISH_{\text{SH}}^{\text{SH}}]_{t_0}\) at time 0. The substrate concentration, \([A^{\text{SH}}B]\), is much higher than the enzyme concentration and is assumed not to change during the experiment. Thus, \([A^{\text{SH}}B]_0 = [A^{\text{SH}}B]_0\).

Combining Equation 2 and Equation 4 leads to Equation 5.

\[
[PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}B] = [PDISH_{\text{SH}}^{\text{SH}}]_{t_0} - [PDISH_{\text{SH}}^{\text{SH}}]_0 \quad (1 + K_1[A^{\text{SH}}B]) \quad (\text{Eq. 5})
\]

Combining Equation 5 with Equation 2 gives Equation 6.

\[
\frac{d[PDISH_{\text{SH}}^{\text{SH}}]}{dt} = k_2 [PDISH_{\text{SH}}^{\text{SH}}][A^{\text{SH}}B] \quad (1 + K_1[A^{\text{SH}}B]) - k_2 [PDISH_{\text{SH}}^{\text{SH}}][B^{\text{SH}}] \quad (\text{Eq. 6})
\]

The \( k_2 \) values are determined as the intercept of the ordinate axis from a linear extrapolation at low substrate concentrations (Fig. 5) (31), and the values are less than \( 10^{-3} \text{s}^{-1} \) for all the substrates. Thus, the second term in Equation 6 can be ignored, and solving the resulting differential equation leads to the expression of the substrate concentration dependence of \( k_{\text{obs}} \).

\[
k_{\text{obs}} = \frac{k_2}{1 + (K_1[A^{\text{SH}}B])} \quad (\text{Eq. 7})
\]

The solid lines in Fig. 5 represent the results of fits using Equation 7. The hyperbolic pattern of \( k_{\text{obs}} \) with the substrate concentration (Fig. 5) is in agreement with Reaction 2 and Equation 7. Thus, the model shown in Reaction 2 can explain the increase in fluorescence caused by the reactions of reduced hPDI, PDISH \( ^{\text{SH}} \), with the substrate, AS–SB.
From Equation 7, the values of $K_1$, $k_2$, and $k_2/K_1$ were obtained (Table II). The ratio $k_2/K_1$ describes the catalytic efficiency. Its magnitude is correlated with specificity in the same way as $k_{cat}/K_m$ of steady-state kinetics. The ranking of the substrates listed in Table II is based on the $k_2/K_1$ value.

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PDI Specificity

The amino acid sequences of the peptides used in the soluble fluorescence assay

The substrates were synthesized as described (22). The quenching amino acid chain of the substrate, Ala-Tyr(NO2)-Cys-Ala, was coupled via a disulfide bond to the sequences listed above in Table I.

### Table II

The amino acid sequences of the peptides used in the soluble fluorescence assay

| VW1* | Abz Gly Ser Cys Thr Phe Ile Met Gly | 210 ± 30 | 0.41 ± 0.05 | 2.0 × 10^{-5} ± 5.10^{-5} |
| VW20* | Abz Gly Arg Cys Val Met Glu Met Gly | 270 ± 20 | 0.40 ± 0.01 | 1.5 × 10^{-5} ± 1.10^{-5} |
| VW6* | Abz Gly Ala Cys Thr Met Ile Met Gly | 560 ± 100 | 0.58 ± 0.06 | 1.1 × 10^{-5} ± 3.10^{-5} |
| VW10* | Abz Gly Gly Cys Met Ala Leu Met Gly | 560 ± 70 | 0.54 ± 0.04 | 9.9 × 10^{-5} ± 2.10^{-5} |
| VW3* | Abz Gly Ala Cys Met Lys Val Met Gly | 500 ± 90 | 0.36 ± 0.03 | 7.6 × 10^{-5} ± 2.30^{-5} |
| VW24* | Abz Gly Leu Cys Pro His Leu Met Gly | 1260 ± 250 | 0.86 ± 0.10 | 7.3 × 10^{-5} ± 2.10^{-5} |
| VW17* | Abz Gly Tyr Cys Ser Gly His Met Gly | 1250 ± 200 | 0.69 ± 0.08 | 5.8 × 10^{-5} ± 1.10^{-5} |
| VW23* | Abz Gly Gly Cys Phe Lys Pro Met Gly | 1650 ± 200 | 0.78 ± 0.07 | 4.9 × 10^{-5} ± 1.10^{-5} |
| VW19* | Abz Gly Pro Cys Ile Lys Leu Met Gly | 1900 ± 400 | 0.26 ± 0.04 | 1.5 × 10^{-5} ± 5.10^{-5} |
| VW21* | Abz Gly Pro Cys Ala Arg Ser Met Gly | 2000 ± 700 | 0.21 ± 0.06 | 1.3 × 10^{-5} ± 7.10^{-5} |
| VW60* | Abz Ala Cys Ala | <10^{-8} |

* The quenching peptide of substrate VW0* was, however, Cys-Ala-Tyr(NO2)-Ala-NH2. This substrate was not picked up when testing the library. The kinetic parameters are calculated from the fits shown in Fig. 5 and Equation 7.

### DISCUSSION

The complexity of PDI and the reactions that it catalyzes have hampered its characterization and quantitative analysis, but PDI has been investigated using a variety of substrates (5, 33). The most widely used assay for analyzing PDI activity has been the reactivation of scrambled RNase, which requires the formation of native disulfide bridges via an unknown number of individual thiol/disulfide steps. The assay is time-consuming, and, because of the heterogeneous starting material, it is not very reproducible. We wished to investigate the activity of PDI using a more quantitative assay. Internally quenched fluorescent peptides have been used extensively as substrates to characterize proteases (34). We employ them here as a new (22) quantitative and highly sensitive tool for the analysis of PDI activity. Screening a library of internally quenched peptides we find certain substrate specificities. There is a preference toward sequences containing a helix breaker/small residue at the C1 position, a basic residue at the C2 position, and a hydrophobic one at C3 (Table I). The C1 position seems not to be subject to a restriction in the amino acid residue. Substrates that had not reacted after 3 days of incubation with reduced hPDI contained many bulky aromatic amino acid residues. Much the same substrate specificity is found in another ER chaperone, the heat-shock protein BiP. Aliphatic side chains are preferred throughout the binding site of BiP. However, aromatic residues, such as phenylalanine and tyrosine, are only tolerated at certain positions, presumably due to steric hindrance (35).

A quantitative analysis of the reduction of a number of peptides was performed. The proposed reaction of reduced PDI with peptide is shown in Reaction 2. The values of kobs obtained from fits using Equation 1 were analyzed according to this model. The data set fit the equation very well, but the deviation for the poorer substrates, VW19* and VW21*, is greater, which is expected since the K1 is far from the substrate concentrations used.

There are some limitations in our understanding of the reactions involved. The enzyme hPDI contains two active sites and nothing is known about the relative specific activities of the two active sites toward these substrates. The observation that there is indeed a reasonable fit according to Reaction 2 would suggest either that the reaction is carried out primarily by one of the active sites, or that reactivity of the two active sites is very similar. A distinction between these two situations must await further analysis involving mutant forms of PDI. Also, we do not know to what extent the mixed disulfide between PDI and the short peptide (Ala-Tyr(NO2)-Cys-Ala) is formed. However, this mechanistic detail does not affect the K1 value. The very slow reduction of VW0* would suggest that the contribution of the short peptide to binding is minimal. Thus, we assume that it is the long peptide containing the randomized amino acid residues that are primarily responsible for the differences in PDI activity between the individual substrates. There is a formal possibility that the reduction of substrate takes place but that two peptides are still bound to PDI at a proximity that would result in quenching. We do, however, find such a slow release unlikely, a view that is supported by the fact that the model presented fits well with the observed kinetics.

It is worth noticing that K1 and k2 are crucial parameters in the initial steps of an isomerization event in which a protein substrate with incorrect disulfide bonds is attacked by reduced PDI to form a mixed intermediate as outlined in Scheme I.

For many proteins, disulfide rearrangement is required to activate the native structure (36–38). The rate of mixed intermediate I formation is probably rate-limiting in many instances, and the rate of this step is completely defined by the catalytic parameters identified in the present analysis.

The dependence of the first-order rate constant, kobs, on the concentration of the substrates, [S]0 (Fig. 5), is in agreement with a mechanism containing at least two steps as shown in Reaction 2. The presteady-state kinetic parameters obtained from measurements of the increase in fluorescence using Equation 7 are listed in Table II. They show a factor of 15 between the best and the poorest of the identified heptameric substrates. When comparing the rate constants for substrates in solution with the time at which a bead lit up in the screen, the reaction on the beads was generally much slower as compared with the reaction in solution. There are clearly many aspects of diffusion and steric factors which distinguish the two situations and which may change the rate of reduction. However, the first bead which was picked up in the screen (VW1) contains the same sequence as the best substrate in solution (VW1*).

The number of human proteins for which the disulfide bonding pattern is known is fairly limited. Furthermore, the in vitro formation of any given disulfide bond may or may not require PDI. It is therefore difficult directly to compare the sequences identified in the present analysis with possible in vivo substrates. Nevertheless, when looking at the C1 position of the substrates, the majority contain a relatively small amino acid residue. In fact, many human disulfide-bond-containing proteins of the secretory pathway have a small amino acid residue just NH2-terminal to the disulfide bonds. Even though we have a few data to support this, it is striking that the substrates with a specificity constant of 1–2 × 10^5 s^{-1} M^{-1} all have a hydrophobic amino acid residue at the C2 position (VW1*,
C2 be interpreted in much the same way. Thus, the main differ-

gest that cysteine residues contribute significantly to the in-

activity than peptides without cysteine residues (17). This sug-

With PDI are not due to differences in susceptibility of the

shown to play an important role in the initial binding of pep-

etaproteins, is more than 100 fold less efficient as a substrate

length (17).

Correlates with inhibition of PDI appears to be the peptide

For non-cysteine-containing peptides, the major factor that

lyzes the reduction of insulin by glutathione, a reaction inhib-

VW20s, VW6s, and VW10s), whereas the rest of the substrates

sequences of the following residues: small/helix-

make the interpretations of the substrate specificity more com-

various amino acid residues at the various positions, which

Peptides with cysteine residues are not due to differences in susceptibility of the

PDI and the substrates are used in nanomolar concentrations, and the assay is accurate and fast. The system described in the present study contains only enzyme and substrate, and the measured activity is not a function of the re-reduction of the enzyme in a redox buffer that often becomes rate-limiting in other systems. This makes the assay a suitable tool for analysis of various forms of PDI and PDI homologs. Furthermore, the results presented in this study naturally open the possibility

PDI Specificity

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