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COMPREHENSIVE INVITED REVIEW

Protein Disulfide Isomerase: A Critical Evaluation of Its Function in Disulfide Bond Formation

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Abstract

Disulfide bond formation is probably involved in the biogenesis of approximately one third of human proteins. A central player in this essential process is protein disulfide isomerase or PDI. PDI was the first protein-folding catalyst reported. However, despite more than four decades of study, we still do not understand much about its physiological mechanisms of action. This review examines the published literature with a critical eye. This review aims to (a) provide background on the chemistry of disulfide bond formation and rearrangement, including the concept of reduction potential, before examining the structure of PDI; (b) detail the thiol-disulfide exchange reactions that are catalyzed by PDI *in vitro*, including a critical examination of the assays used to

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determine them; (c) examine oxidation and reduction of PDI *in vivo*, including not only the role of ERo1 but also an extensive assessment of the role of glutathione, as well as other systems, such as peroxide, dehydroascorbate, and a discussion of vitamin K–based systems; (d) consider the *in vivo* reactions of PDI and the determination and implications of the redox state of PDI *in vivo*; and (e) discuss other human and yeast PDI-family members. *Antioxid. Redox Signal.* 11, 2807–2850.

I. Introduction

Disulfide bonds are covalent linkages formed between the side chains of cysteine residues. Their usual function is to stabilize protein structures, although they may also play other roles, including redox regulation of enzymatic activity. Disulfide bond formation is one of the key rate-limiting steps in protein folding. For example, the *in vitro* refolding of lysozyme, a monomeric protein of 129 amino acids that contains four disulfide bonds in the native state, has been extensively studied. Refolding from the oxidized state (*i.e.*, with all four disulfide bonds in their native state) is rapid. The α-domain of lysozyme folds first, with the formation of the β-domain being rate limiting with a half-time of $\sim$350 ms; folding is essentially complete for the majority of oxidized lysozyme within 1 s (see ref. 200 for a review). In contrast, noncatalyzed folding from the reduced state (*i.e.*, with no disulfide bonds) is much slower. Even in an optimal glutathione redox buffer, it takes $\sim$2 h to regain 50% lysozyme activity (308). The slow kinetics of noncatalyzed disulfide bond formation provides a strong imperative for the evolution of catalytic mechanisms to enhance these rates.

In several compartments, catalyzed disulfide bond formation occurs, including the endoplasmic reticulum (ER), mitochondria (118, 172), and the bacterial periplasm (141). Although many similarities appear between disulfide bond formation in these different compartments, many differences are noted, and this review focuses only on disulfide formation in the ER, the cellular compartment in which protein disulfide isomerase (PDI) resides.

PDI was the first folding catalyst ever reported (101, 316). It is an ER-located protein with a classic KDEL-ER retrieval motif (234) and is present in the ER (177) in high concentrations. It is a multifunctional protein, being able to catalyze disulfide bond formation, breakage, and rearrangement in all nonnative protein and peptide substrates reported to date. In addition to its role in disulfide bond formation, PDI is the β-subunit of prolyl-4-hydroxylase (161) and microsomal triglyceride transfer protein (336). PDI is also implicated in peptide loading onto MHC class I (232), and in regulating NAD(P)H oxidase (133) in the ER. It has also been reported to be involved in a wide range of other biological functions in nearly every cellular compartment (305), although it is unclear for many of these reactions how it escapes the ER. Recently, a number of reports indicated that PDI is, in effect, a marker for the release of intracellular contents from damaged cells (for example, activating tissue factor *via* catalysis of thiol-disulfide exchange and thus initiating the blood-clotting cascade at the site of wound damage) (253). This review focuses only on its role in disulfide bond formation in the ER.

After 30 years of trials, structural studies on full-length PDI-family members are starting to bear fruit with two crystal structures of human PDI-family members, two full-length yeast PDI-family members, as well as the crystal and NMR structures of the **b**′ domain of human PDI all having been published in the space of just a few months (16, 60, 66, 221, 297, 326). Undoubtedly this will trigger a new wave of studies into structure–function relations of the PDI family. This is also combined with new discoveries and ideas on physiological mechanisms of action over the past decade. This is an exciting time in the PDI field. However, more than four decades of studies exist on PDI. New research must build on what is already known and not reinvent the wheel. In addition, the many years of study means that a considerable amount of baggage is associated with the field, of misinterpretations that have been or can be corrected in light of subsequent articles, and of the use of assays that, although at the cutting edge at their time of conception, have now grown long in the tooth. Hindsight is a wonderful thing; it enables us to look back and critically reevaluate preceding data, to enable us to make sensible decisions on the best way to go forward from here. We should begin with the identification and initial characterization of PDI.

II. Identification and Initial Characterization of PDI

The first identification of PDI-like activity was independently published by two groups in 1963. The group of Brunó Straub (315, 316), later president of Hungary, found that extracts from both pigeon and chicken pancreas were able to stimulate the reoxidation of reduced ribonuclease. In parallel, Anfinsen and co-workers (101), as part of the work on ribonuclease that earned Anfinsen the 1972 Nobel Prize in Chemistry with Moore and Stein, detailed studies showing acceleration of reactivation of ribonuclease by a microsomal system from rat liver. Anfinsen's group subsequently partially purified the enzyme responsible (99, 100, 102) and showed that it was able to catalyze thiol-disulfide exchange reactions. The material they obtained contained several protein components and was "a faint yellow" (99). In addition, a low-molecular-weight component that was essential for activity and was lost during dialysis could be replaced by the addition of FAD (101). These results predate the discovery and characterization of the FAD-cofactor containing enzyme Ero1, which works with PDI, by three decades (see Section VIII). Subsequently, the "sulfhydryl-disulfide exchange enzyme" was purified to near homogeneity from beef liver (57) and was found to contain at least one disulfide bond that was sensitive to reduction by dithiothreitol (92). PDI was given the enzyme classification number EC 5.3.4.1 in 1972, along with its subsequent officially accepted name, a name that was used for the first time in a publication in 1975 (113).

For 20 years, the significance of PDI was not widely recognized, nor were its activities well defined. However, other proteins were identified as substrates of PDI *in vitro*, including soybean trypsin inhibitor (284), insulin (313, 314),

immunoglobulins (58, 217, 256), vasopressin and oxytocin (314), bovine serum albumin (295), cholera toxin (216), bovine pancreatic trypsin inhibitor (BPTI) (43), ricin (17), and procollagen (82). In addition, the subcellular localization of PDI to the ER was confirmed (177). Furthermore, its detailed action in catalyzing a specific folding pathway was defined (44), and confusion between PDI (EC 5.3.4.1) activity and glutathione-insulin transhydrogenase (EC 1.8.4.2) activity was resolved with the finding that PDI is one of the cellular enzymes that has glutathione-insulin-*trans*-hydrogenase activity (25, 120).

The major breakthroughs in the study of PDI came in the mid-1980s with the publication of the first highly cited review specifically on the enzyme (87), which raised awareness of the enzyme, and the publication of the sequence of rat PDI (70). This sequence predicted two regions with a high degree of homology to thioredoxin, a small cytoplasmic enzyme involved in thiol-dependent redox reactions (see refs. 93, 180, and 182 for recent reviews of different aspects of thioredoxin). In particular, it showed that, as previously postulated (121), the active sites of PDI contain vicinal thiol groups, in particular, the sequence WCGHC, which changes between the dithiol and disulfide states during the catalytic cycle of PDI.

Before discussing the role of the active-site cysteines and the structure of PDI in its mechanisms of action, we must look first at the chemistry of disulfide bond formation.

III. The Chemistry of Disulfide-Bond Formation and Rearrangement

The chemistry of disulfide bond formation is the chemistry of redox reactions and of thiolates. Redox reactions, or reduction–oxidation reactions, are, as the name suggests, reactions in which one chemical species attains a higher oxidation state through the loss of electrons or gain of oxygen, while another is reduced, *i.e.*, attains a lower oxidation state, through the gain of electrons or a loss of oxygen. For disulfide bond formation, the starting species is the thiol group found on the side chain of cysteine residues in proteins. The thiol group represents the −2 oxidation state of the sulfur atom. When two thiol groups are oxidized to form a disulfide, both of the sulfur atoms involved attain the −1 oxidation state, and two electrons and two protons must be transferred to the oxidant. The simplest oxidant that can be used to form a disulfide bond is molecular oxygen, which, in the process of forming the disulfide, is itself reduced to hydrogen peroxide (see Fig. 1A). The formation of disulfide bonds by using molecular oxygen as the oxidant is a thermodynamically favored process, and so it occurs spontaneously, as long as the two thiol groups can be juxtaposed. However, this oxidation process is kinetically slow, but it is used, in combination with metal ion catalysis, *in vitro* for the refolding of proteins produced as insoluble inclusion bodies (73). This reaction also mirrors what happens, at least in part, *in vivo*. However, *in vivo*, the reaction is catalyzed by enzymes known as sulfhydryl oxidases, including the ER-resident protein Ero1 (see Section VIII). Other oxidants can also be used to form disulfides, including reactive oxygen species (ROS) (for example, hydrogen peroxide, and other species such as dehydroascorbate, the oxidized form of vitamin C) (see Section VIII). Another category of oxidants is proteins, for example, PDI, peptides, for example, oxidized glutathione (GSSG), and other molecular species, for example, dithionitrobenzoate, which contain a disulfide bond and transfer this to a folding protein, becoming themselves reduced to the dithiol state in the process (see Fig. 1B). PDI is able to act in such a way as an oxidant of protein dithiols *in vivo* and *in vitro* (see Sections VI and VIII). In addition to the disulfide state, other oxidized forms of cysteine thiol groups can and do exist *in vitro* and *in vivo*. These include cysteine sulfenic acid (−SOH, oxidation state 0), cysteine sulfinic acid ($-SO_2H$, oxidation state +2), and cysteine sulfonic acid ($-SO_3H$, oxidation state +4). All of these higher oxidation states are reached by the reaction of ROS with cysteine thiols. Cysteine sulfenic acid has a number of biological functions. For example, it is used in intracellular signaling as well as being an intermediate in some enzymatic reactions (38, 240). It is also a potential intermediate in disulfide bond formation in the ER (see Section VIII). In proteins, cysteine sulfinic acid has no reported function. It is thought to be a nonfunctional product caused by the action of ROS on reactive cysteines and requires the action of sulfiredoxin to reduce back it to the free cysteine thiol (24). Cysteine sulfinic acid is, however, an important cellular metabolite lying at the branch point between the formation of sulfate and taurine in cysteine metabolism (285). It has also been reported to be an agonist of various cellular receptors (for example, see ref. 154). In contrast to these oxidation states of cysteine, cysteine sulfonic acid has no known biological functions.

Any two cysteine residues in a protein have the potential to form a disulfide bond. With two cysteines in a protein, only one intramolecular disulfide can be formed; with three cysteines, three different disulfides could be formed; with four cysteines, six different disulfides, *etc*. Because the protein could have none, some, or all of its cysteines in disulfide bonds, this gives 10 different intramolecular thiol/disulfide redox states for a protein with just four cysteine residues and >13,000 different intramolecular redox states for a protein with 10 cysteine residues. Usually a protein will have only one pattern of disulfide bonds and free thiols in the native state (unless it is involved in redox processes *via* thiol chemistry, such as PDI, or it has redox-regulated activity).

How does a protein attain its native disulfide state? Some proteins [for example, bovine pancreatic trypsin inhibitor

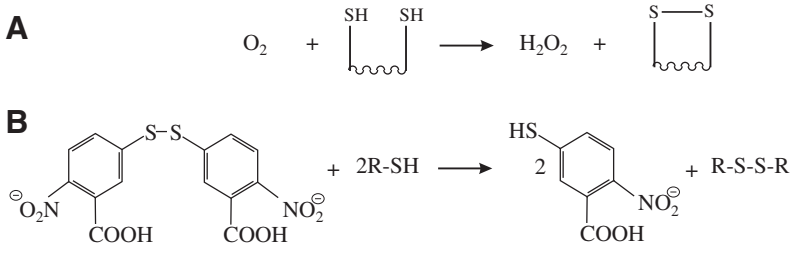

FIG. 1. Schematic representation of the formation of a disulfide bond from two cysteine thiol groups. (**A**) Molecular oxygen as the electron acceptor, which results in the formation of hydrogen peroxide. (**B**) Ellman reagent (dithionitrobenzoate) as the electron acceptor.

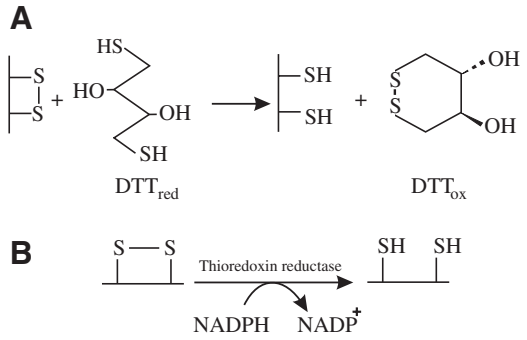

FIG. 2. Schematic representation of the reduction of a disulfide bond to form two cysteine thiol groups. (**A**) DTT as the electron donor. (**B**) NADPH as the electron donor with thioredoxin reductase as the catalyst.

(BPTI)] appear to proceed *via* a folding pathway where predominantly native disulfide bonds are formed in the folding protein (331). This is presumably linked to the formation of regular secondary structure, which limits the juxtaposition of cysteine residues in the folding intermediates to native-like combinations. However, many proteins presumably form both native and nonnative disulfide bonds during oxidation, and processes are necessary to convert the nonnative disulfide bonds into native ones. This can occur *via* two distinct pathways: (a) cycles of reduction–oxidation, and (b) direct isomerization. PDI is able to catalyze both of these pathways (see Section VI). Cycles of reduction–oxidation are as described. First, the incorrect nonnative disulfide bonds are reduced back to the dithiol state, and then a second oxidation step occurs, as described earlier. The reduction of nonnative disulfide bonds can be done by a variety of reducing agents, which themselves become oxidized in the redox reaction. For example, *in vitro*, strong chemical reductants can be used, such as sodium borohydride. *In vivo,* disulfide bond reductants tend to be based on thiol chemistry [for example, reduced glutathione (GSH), equivalent to the use of β-mercaptoethanol or dithiothreitol (DTT) *in vitro* (see Fig. 2A)] or linked to NAD(P) or FAD redox cycles [for example, thioredoxin reductase (see Fig. 2B; 218) or glutathione reductase (11)]. In contrast to the two-step reduction–oxidation cycle, intramolecular isomerization of disulfide bonds is a one-step process based on nucleophilic attack by a free thiol group on a disulfide bond to form a new disulfide and releasing a new thiol group (see Fig. 3A). Intermolecular isomerization is similarly based on nucleophilic attack by a free thiol group on a disulfide bond to form a new disulfide, but this time, a transient mixed disulfide is formed, which in turn undergoes nucleophilic attack by another free thiol group (see Fig. 3B). In intra- or intermolecular isomerization reactions, no net changes occur in the number of disulfide bonds or free thiol groups at any stage of the reaction. Note that this is also true of oxidation reactions, in which a disulfide bond–containing species (*e.g.*, GSSG or PDI) transfers its disulfide bond to the folding protein (see earlier). The same overall reaction can be called an oxidation reaction or an isomerization reaction, depending on the viewpoint used. Usually the net effect on the folding protein is used to determine which terminology to adopt, but this can cause confusion, for example, when examining *in vivo* routes for oxidation of PDI (see Section VIII).

Although we said earlier that isomerization is based on a nucleophilic attack by a thiol group on a disulfide bond, this is not usually the case in practice. Whereas thiol groups are nucleophiles, thiolate anions (*i.e.*, the $-S^-$ species) are much more potent nucleophiles. Given this, it is important to consider factors that change the ionization state of cysteine thiols (*i.e.*, factors that modulate the pK_a of these groups). The pK_a of a chemical moiety is defined as the pH at which 50% of the species is in the protonated state, and so 50% is in the deprotonated state (see Fig. 4A). The proportion of the moiety in the protonated/deprotonated state at any pH can be linked to the pK_a value by the Henderson–Hasselbalch equation. The typical pK_a of cysteine thiols in proteins is often cited as being around 8.3; however, considerable variations occur in this depending on the local microenvironment of the thiol group. Because buried unpaired charged groups are thermodynamically unfavorable, a buried cysteine side chain would be relatively more stable in the thiol form, and so the pK_a would be higher than average. Local electrostatics also play a major role in modulating pK_a values. For example, a positive charge adjacent to the thiol group would provide a stabilizing electrostatic attraction between this charged species and the deprotonated thiolate state, stabilizing the thiolate form and lowering the pK_a value for the cysteine (see Fig. 4B). Similarly, adjacent negative charges would result in electrostatic repulsion with the thiolate and would destabilize its formation, resulting in an increase in pK_a. The pK_a of the thiol group in GSH is 8.75, higher than normal value cited for cysteine thiol groups in proteins and peptides because of the local negative charge density on this tripeptide.

The kinetics of noncatalyzed folding pathways of disulfide bond–containing proteins are probably determined by a combination of the pK_a values of individual cysteines in the folding protein, along with accessibility and the constraints put on the juxtaposition of pairs of cysteine residues by the formation of regular secondary structural elements. Because the pK_a values of the cysteine residues will depend on the conformation of the folding intermediates, which cannot

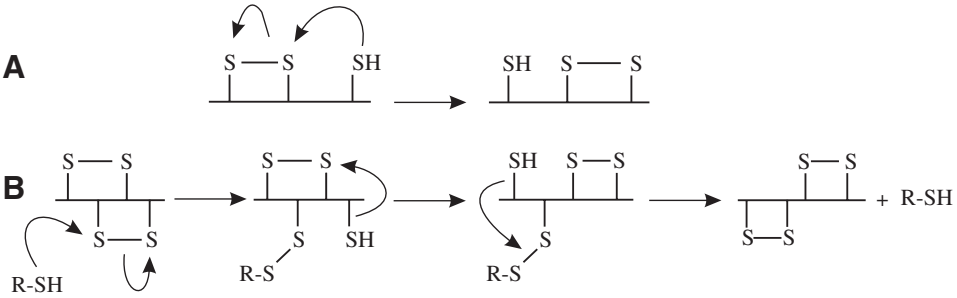

FIG. 3. Schematic representation of disulfide isomerization. (**A**) Intramolecular rearrangement of a three-cysteine system containing one disulfide bond. (**B**) Rearrangement of a four-cysteine system containing two disulfide bonds based on the transient formation of an intermolecular mixed disulfide.

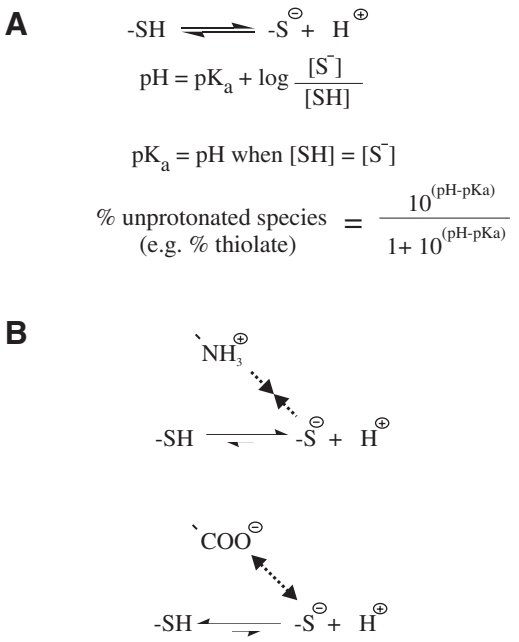

FIG. 4. The pK_a of cysteine thiol groups. (**A**) Schematic representation of the dissociation process. (**B**) Modulation of the pK_a of a thiol group by adjacent charged groups. A positively charged group makes favorable interactions with the thiolate state, stabilizing it and reducing the pK_a of the cysteine side chain. In contrast, an adjacent negatively charged group has electrostatic repulsion with the thiolate group, resulting in an increase in the pK_a of the cysteine.

easily be determined, this cannot be easily verified. However, it is noteworthy that the nonnative disulfide bonds observed by Creighton and co-workers (43) in the refolding pathway of BPTI (43) all contain cysteine residues with adjacent positively charged residues (Lys15 adjacent to Cys14, and Arg39 adjacent to Cys 38). A low pK_a value for these cysteines may be responsible for the rapid intramolecular rearrangements that occurred during quenching (see Section VI).

Because PDI is a catalyst of thiol-disulfide isomerization, we would expect the pK_a of at least one of its reactive thiols to be lower than average to increase its nucleophilicity for the first step of intermolecular-based thiol-disulfide isomerization. This is indeed the case, with the pK_a of the N-terminal active-site cysteine being <5.5 (see Section V). The fundamental basis of the structure of the catalytic domains of PDI seems to be attaining the correct pK_a values for the two active-site cysteines at different steps of the catalytic process (see Sections V and VII). However, before we look at PDI itself, we must consider first one more important aspect of thiol-disulfide chemistry, reduction potential.

IV. The Reduction Potential of PDI

The term "redox potential" is used variably in the field of catalyzed thiol-disulfide exchange. The term itself is imprecise, with reduction potential (E_r^0) or oxidation potential (E_o^0) (the tendency of a chemical species to either gain or lose electrons when it is subject to change by the addition of another chemical species) being the more-accurate terms. A chemical species with a higher reduction potential will have a tendency to gain electrons from a species with a lower reduction potential (*i.e.*, to become reduced by oxidizing the lower-reduction-potential species). Reduction potentials are measured in volts, or more usually for biochemical systems in millivolts, defined relative to a standard hydrogen electrode. More usually, the standard reduction potential (E_r^0) rather than the reduction potential is reported. This is the reduction potential under standard temperature, pressure, and concentration. However, the standard reduction potential implies that $[H^+] = 1.0\,M$ (equivalent to pH 0), which is clearly inappropriate for biological systems. Therefore, the most commonly used system in biochemistry is the biochemical standard reduction potential ($E_r^{0\prime}$, often written just as $E^{0\prime}$), which is defined at $[H^+] = 10^{-7}\,M$ (*i.e.*, pH 7.0). In the rest of this review, when we use the phrase "reduction potential," we will mean the biochemical standard reduction potential. Measuring $E_r^{0\prime}$ is problematic; for many biochemical systems, the reduction potential is measured indirectly by measuring the relative reduction potential against a species with defined $E_r^{0\prime}$. Usually in the case of thiol-disulfide exchange systems, the relative reduction potential is defined against glutathione (see Fig. 5A; for example, see refs. 103, 111, and 129). However, where the protein thiol-glutathione mixed disulfide is a longer-lasting species, direct protein–protein redox equilibria (see Fig. 5B) (346) is a more accurate method to determine relative reduction potential. The currently accepted reduction potentials of various species, including a range of thioredoxin-superfamily members, such as PDI, are shown in Fig. 6.

A number of problems and misunderstandings are associated with the reduction potential of PDI. First, because reduction potentials are negative numbers for thiol-disulfide exchange reactions, the terminology can become confusing.

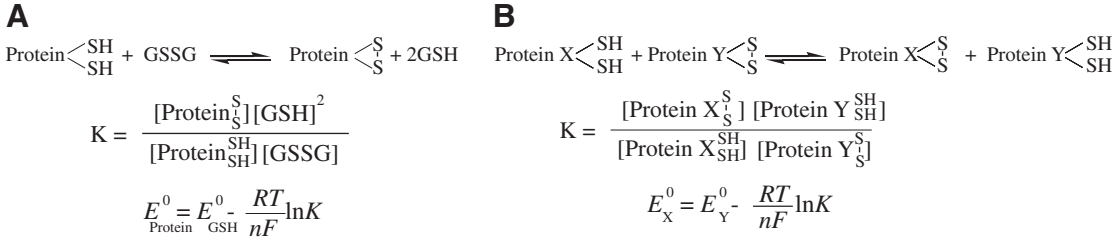

FIG. 5. Schematic representation of the determination of thiol-disulfide reduction potential. (**A**) With glutathione. (**B**) With protein–protein redox equilibria. In both cases, the relative proportions of reduced and oxidized material can be determined by any appropriate biophysical method (for example, reverse-phase HPLC).

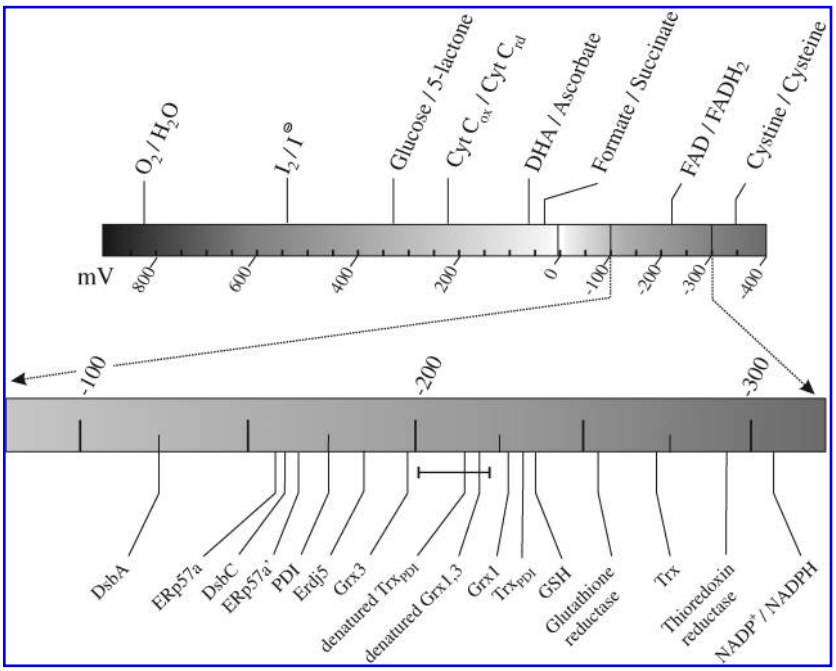

FIG. 6. Biochemical standard reduction potentials, including thioredoxin-superfamily members (adapted from ref. 346). Trx_{PDI} refers to thioredoxin with the active site mutated to CGHC. The horizontal bar represents the range of values cited for denatured proteins and unstructured peptides (31, 111, 129, 168, 186, 189, 249, 254, 275, 346).

For example, a species with a reduction potential of −180 mV (*e.g.*, PDI) (189) has a higher reduction potential than a species with a reduction potential of around −220 mV (*e.g.*, denatured proteins and peptides) (for example, see refs. 275 and 346) and so will be reduced by it. In addition, when talking about the reduction potential of a protein oxidant such as PDI, it can be easy to become confused about which reaction is being discussed. Combining these two issues, PDI ($E_r^{0\prime} = -180$ mV) has a higher reducing potential than denatured proteins ($E_r^{0\prime} \approx -220$ mV), which means it will be preferentially reduced, and the equilibrium shown later will tend to the right (*i.e.*, the higher reducing potential of PDI implies that it will act as a protein dithiol-disulfide oxidant toward the denatured protein).

$$\mathrm{PDI_{disulfide}} + \text{denatured protein}_{\mathrm{dithiol}}$$
$$\updownarrow$$
$$\mathrm{PDI_{dithiol}} + \text{denatured protein}_{\mathrm{disulfide}}$$

Second, the reduction potential is a thermodynamic property; it measures the potential direction of the system, but does not tell us anything about the kinetics of the system. The reduction potential of NADPH is −315 mV, whereas that of glutathione is −240 mV (254, 346). This implies that, based on the thermodynamics of the system, NADPH should spontaneously reduce GSSG. However, the reaction is kinetically very slow unless catalyzed, for example, by glutathione reductase. Two enzymes that catalyze thiol-disulfide exchange reactions may have the same reduction potential but have very different kinetics or specificities or both.

Third, the reduction potential of a species cannot be seen in isolation. By itself, the number is meaningless; it must always be seen in the context of reaction with another species (*i.e.*, a comparison of reduction potentials). Reaction schemes are often erroneously simplified to consider only two species, ignoring contributions from other species present. *In vivo*, the reduction potential of the cytoplasm for thiol-disulfide exchange reactions is primarily defined by the major redox species present, reduced glutathione (and the NADPH produced by the pentose phosphate pathway to keep glutathione reduced). Similarly, the reduction potential of the ER is defined primarily by the ratio of the major redox species present, GSH and GSSG (20, 130) and, in particular, the $[GSH]^2/[GSSG]$ ratio, assuming limited glutathione-protein mixed disulfides, although all redox species present in the ER contribute.

Fourth, and linked to the previous point, the reduction potential is an equilibrium measurement, implying both that all thiol-disulfide redox reactions are equilibrium reactions (*i.e.*, they proceed in both directions) and that the relative concentrations of the species present plays a major role in the net reaction. For example, the cytoplasmic enzyme thioredoxin has the lowest reduction potential of the thioredoxin-superfamily members (*i.e.*, it is the most reducing enzyme). Thermodynamically, it reduces disulfide bonds in proteins, and this is its primary physiological role *in vivo* (see ref. 182 for a review). The reduction potential of thioredoxin is −270 mV (168), 30 mV lower than that of glutathione (254, 346) and *circa* 50 mV lower than that of denatured proteins and peptides (for example, see refs. 275 and 346). However, *in vitro*, it is able to catalyze the oxidation of dithiols to disulfides in folding proteins by using GSSG as the net electron acceptor (see Fig. 7A; 190). Thus all thioredoxin-superfamily members have the potential to act as catalysts of protein disulfide bond formation, reduction, and isomerization (see Fig. 7). However, the relative efficiencies of these will vary; in part dependent on the reduction potential of their active sites, but also dependent on their kinetic properties and on their physiological redox environment.

Fifth, the reduction potential should not be seen as being an immutable value. What is cited is the standard reduction potential. The actual reduction potential of a system varies with biophysical conditions, including temperature and, for systems such as ours that include a proton, the pH of the

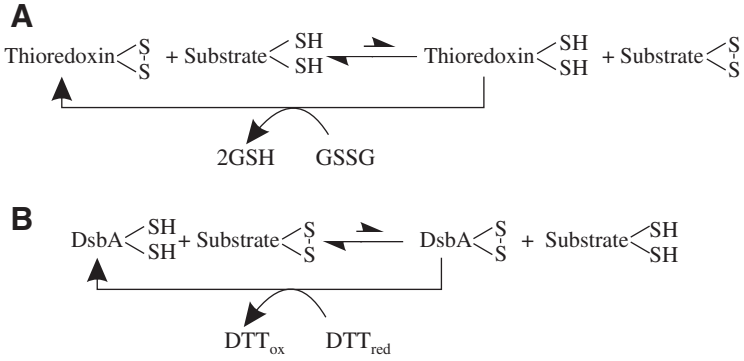

FIG. 7. Catalysis of thiol-disulfide reactions depends on the redox environment as well as on the reduction potential of the catalyst. (**A**) Catalysis of protein dithiol oxidation by thioredoxin by using GSSG as the electron acceptor. In this system, reduced thioredoxin is readily oxidized by GSSG. Whereas the potential for oxidized thioredoxin ($E_r^{0\prime} = -270$ mV) (168) to act as electron acceptors is lower than that of other thioredoxin-superfamily members [for example, PDI ($E_r^{0\prime} = -180$ mV) (189) or DsbA ($E_r^{0\prime} = -125$ mV) (129)], it is in an *equilibrium* with the denatured protein substrate thiol-disulfide ($E_r^{0\prime}$ for denatured proteins is in the range from 210 to −220 mV) (346 and references therein), and so some oxidized thioredoxin will transfer its disulfide to the substrate protein. Once reduced thioredoxin is generated, this is rapidly oxidized by GSSG, and the catalytic cycle continues. (**B**) Catalysis of protein disulfide reduction by DsbA by using DTT as the electron donor. In this system, oxidized DsbA is readily reduced by DTT. Whereas the potential for reduced DsbA ($E_r^{0\prime} = -125$ mV) (129) to act as an electron donor is lower than that of other thioredoxin-superfamily members, it is in an *equilibrium* with the substrate protein disulfide, and so some substrate will be reduced, generating oxidized DsbA. Once oxidized DsbA is generated, it will be rapidly reduced by DTT, and the catalytic cycle continues.

solution. The reduction potential of enzymes, such as PDI, will also depend on the conformation of the protein. Intrinsic conformational changes in PDI (see Section VII) or conformational changes due to substrate binding or due to changes in the biophysical conditions can change the reduction potential of the active sites.

So what determines the reduction potential of PDI? Because the reduction potential is a measurement of the relative stability of the reduced (dithiol) and oxidized (disulfide) states of the active site, factors that stabilize the disulfide state will lower the reduction potential and will make the enzyme a better reductant: the enzyme is stabilized by having the active-site disulfide, and so it is preferential to take it from nonnative protein substrates (*i.e.*, to reduce them). Similarly, factors that stabilize the dithiol state will increase the reduction potential and will make the enzyme a better oxidant—the enzyme is destabilized by having the active-site disulfide, and so it is preferential to donate it to a nonnative protein substrate (*i.e.*, to oxidize it).

Relatively little work has been done on determining the factors that determine the reduction potential of PDI. However, a considerable amount of work has been done on other thioredoxin-superfamily members, particularly thioredoxin and DsbA (for examples, see refs. 95, 103, 129, 168, 191, 220, and 345), and it is generally assumed that the same factors determine the reduction potential of PDI. Two main factors influence the reduction potential of thioredoxin-superfamily members, the pK_a of the N-terminal cysteine thiol and the effects of the two intervening residues between the active-site cysteines on stabilizing/destabilizing the disulfide state. Factors that reduce the pK_a of the N-terminal cysteine will favor the formation of the thiolate, which in turn favors the formation of the dithiol state; the net effect is to increase the reduction potential of the active site (*i.e.*, to make the enzyme more oxidizing). Factors that modulate the pK_a of the N-terminal cysteine of PDI are discussed in more detail later (see Section VII), but it is widely accepted that the pK_a of the N-terminal cysteine of PDI lies between that of the more-oxidizing DsbA (pK_a, 3.3 to 3.5) (103, 129, 220) and that of the more-reducing thioredoxin (pK_a, 6.3 to 7.1) (69, 79). As mentioned earlier, the pH of the solution changes the reduction potential of thioredoxin-superfamily members by altering either the thiol-thiolate equilibria for the active-site cysteines (36) or the conformation of the protein, including at extremes of pH by denaturation.

The role of the other amino acids in the C*XX*C active site motif is twofold. First, these amino acids are juxtaposed to the active-site cysteines and so can have a major effect on the pK_a values of the active-site thiols. Second, they can directly affect the stability of the disulfide state and its relative stability to the reduced state. For example, the active site of DsbA contains the motif CPHC. The histidine, also found in the active sites of PDI, is thought to modulate the pK_a of the N-terminal cysteine (see refs. 117 and 165 for effects on PDI), whereas the proline introduces strain into the disulfide state, destabilizing it. The net effect of these two residues is reported to make the C*XX*C motif into a rheostat (36), although their function goes beyond this (245), because they must at least also form part of the substrate interaction site.

The reduction potential of the active sites of PDI is about −180 mV (189). The reduction potential of the glutathione present in the ER depends on the $[GSH]^2/[GSSG]$ ratio. An agreement exists in the field that the ratio of [GSH] to [GSSG] in the ER is lower than in the cytoplasm (*i.e.*, that the ER is a more-oxidizing redox environment), and a consensus that the ratio is 3:1 (20, 130); however, this has recently been disputed (65). The absolute concentration of glutathione in the ER is unknown, but it has been estimated to be around 9 m*M* (20), and this is consistent with typically cited values for total cellular glutathione concentration. Because the reduction potential of glutathione depends on the $[GSH]^2$ to [GSSG] ratio, it is critical to know the absolute concentrations of the two species and not just their relative ratios. However, a ratio of 3:1 and a total glutathione concentration of 10 m*M* would give a reduction potential for ER glutathione of −191 mV (see Fig. 8). This value is sufficiently oxidizing to allow the formation of disulfide bonds because $E_r^{0\prime} \approx -220$ mV for denatured protein thiol/disulfides. In addition, this value is around the optimal value found for native disulfide bond formation *in vitro* (see Section VI). These concentrations are also sufficiently high for glutathione to act as a redox buffer (see Section VIII). In addition, this reduction potential for the glutathione redox

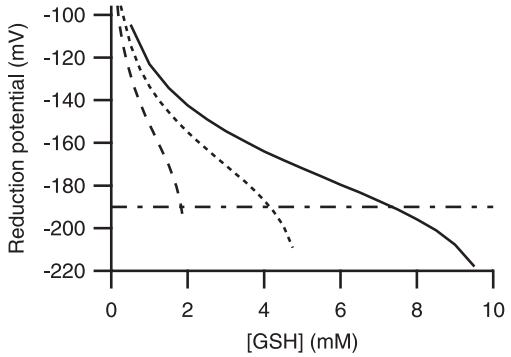

FIG. 8. Reduction potential of glutathione as a function of concentration. Calculated reduction potential under standard conditions (pH 7; 25°C; 1 atmosphere pressure) for total glutathione concentrations of 10 m*M* (*solid line*), 5 m*M* (*dotted line*), and 2 m*M* (*dashed line*). The [GSH]/[GSSG] ratio is varied from 1:19 to 19:1. The horizontal line represents a reduction potential of −190 mV, sufficiently above $E_r^{0\prime} \approx -220$ mV for denatured protein thiol/disulfides to allow efficient disulfide-bond formation and consistent with *in vitro* requirements (see Section VI) and *in vivo* measurements of the redox state of PDI (see Section X). Note that at 10 m*M* total glutathione concentration, a concentration of [GSH] of 7.35 m*M* and [GSSG] of 2.65 m*M* (*i.e.*, a [GSH]/[GSSG] ratio of around 3:1) is required for this reduction potential, whereas at 2 m*M* total glutathione, a ratio of 9:1 is required.

buffer present in the ER would imply that at equilibrium in the ER, we would expect the active sites of PDI to be present in both the oxidized and reduced states, allowing PDI to act as a catalyst of protein dithiol-disulfide oxidation and isomerization reactions. PDI is found in this mixed state (8; see also see Section IX).

The understanding of the reduction potential(s) of PDI helps us to understand the physiological relevance of the *in vitro* and *in vivo* reactions that PDI can catalyze. However, before examining these, it is worthwhile to look at what is known about the domain architecture and structure of PDI.

V. The Architecture and Structure of PDI

The mature form of human PDI is 491 amino acids. Like most proteins larger than a couple of hundred amino acids, PDI has a multidomain structure. PDI is currently recognized as having four distinct domains, **a**, **b**, **b′**, and **a′**, plus a highly acidic C-terminal extension **c** and a 19-amino-acid long interdomain linker between the **b′** and **a′** domains named **x** (see Fig. 9). This domain structure was revealed by a combination of intron-exon analysis, comparison with other superfamily members (*e.g.*, thioredoxin), partial proteolysis studies, and the expression and characterization of isolated domains and combinations of domains (see refs. 1, 55, 70, and 88 as examples). The **a** and **a′** domains share 36.8% identity with each other and significant homology with thioredoxin, but not with the **b** and **b′** domains. The **b** and **b′** domains are also said to

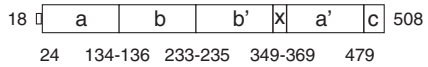

FIG. 9. Domain architecture of human PDI. Numbering is for full-length human PDI.

show homology with each other, but for human PDI, they have only 19.3% identity based on structural alignment (*i.e.*, they are not particularly similar). After the first publication of the sequence of PDI, an additional domain, the **e** domain, was speculated to exist between the **a** and **b** domains (89, 301). This speculation arose as residues 118 to 161 of PDI have homology to residues 350 to 392 of the estrogen receptor (with 29.5% identity for human PDI and human ESR1); PDI was reported to be inhibited by estrogens (301), and the C-terminal 25 amino acids of the **a** and **a′** domains of PDI have only 20% identity (*i.e.*, they show less similarity than the N-terminal regions), resulting in an initial belief that these domains were shorter than they actually are (70), with a number of subsequent studies using these incorrect boundaries. Functional and structural studies on isolated domains and combinations of domains (see later) have shown that the **e** domain does not exist, but rather, this region comprises the C-terminal part of the **a** domain, the short interdomain linker, and the N-terminal part of the **b** domain.

No published structure exists for full-length mammalian PDI, despite decades of trials from multiple groups. The structures of the isolated **a** (PDB: 1mek) (148, 150), **b** (PDB: 2bjx) (149), **b′x** (PDB: 3bj5) (221), and **a′** (PDB: 1x5c (N. Tochio, S. Koshiba, M. Inoue, T. Kigawa, and S. Yokoyama, unpublished data) along with the **bb′** double-domain construct (PDB: 2kl8) (60) of human PDI have been solved. These, together with the recent publication of a 2.4-Å crystal structure for Pdi1p from the yeast *Saccharomyces cerevisiae* (PDB: 2b5e) (298) and small x-ray scattering studies of PDI in solution (181) give us some idea of the overall structure of the protein and links between structure and function. However, before proceeding to examine the structures, it is worthwhile to raise two issues.

One problem associated with the study of catalyzed disulfide bond formation is the poor use of nomenclature and the concomitant cross-correlations between different proteins that may be part of the same family but may not have the same physiological function. For example, PDI-family members, especially ERp57 (see Section XI), are sometimes referred to as PDI, despite clear physiological differences. Similarly, PDIs from different species are often bundled together without full consideration of the potential differences in sequence, structure, or function. For example, the most highly studied PDIs are bovine PDI, human PDI, and the yeast *S. cerevisiae PDI1* gene product Pdi1p. Although bovine and human PDI are very similar (94.9% identity), human PDI and yeast Pdi1p are not, although they share the same overall domain architecture. Pdi1p is 14 (or 22 for the longer variant found in some *S. cerevisiae* strains) amino acids longer than human PDI. Furthermore, human PDI is not N-glycosylated, whereas Pdi1p is heavily glycosylated. Overall, the percentage identity between the two mature proteins is reasonably high, given the species difference at 29.1%. However, whereas the catalytic domains show 40.9% (**a** domain) and 40.8% (**a′** domain) identities, the noncatalytic **bb′** domain fragment shows only 17.7% identity. This identity is significantly lower than that of human PDI and human ERp57 **bb′** (22.0%), yet PDI and ERp57 have different physiological functions based, at least in part, on the differences in this region (see Section XI). A BLAST search using Pdi1p against a human protein database gives the PDI-family member ERp72 as the best hit, whereas a search using the **bb′** domains of Pdi1p gives ERp44 as the only

human PDI-family member hit. It is also noteworthy that Pdi1p was identified by one group (175) based on similarity with chicken glycosylation-site binding protein (GSBP), and yet, whereas chicken GSBP is clearly a PDI-family member (97), chicken PDI (Swiss-Prot ID: P09102) and chicken GSBP (Swiss-Prot ID: P12244) have only 70% identity, and GSBP is N-glycosylated (144), whereas chicken PDI is not. Furthermore, because protein–protein interactions, which define physiological function, are poorly defined between species unless high levels of sequence similarity exist (210), why should human PDI and yeast Pdi1p be automatically assumed to have identical physiological functions? The ability of mouse or rat PDI to be able to complement the essential function of Pdi1p in *S. cerevisiae* (35, 108) is insufficient evidence to say that they are physiologically identical, especially because other PDI-family members and even an active-site mutant thioredoxin are also able to complement (see Sections X and XI). In this review, we use the term PDI to represent mammalian protein disulfide isomerase, in particular, human PDI, Pdi1p to represent the *S. cerevisiae PDI1* gene product, and the term "PDI family" when making more-generic statements.

Another confusion that arises is connected with the numbering of the protein sequence. A significant amount of experimental work was done on PDI-family members before the full amino acid sequence was known or before the cleavable signal sequences were defined. Therefore at least two sets of numberings exist, those based on the full-length protein and those based on the mature protein. Unfortunately, because of differences of opinion on the length of the signal sequence, some family members have different numbering systems even for the mature protein. Because the length of the signal sequence has not been experimentally determined for most PDI-family members, we will use full-length protein numbering throughout this review. For those who wish to convert to the mature numbering, the signal sequence of human PDI is 17 amino acids in length.

Given the high degree of sequence homology between the catalytic domains of PDI and thioredoxin, it is perhaps unsurprising that they share very similar structure. In addition to being the founding member of the thioredoxin superfamily of proteins, the structure of thioredoxin forms the foundation of a protein fold known as the thioredoxin fold. This fold is found in a wide range of proteins including thioredoxin, DsbA, PDI, glutathione peroxidase, glutathione S-transferase, arsenate reductase, calsequestrin, and circadian oscillation regulator, many of which are involved in thiol metabolism or use thiol-based chemistry. The thioredoxin fold is defined as a three-layer α–β–α structure with two α-helices packing onto one side of a four stranded β-sheet, of which strand three is antiparallel to the others, and with an additional α-helix packing onto the other side of the β-sheet. In thioredoxin, the structure is β–α–β–α–β–β–α. However, α3 is sometimes missing and, allowing for this, and for circular permutations of the fold, Qi and Grishin identified a significant number of other thioredoxin-fold–containing proteins and protein families, including tubulin C-terminal domain, cytidine deaminase, chorismate mutase, RNA 3′-terminal phosphate cyclases, and phospholipase D (243).

Although the thioredoxin fold is defined by the core three α-helices and four β-strands, this is not the structure of thioredoxin. Instead, thioredoxins, including human thioredoxin (80) and *Escherichia coli* thioredoxin (124, 147), show a β–α–β–α–β–α–β–β–α structure (*i.e.*, they have an additional β–α before the thioredoxin-fold). This fact causes some problems connected with nomenclature. For example, the active site of thioredoxin lies at the N-terminus of the first α-helix of the thioredoxin fold (α1), but this is the second α-helix of thioredoxin (the first α-helix is α0). In this review, we use the standard nomenclature in the field (*i.e.*, that the active site of PDI lies at the N-terminus of α2), and the phrase "thioredoxin-like" fold to describe structures with a β–α–β–α–β–α–β–β–α structure.

As stated, the active site of thioredoxin and of the catalytic domains of PDI lie at the N-terminus of the α2 helix, with the N-terminal active-site cysteine being at the N-terminus of the helix, and the C-terminal active-site cysteine having limited solvent exposure. This arrangement is important in determining the pK_a of the active-site cysteine residues. The pK_a of the N-terminal active-site cysteine must be low both to have a sufficiently high reduction potential so as to act efficiently in protein dithiol oxidation (see Section IV) and to have efficient kinetics for the initial steps of catalysis of thiol-disulfide exchange (see Section III). The pK_a of the N-terminal cysteine of the catalytic domains of PDI has been reported to be in the range 4.4 to 6.7 (114, 165, 260), considerably lower than the normal pK_a of a protein cysteine thiol. This low pK_a results from multiple effects. In part, it results from the N-terminal active-site cysteine being located at the N-terminus of an α-helix. α-Helices have a permanent dipole associated with them, with the N-terminus of the helix having a positive dipole moment, whereas the C-terminus has a negative dipole moment. This leads to a decrease in the thiol pK_a of up to 1.6 pH units for cysteine residues at the N-terminus of α-helices in model peptides (164). This by itself its not sufficient to explain the abnormally low pK_a value of Cys 53 and Cys 397 of PDI, because thioredoxin has a similar structure, and the pK_a of the N-terminal active-site cysteine of thioredoxin is usually cited as being 7.1 (69). Other electrostatic effects must be around the active site of PDI. The primary effector is probably the histidine residues, His 55 and His 399, which lie between the active-site cysteines. However, other charged residues are within 7 Å of the active-site cysteines in the NMR structure of the **a** domain of PDI, including Glu 47, Lys 57, and Lys 81, and even distant residues, such as the side chain of Arg 120, which is more than 17 Å away from the active site, may approach the active site during conformational exchange (see Section VII).

The requirements for the pK_a of the C-terminal active-site cysteine residue, Cys 56 and Cys 400 in PDI, are more complex. However, for much of the catalytic cycle of protein dithiol oxidation or isomerization, this group must be in the protonated thiol state, so these cysteines require a higher than average pK_a value. The pK_a of this group is so high that it is difficult to measure, without competition from protein denaturation, but general agreement in the field indicates that it is >10, and it has a calculated value of 12.8 (178). However, at one step of the catalytic cycle for protein dithiol oxidation, this group must be a thiolate, and a conformational change within the catalytic domain lowers the pK_a of this group to <7 (see Section VII).

Other structural features that are conserved between other thioredoxin-superfamily members and the catalytic domains of PDI include (a) a conserved proline residue in the middle of α2, Pro 61, and Pro 405 in PDI, which introduces a kink into

the helical structure and allows it to wrap around the core β-sheet; (b) a *cis*-proline residue, Pro 100 and Pro 441 in PDI, found at the start of β4 and juxtaposed to the active site. This residue, forming part of what was known as the "Egypt motif," as the sequence is GYPT in the **a** domain of PDI, has been implicated in substrate binding in other thioredoxin-superfamily members (71). This residue has not been mutated in PDI. In Pdi1p, mutation of the proline to alanine in either catalytic domain abolished enzymatic activity, but it is not known whether the *cis*-peptide bond is retained in the mutated protein (298); and (c) a buried-charge pair, Glu 47 and Lys 81 in the **a** domain and Glu 391 and Lys 424 in the **a**′ domain of PDI, lying under the active site. This pair, which is conserved in many of the catalytic active domains of human PDI-family members, has been implicated in proton-transfer reactions required for catalysis (71) and has been shown to be important for the catalytic activity of thioredoxin (69). However, it is not conserved in the **a** or **a**′ domains of Pdi1p (298), implying that it is not essential for PDI-like activity.

Whereas the **b** domain of PDI shows little sequence similarity with the catalytic domains, it too has a thioredoxin-like fold (149), although it lacks the active site, both prolines, and the buried-charge pair. Given the very low level of sequence identity, 13% based on structural alignment, between the **a** and **b** domains, it is unclear whether the noncatalytic domain modules evolved through internal gene duplication in the way that duplications of the catalytic domains occurred to generate the current range of different eukaryotic PDI-family members (143, 204). Similarly, the **b**′ domain of PDI shows little sequence similarity with either the **a** or **b** domains, but it too has a thioredoxin-like fold that lacks the conserved features of the catalytic domains (221).

No multidomain structures are available for human or any other mammalian PDI. However, in the last few years, crystal structures have been reported for yeast Pdi1p (297, 298) and the human PDI-family members ERp57 (in complex with tapasin; PDB: 3f8u) (66), ERp29 (a structure that contains only one thioredoxin-like domain; PDB: 2qc7) (16) and ERp44 (PDB: 2r2j) (326), along with structures for the **bb**′ double-domain constructs of PDI (PDB: 2k18) (60) and ERp57 (PDB 2h8l) (166). The structure of Pdi1p shows the four domains in a "twisted U" structure (see also Section VII), with one molecule of Pdi1p occupying the putative substrate-binding site formed between the **a**, **b**, **b**′, and **a**′ domains. The **a-b-b**′ fragment shows considerable structural conservation with the three redox-inactive domains of rabbit calsequestrin, including domain orientation. At the time of writing, the PDB file for the crystal structure of ERp57 had not yet been released, but the overall structure of human ERp57 is broadly similar to that of Pdi1p. In effect, both the Pdi1p and ERp57 structures are structures with "bound substrate" (either another Pdi1p molecule or tapasin), and interdomain flexibility in the unliganded structures may be why decades of trials were previously unsuccessful in obtaining high-quality protein crystals. The structure of ERp57 in the tapasin complex shows a significant rotation of up to 14 degrees between the **b** and **b**′ domains compared with the previously published structure of the isolated **bb**′ fragment. It is not clear whether this rotation of domains is linked to one structure being of an ERp57 fragment, or if it is due to one structure being in a complex. The structure of the shorter human PDI-family member ERp44 (see Section XI) has only three thioredoxin-fold–containing domains, which again show an arrangement similar to that of calsequestrin. Although it is tempting to extrapolate these data on domain–domain orientations to other members of the PDI family, the recent structure of the yeast PDI-family member Mpd1p (PDB: 3ed3) (318) warns of the dangers of doing this, because the two thioredoxin-fold domains of Mpd1p have a unique orientation with respect to each other.

With structural information at hand, it is time to move on to examining the reactions that PDI can catalyze and the methods used to characterize its mechanisms of action, starting with *in vitro* analysis.

VI. *In vitro* Thiol-Disulfide Exchange Reactions of PDI

A long and sometimes contradictory history is associated with the study of the reactions that PDI is able to catalyze. This is best presented as a summary of the reactions PDI is able to catalyze *in vitro*, in line with Section III, the assays used to examine these activities, and then the roles of individual domains in these processes.

PDI is oxidized by molecular oxygen relatively slowly, and therefore, it should not be categorized as an oxidase (296). Therefore, all of the reactions catalyzed by PDI are thiol-disulfide exchange reactions. However, it is important to distinguish at least three categories of reactions that PDI can catalyze (see Fig. 10).

1. Oxidation reactions in which a protein or peptide substrate dithiol is oxidized to the disulfide state, with the concomitant loss of an active-site disulfide from PDI. To complete the catalytic cycle, PDI must be reoxidized. *In vitro,* GSSG is usually used as the terminal electron acceptor. This generates GSH and results in a change in the reduction potential and redox-buffering capacity of the buffer. Care must be taken to ensure that sufficient GSSG is present to complete the reaction and that the change in reduction potential during the reaction does not significantly affect the folding efficiency or yield. If no GSSG or equivalent electron acceptor is present, PDI is also able to reduce a disulfide bond in one nonnative protein molecule to form a disulfide in another nonnative protein.
2. Reduction reactions in which a protein- or peptide-substrate disulfide is reduced to the dithiol state, with the concomitant gain of an active-site disulfide in PDI. To complete the catalytic cycle, PDI must be reduced. *In vitro,* GSH or DTT is often used as the electron donor. Again, this results in a change in the reduction potential and redox-buffering capacity of the buffer.
3. Isomerization reactions in which the disulfides and thiols in a protein or peptide substrate are rearranged to give a different disulfide-bonding pattern. In direct isomerization, no net change exists in the redox state of the active site of PDI, and so no other redox reagents are required.

All of these reactions can involve intra- or intermolecular substrate disulfides, with the focus to date being on the former. In addition, certain reactions also have specialist names (*e.g.*, the reduction of a protein or peptide mixed disulfide with glutathione by using GSH as the electron donor to generate GSSG and a protein thiol is known as a deglutathionylation reaction). Similarly, the formation of a protein or

peptide mixed disulfide with glutathione is known as a glutathionylation reaction.

A. Assays for PDI-like activity

Multiple different assays have been used to determine the activities of PDI, all of which have advantages and disadvantages. A consensus is found in the field that it is better to use "natural" substrates (*i.e.*, those based on proteins and peptides) rather than artificial ones, although the use of extrinsic fluorophores can give highly sensitive systems (251). However, only rarely is a physiological substrate used. Instead, a fairly narrow range of "well-behaved" proteins and peptides are used. It should also be remembered that *in vitro,* we usually deal with refolding of proteins in dilute solutions with PDI present in catalytic amounts, whereas *in vivo,* folding occurs co-translationally at high protein concentrations and with PDI present in very high concentrations and probably in excess over substrate. The *in vitro* systems may give a distorted picture of the physiological functions of the enzyme. However, given the dearth of methods for dissecting mechanisms of action in natural systems, the only realistic approach is to combine *in vitro* and *in vivo* data, accepting that both may be prone to artifact.

Some *in vitro* assays are based on the gain of activity of a substrate protein. Here the classic example is the use of a ribonuclease; usually RNase A. RNase A is a one-domain protein that contains four disulfide bonds in the native state. The RNase assay comes in many different formats. The starting material can be (a) reduced RNase, to give an assay that looks primarily at oxidation; or (b) "scrambled" RNase, in which the protein has been reduced and allowed to oxidize under denaturing conditions, which produces a set of proteins in which the disulfide bonds are predominantly nonnative, to give an assay that looks primarily at isomerization, or (c) glutathionylated RNase T_1 (261, 262), in which all of the cysteines in the protein are glutathionylated at the start, in an assay that looks at isomerization, including the deglutathionylation subsystem. Assays that are based on the gain of activity of a substrate protein are widely used, in part because of their relative simplicity. The major advantage that drove their development was their sensitivity, allowing detection after only a very small percentage of the refolding protein had reached the active state. However, a number of problems are associated with them. The first problem is that the assay does not directly measure disulfide-bond formation in the substrate protein. A gain of activity does not directly correlate with disulfide-bond formation because many folding intermediates may have biological activity, with the degree of activity depending on the structure of individual folding intermediates and being protein dependent. In addition, for mechanistic studies, the biophysical conditions of the system are often varied, and care must be taken that any changes observed relate to changes in the activity of PDI and not to changes in activity or stability of the folded substrate or of its folding intermediates.

The second problem relates to the complexity of the material: 764 different disulfide-bonded states are possible for RNase A. Whereas only a small fraction of these may be

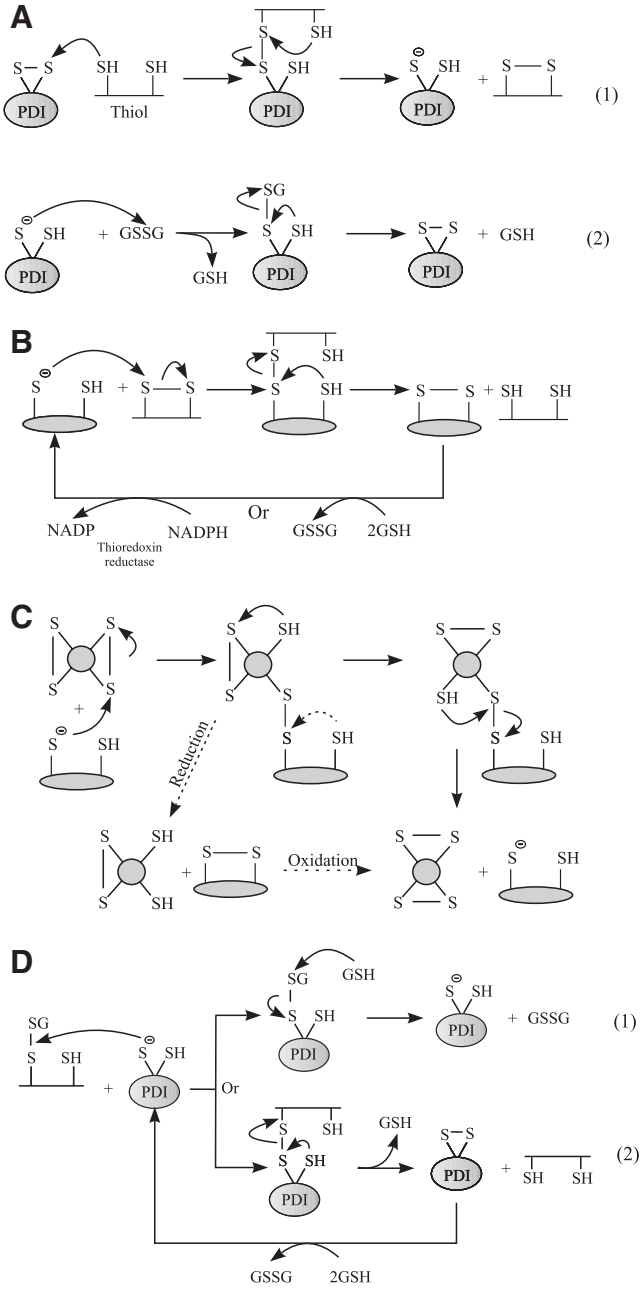

FIG. 10. Schematics of the reactions catalyzed by PDI *in vitro*. (**A**) Oxidation of a protein or peptide dithiol with glutathione as the electron acceptor. (**B**) Reduction of a protein or peptide disulfide by using GSH or NADPH (catalyzed by thioredoxin reductase) as the electron acceptor. (**C**) Disulfide isomerization of a protein or peptide by both reduction–oxidation cycles and by direct isomerization. (**D**) Deglutathionylation of a protein or peptide substrate. The two possible reaction intermediates are the mixed disulfide between PDI and glutathione (reaction 1) and the mixed disulfide between PDI and the protein (reaction 2). Deglutathionylation by glutaredoxin occurs primarily *via* reaction 1, whereas deglutathionylation by the **a** domain of PDI occurs primarily *via* reaction 2 (235). Note that in all panels, thiol species are shown in their predominant ionization state; only the N-terminal active-site cysteine of PDI is shown as a thiolate. Although thiol groups are nucleophiles, the thiolate is the usual reactive species. Because PDI is able to catalyze these reactions, even at pH values as low as pH 4 (260), it must have mechanisms in place to reduce the pK_a of substrate thiols. These mechanisms are currently unknown.

experienced during refolding, each intermediate is in effect a different substrate for PDI with different requirements for catalysis, and a global assay, such as the regain of activity, will miss many of the subtleties. This problem is exacerbated for "scrambled" RNase in that the starting material itself is not homogeneous (and shows considerable batch-to-batch variation).

The third problem is related to assaying the activity of RNase. Essentially, two different assays are used: (a) the hydrolysis of RNA (102, 133, 173, 288), which uses a natural substrate but which requires very small changes in absorbance to be accurately measured with time, necessitating the use of a good double-beam spectrometer; or (b) the hydrolysis of cCMP (193, 322, 323, 340), an assay that, for detailed kinetics, requires the accurate measurement of very high absorbance values. The cCMP variant has also been used in a continuous variant of a PDI assay. This is based on taking the first derivative of the absorbance with respect to time and correcting for the depletion of cCMP and product inhibition of RNase by CMP (193, 340). Because of these problems with the assays, often a less-comprehensive measurement is used: either the use of measuring the "lag-phases" (the time taken before activity of the substrate enzyme starts to appear), or of measuring the initial rate of appearance of RNase activity after the "lag-phase," sometimes based on just two time points. Although these methods can be used to measure the relative order of activity (*i.e.*, protein X is more active than protein Y), they are poor methods to determine PDI activity quantitatively, given the complexity of the system and the heterogeneity of the starting material in the "scrambled" RNase assay. A recent addition to this category of assays is the use of riboflavin-binding protein (250), a protein that contains nine disulfide bonds. Quenching of riboflavin fluorescence on binding by functional riboflavin-binding protein allows continuous monitoring of the system, even at high protein concentrations, making it potentially a very powerful assay. However, as with most assays in this category, it is a measure of gain of some function and not necessarily a measure of gain of the native state, and so care must be taken in interpretation of the results.

A second category of assays is based on changes in the biophysical properties of a substrate. An early assay in this category was the insulin-reduction assay (202, 224). In this assay, insulin reduction by GSH or DTT results in the aggregation of the B chains of insulin. This aggregation event is followed by changes in light scattering, usually by monitoring the apparent increase in absorbance at $\sim$600 nm. Again, this assay is very useful to determine the relative order of activity of different catalysts, but it is a poor method for quantification. Because the assay measures aggregation properties of the product, it is also very sensitive to changes in biophysical conditions. However, this method forms the basis of a proposed high-throughput screen for PDI inhibitors (278). A more quantitative version of this assay is the coupled insulin-reduction assay (176, 244, 245). In this assay, the GSSG formed by the reduction of insulin by GSH is reduced by glutathione reductase, with the concomitant oxidation of NADPH and a decrease in absorbance at 340 nm. This assay is quantitative, but only up to time points at which insulin aggregation starts to contribute to the apparent changes in absorbance at 340 nm due to light scattering. A similar assay is also possible by using thioredoxin reductase in place of the GSH and glutathione reductase, because PDI is a substrate for thioredoxin reductase (190). Two additional drawbacks of the insulin-reduction–based assays are that (a) many other enzymes show significant activity in this assay; and (b) the assay looks at the reduction of a substrate disulfide that is neither the main physiological function of PDI (see Section X) nor the main defining reaction of the enzyme.

Other assays that fall into this category are peptide-based thiol-disulfide exchange assays in which an associated change in fluorescence occurs. The first of these published was an oxidation assay in which disulfide-bond formation is accompanied by a change in intrinsic fluorescence of a tryptophan residue in the substrate peptide (260). This is a quantitative assay, and the designed peptide allows a wide range of biophysical conditions to be used, but being based on intrinsic fluorescence, it has limitations, especially because the change in signal is only $\sim$18%. This assay also formed the basis for similar assays to examine glutathionylation and deglutathionylation activity (235). Subsequently Winther and coworkers (335) introduced a series of substrate peptides with a fluorescent aminobenzoic acid residue and a nitrotyrosine quencher, which on reduction (37, 335) or oxidation (37) show very significant changes in fluorescence, with spectral parameters that are not affected by the intrinsic fluorescence of proteins. Another recent addition is a peptide based on tachyplesin I that allows examination of isomerization reactions in a homogeneous substrate *via* donor-quencher–based fluorescence assays (151). This very useful and welcome assay does, however, have complications relating to the properties of the peptide, including its adsorption to cuvettes.

A third grouping of assays is based on quenching dithiol-disulfide exchange reactions and then subsequently monitoring the disulfide bond state of the substrate. These quenching-based assays have the disadvantage that they are considerably more time consuming than the other assays presented here, and they are always discontinuous. However, they have the potential to provide much more detailed kinetic analysis of even complex systems. Although various peptides and proteins have been used, the classic substrate in this type of assay is BPTI (44). BPTI is a small one-domain protein that contains three disulfide bonds. Its use has the advantages that the folding pathway has been extensively characterized (but see later) and that all of the folding intermediates are soluble. These assays are based on quenching or freezing the dithiol-disulfide exchange reaction at discrete time points. Quenchers fall into two categories: acidification and chemical modification (see Fig. 11). Acidification, the reduction of the pH of the solution by the addition of acid, has two advantages: it is very rapid, and it results in denaturation of the enzyme and the substrate. However, it also has disadvantages. Specifically, it does not permanently quench thiol-disulfide exchange reactions, it only slows them (see Fig. 11A). For example, PDI has residual activity under the most commonly used acid-quenching conditions (A.-K. Lappi and L.W. Ruddock, unpublished results). In contrast, chemical modification of the thiol groups will permanently stop thiol-disulfide exchange reactions. Typical chemical quenchers include iodoacetamide, iodoacetate, and maleimides such as *N*-ethyl maleimide (NEM), but any thiol-reactive compound can potentially be used. However, care must be taken in the choice of chemical modifier used. For example, thiosulfonates such as *S*-methyl methanethiosulfonate have been used as quenching reagents (132, 187, 233); however, these reagents can result in

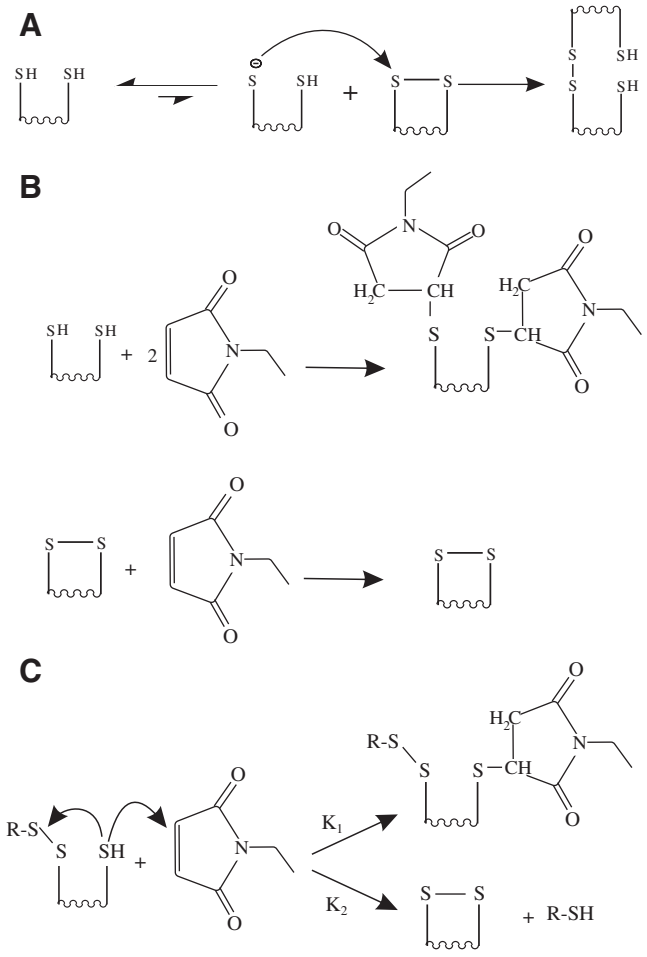

FIG. 11. Schematic representation of quenching thiol disulfide exchange reactions with PDI as an example. (**A**) Quenching by acidification. Because thiol-disulfide exchange reactions occur predominantly *via* thiolate species, reducing the pH of the solution has a strong inhibitory effect, as it drives the thiol-thiolate equilibrium toward the thiol state. However, the equilibrium still exists, implying the presence of some thiolate at all pH values. In addition, thiol groups are nucleophiles, and so thiol-disulfide exchange can still occur, although with much slower kinetics. (**B**) Quenching by chemical modification, by using NEM as an example. NEM is able to react quickly with surface-exposed thiol groups (predominantly *via* thiolate chemistry), inhibiting their thiol-disulfide exchange. (**C**) However, buried or partially buried thiol groups, for example, the C-terminal active-site cysteine residue of PDI, will have much slower rates of reaction. For these groups, kinetic partitioning exists. Either they react with the chemical quencher (reaction 1) or they undergo thiol-disulfide exchange reactions (reaction 2). Reaction 2 occurs with a rate constant of 0.28 per second (146), c.f. 0.5 per second (extrapolated value, see text) for a surface-exposed cysteine with an average pK_a at pH 7 reacting with 1 *M* iodoacetamide (reaction 1). It takes only a small reduction in the kinetics of reaction due to inaccessibility for reaction 2 to predominate (*i.e.*, for inefficient chemical quenching to occur).

a change of the thiol-disulfide state of the system (145). Similarly any iodo-based compounds must be high grade, because any contaminating iodine can catalyze disulfide-bond formation. The use of chemical quenchers has one major disadvantage: the reactions of thiol groups with these compounds are nucleophilic reactions, which are in direct kinetic competition with the nucleophilic reactions involved in thiol-disulfide exchange by the same thiol groups (see Fig. 11B). For surface-exposed thiol groups, this problem can be minimized by the use of a large excess of quenching reagent. Iodoacetamide is often used as a quencher at molar concentrations. For PDI, the surface-exposed N-terminal active-site cysteine has a second-order rate constant of 11 $M^{-1}s^{-1}$ for reacting with iodoacetate when in the thiolate state, and circa 7 $M^{-1}s^{-1}$ at pH 7 (114). This implies a half-time for reaction at pH 7 of around 0.1 s when 1 *M* iodoacetamide is used. This time-scale is fast enough to trap most thiol-disulfide exchange reactions. However, the pK_a of this thiol is unusually low and, assuming a similar second-order rate constant with a typical cysteine thiol pK_a of 8.3, the half time for quenching under the same conditions becomes 1.3 s. In addition, buried thiols in folding proteins may be poorly accessible to chemical quenchers and may continue to undergo intramolecular thiol-disulfide exchange reactions rather than reacting with the chemical quencher. This is a serious problem. For example, in BPTI with chemical quenchers, Creighton and co-workers (43) saw major populations of nonnative two-disulfide bonded states, whereas Weissman and Kim (330, 331) saw predominantly only native-like two-disulfide states by using acid quenching. The difference is probably due to the inaccessibility of buried thiol groups to chemical quenchers (see also 42, 332). Similarly mixed disulfides between PDI and substrates or glutathione are very difficult to trap, as the C-terminal active site cysteine is very inaccessible to chemical quenchers (see Fig. 11) (53; A.-K. Lappi and L.W. Ruddock, unpublished observations).

After the quenching, reaction analysis of the thiol-disulfide state of the protein is often done by reverse-phase HPLC or by mass spectrometric methods. Mass spectrometric methods are more rapid. However, they give information only on the populations of different disulfide-bonded states (*e.g.*, no disulfides *vs*. one disulfide *vs*. two disulfides). They do not give information on subpopulations (*e.g.*, for folding intermediates that contain two disulfide bonds, what proportion of these are native). In contrast, under ideal conditions, reverse-phase HPLC allows the separation and relative quantification of all of the different species present in the sample. Although it is a much more powerful technique, it requires extensive optimization and identification of the different eluting species, something that is often complicated by the presence of one or more species co-eluting.

Given the narrow range of substrates used to assay PDI-like activity, it is possible that a significant bias exists in the data set obtained to date, especially for PDI-family members that are not as promiscuous as PDI.

B. Domain contribution to thiol-disulfide exchange activity

Because PDI has two catalytic domains, along with two domains that lack active sites but that may contribute to the overall catalytic activity of PDI, it is useful to dissect the molecule to examine the roles of each domain in isolation and in combination. Similarly, the use of mutants, either in full-length PDI or in domain constructs, allows a dissection of mechanisms of action. However, in both cases, it is essential to ensure that the structure of the construct is not compromised.

The minimal unit required for catalysis of thiol-disulfide exchange reactions is usually considered to be the isolated **a** or **a′** domain of PDI. These are able to catalyze oxidation reactions efficiently in peptide substrates (4, 52) and the early stages of protein oxidation (52), with the N-terminal active-site cysteine forming the mixed disulfide reaction intermediate with the substrate, and the C-terminal active site cysteine being involved in reforming the active-site disulfide bond (see Fig. 10A). Both active-site cysteine residues are required to catalyze oxidation and reduction reactions (see Fig. 10B). In contrast, as shown in Fig. 10C, the C-terminal active-site cysteine is not essential for the catalysis of isomerization reactions, although it is required for isomerization *via* cycles of reduction and oxidation and for the "escape pathway"—the release of a kinetically trapped PDI-substrate mixed disulfide (322) (see also Section VII). The relative contributions of direct isomerization *versus* isomerization *via* cycles of reduction and oxidation is difficult to judge and is probably substrate dependent. However, C-terminal active-site cysteine mutants retain at least some isomerization activity (323), and cycles of reduction and oxidation during isomerization can be observed (269), implying both mechanistic pathways exist *in vitro*. The cysteine residues found in the **b′** domain do not contribute to catalytic activity (56). Not surprisingly, given the effects of reduction potential on the thermodynamics of the system (see Section IV) and the requirement for the oxidized active-site state of PDI to catalyze disulfide-bond formation and the reduced form to catalyze disulfide-bond isomerization, the kinetics and yield of oxidative folding of proteins are dependent on the composition of the glutathione redox buffer used. For PDI-catalyzed refolding of ribonuclease A at pH 8.0, the optimal activity was observed at [GSH] = 1.0 m*M* and [GSSG] = 0.2 m*M* (193), which gives a reduction potential close to that observed *in vivo* (see Section IV).

In a landmark study, Darby and co-workers (56) dissected out the contributions of domains to the relative activities of PDI by using a range of substrates including two different kinetically trapped nonnative disulfide-containing BPTI-refolding intermediates, a 28-amino-acid peptide based on the sequence of BPTI, and the insulin-reduction assay (56). This study, and others (162, 325), showed (a) that deletion of the **c** region had no effect on activity; (b) that the isolated catalytic domains had poor activity toward protein substrates, but that they were able to catalyze oxidation, reduction, and isomerization; (c) that all domains contributed to the thiol-disulfide exchange activities of PDI; and (d) that the minimal constructs required for efficient catalysis were either **a-b-b′** or **b′-a′-c** (*i.e.*, that the inclusion of the **b′** domain in a linear-domain construct is important for activity; see later for the function of the **b′** domain). Perhaps surprisingly, a full-length PDI, with all four active-site cysteine residues mutated, still had residual activity (194, 323). This may be linked to one of two poorly characterized functions that PDI must possess: (a) the ability to reduce the pK_a of substrate thiols. Because PDI has significant activity as a catalyst of oxidation of a peptide dithiol by using GSSG as the electron acceptor down to pH 4 (4, 260), it must have mechanisms in place to reduce the pK_a of substrate thiols and thereby increase their nucleophilicity; or (b) the ability to access buried disulfides and thiol groups in nonnative proteins. This must occur *via* the induction of conformational change in the substrate proteins (see Section VII). Because of the asymmetry of the molecule, the active sites in full-length PDI may not be equivalent in catalyzing thiol-disulfide exchange reactions when using a glutathione redox buffer *in vitro*, although contradictory results were found on the relative importance of the two active sites (56, 194, 325). This may be related to substrate specificity. However, for Pdi1p with multiple well-characterized systems, the **a′** domain active site was shown to contribute more to all thiol-disulfide exchange reactions examined *in vitro* (334).

For oxidative protein folding *in vitro*, the rate-limiting steps are thiol-disulfide exchange reactions in folding intermediates that contain substantial regular secondary structure. For example, for some two-disulfide BPTI-folding intermediates, the noncatalyzed half-times to reach the native state in a glutathione buffer are measured in hours or even days. It is for these reactions involving quasi-native intermediates that PDI would be expected to have the greatest catalytic effects. The relative contribution of PDI catalysis to oxidation, reduction, and isomerization is difficult to gauge and is probably substrate dependent, but it is clear that PDI is efficient at catalyzing all three thiol-disulfide exchange reactions. Although PDI is able to catalyze all thiol-disulfide exchange reactions, it is reactions in late-stage folding intermediates that are the rate-limiting steps in oxidative folding *in vitro*. How efficient is it at catalysis? Values from the literature for which a defined transition is looked at can be examined for evidence. For example, for BPTI refolding at pH 7.3 in a buffer containing 2.0 m*M* GSH and 0.5 m*M* GSSG, the acceleration for transitions were 3,500- to 6,000-fold (330). For the oxidation of a kinetically trapped state of RTEM-1 β-lactamase, catalysis was 500-fold faster than the noncatalyzed GSSG reaction at pH 8.0 (321). Similarly, when using 0.5 m*M* GSSG as the electron acceptor for oxidation of a simple peptide substrate at pH 7.0, a *circa* eightfold difference in rate of refolding was found in the presence and absence of 0.74 μM PDI (260), which, given the relative concentrations, implies that PDI was >5,000-fold more efficient at introducing the disulfide bond into the peptide than was GSSG. As a final example, at pH 7.3, a larger and unrelated peptide substrate PDI, with GSSG as the net electron acceptor, was ~100- to 120-fold faster than GSSG at introducing a disulfide bond (54). It should be noted that both the catalyzed and noncatalyzed reactions show a very strong pH dependence, and that the major effect on catalysis is the unusually low pK_a of the active-site cysteine of PDI, which allows it to be an efficient nucleophile at physiological pH values. At pH 7, the proportion of N-terminal active-site cysteine that is in the thiolate state (assuming a pK_a of 5.1) is 98.8%, whereas the proportion of a normal protein or peptide cysteine in the thiolate state (assuming a pK_a of 8.3) is 4.8%. This 20-fold difference certainly contributes to the catalytic activity of PDI at pH 7. At higher pH values, the proportion of the N-terminal active-site cysteine in the thiolate state cannot significantly increase, whereas that of a normal cysteine can and does. As the pH increases above 7.3, the difference between the rates of the catalyzed and uncatalyzed reactions decreases. For this reason, and for reasons connected to the structure and stability of PDI and of folding intermediates, the study of catalysis should be done, wherever possible, either at pH 7.3 (physiological pH) or at pH 7 (the pH for determining standard biochemical reduction potential).

Very little work has been reported on the substrate specificity of catalysis of thiol-disulfide exchange by PDI. In a landmark article, Westphal and co-workers (335) screened a library of random peptides and found and characterized 13 different

peptides whose reduction could be catalyzed by PDI and two that could not. Although this is a small dataset, a preference for small amino or imino acids existed before the cysteine to be reduced (Ser, Ala, Gly and Pro were found in 10 of 13 positive peptides), and a preference for basic amino acids at a position two amino acids after the cysteine (His, Arg, Lys were found in seven of 13 positive peptides), which may be linked to modulation of the pK_a of one or more of the cysteines in the system. Substrates that could not be reduced by PDI contained multiple aromatic groups in positions adjacent to the peptide cysteine, suggesting that steric hindrance was the problem.

C. Substrate binding by PDI

To be able to catalyze thiol-disulfide exchange reactions in folding proteins, PDI must be able to bind them. Initial studies on catalysis showed that the K_M of PDI for peptide substrates was $<3\ \mu M$ (54). To identify substrate-binding sites directly, a cross-linking–based approach has been used, in which a radiolabeled peptide is added to PDI, usually in a lysate to minimize potential artifacts. This approach first identified PDI as being equivalent to the glycosylation-site binding peptide (97), as it was able to bind to a radiolabeled peptide Asn-Lys-Thr, although from subsequent analysis of PDI specificity, it is possible that the actual groups bound were the aromatic labels and not the tripeptide itself. This same probe was used to locate the interaction site in PDI (223). The interaction site was found to be in a 26-amino-acid fragment that included part of the last helix of the **a′** domain along with the first half of the **c** region. Subsequently, a range of other peptides, including Δ-somatostatin and mastoparan (156), were introduced, and peptide binding was found to be reversible and based primarily on hydrophobic interactions. Studies on isolated domains and domain constructs of PDI revealed that the **b′** domain provides the principal binding site of PDI (158), being essential and sufficient for peptide binding. However, whereas the **b′** domain was essential for binding larger substrates, both the **a** and **a′** domains contributed to the binding of misfolded proteins. This ability of the **b′** domain to bind substrates explains its essential role in the efficient catalysis of isomerization reactions (see earlier). Substrate binding by the **b′** domain is sensitive to conformational instability in other domains, so mutations that destabilize the **a′** domain of PDI indirectly affect peptide binding, probably because of the occupancy of the substrate-binding site in the **b′** domain by the disordered or nonnative regions of the mutated **a′** domain (157). The initial characterization of residues comprising the substrate binding site within the **b′** domain, through a combination of modeling and mutagenesis studies (237), was subsequently shown to be incorrect. Rather than directly being involved in substrate binding, the mutants identified instead altered a conformational equilibrium occurring in PDI, in which the **x** region caps the substrate-binding site in **b′,** occluding substrate binding (see Section VII) (221). The substrate-binding site in the **b′** domain is also required for the assembly of PDI with the α-subunit of prolyl-4-hydroxylase. In addition, by cross-comparisons with other thioredoxin-superfamily members, additional sites in the **a** and **a′** domains were identified as being required for assembly (163). The site in the **a′** domain includes Phe 469 in the terminal α-helix, which forms part of the site identified by Noiva and coworkers (223), and these same sites are involved in binding nonnative proteins (H.I. Alanen and L.W. Ruddock, unpublished observations). Thus, substrate binding involves the cooperation of sites in at least three of the domains of PDI. In addition, the **b** domain of Pdi1p has been implicated in substrate binding from the crystal structure (298). This combination of multiple low-affinity sites is probably essential for the physiological function of PDI (see 109 for more detailed arguments).

To date, the specificities of binding by PDI or of individual domains of PDI have not been determined. However, the specificity of substrate binding by the other PDI-family members PDIp (259) and ERp29 (16) have both been shown to have preferences for specific patterns of hydrophobic amino acids (see Section XI): Such a requirement for hydrophobic residues for substrate binding by the **b′** domain of PDI is consistent with published results (156, 158) and with work toward the identification of substrate specificity of PDI by using phage display libraries (A. Pirneskoski and L.W. Ruddock, unpublished observations).

D. Molecular chaperone and antichaperone activity

In addition to its ability to catalyze thiol-disulfide exchange reactions, PDI has also been suggested to have molecular chaperone activity (324). This is manifested during folding of proteins that contain disulfide bonds (242, 339, 342) and the ability to assist in the refolding of proteins with no disulfide bonds (30, 282). This activity does not depend on the catalytic domain active sites and is inhibited by the presence of a peptide substrate for the **b′** domain (244). A truncation to the C-terminus of PDI, which deletes the **c** region and the C-terminus of the **a′** domain, results in a loss of chaperone activity (51). This truncation also probably results in the loss of substrate binding by the **b′** domain due to destabilization of the **a′** domain (see earlier). The chaperone activity was reported to require full-length PDI (288). However, the domain boundaries used in this study were not correct, resulting in a truncated and probably nonfunctional **b′** domain. The action of PDI as a protein-folding catalyst requires it to interact with and bind to nonnative proteins, so a molecular-chaperone like activity must be intrinsic to its function as a protein-folding catalyst, a view backed up by the correlation between the identified substrate-binding sites in PDI (see earlier) and the regions required for the molecular-chaperone–like activity of PDI.

In addition to a chaperone-like activity, PDI has also been reported to have an antichaperone activity (242), which is inhibited by the **b′**-inhibiting C-terminal deletion that prevents chaperone activity (51, 281). This property is probably linked to the substrate binding required to act as a protein-folding catalyst. The mechanism of antichaperone activity of PDI for lysozyme was reported to be due to PDI forming cross-links, of unclear nature, between soluble aggregates of lysozyme to form a large insoluble aggregate containing a constant ratio of PDI and lysozyme (274). However, studies on other soluble, structurally sound, human PDI-family members, including ERp57 and ERp72, on the refolding of soluble BPTI-folding intermediates that do not intrinsically form aggregates, shows the rapid formation of BPTI-ERp57 and BPTI-ERp72 aggregates with no apparent defined stoichiometry (A.-R. Karala and L.W. Ruddock, unpublished results). This effect is probably due to the nature of the interaction between the substrate protein and the PDI-family

member, which disrupts the hydrophobic core of the substrate protein and may result in the exposure of hydrophobic residues on the surface of the complex, leading to aggregation. *In vivo*, or at high concentrations of PDI *in vitro* (242, 274), these exposed hydrophobic regions would be bound by another folding catalyst or molecular chaperone and so would not be exposed, and no aggregation would occur. The antichaperone activity of PDI may not be physiologically relevant.

VII. Conformational Change in PDI

Protein structures are dynamic in nature. All proteins have structures that constantly undergo small "breathing" motions, and many undergo larger conformational changes, such as induced-fit substrate binding, that are directly linked to their function. One of the early models for PDI was that the four domains formed "beads on a string" (*i.e.*, that they were linked by highly flexible regions). Such an arrangement would allow PDI the flexibility to bind to and act on its very wide range of protein substrates. This model slowly lost favor, as it was realized that the interdomain linker regions between the **a** and **b** and **b** and **b**′ domains were only a few amino acids long and so may not allow a very large degree of interdomain flexibility. Furthermore, the structures of broad-range molecular chaperones such as GroEL (75) revealed that although conformational exchange in folding factors occur during their catalytic cycle, they do not adapt themselves to the shape of the substrate. The opposite was shown (*i.e.*, that substrates changed conformation on interacting with GroEL) (344). Because unfolded or partially folded proteins are less stable than those in the folded state, they have intrinsically a greater degree of conformational flexibility; it is more logical that the substrate protein adapts its structure to that of PDI rather than PDI adapting its structure to that of the substrate protein. This is not to say that no conformational changes are found in PDI; at least four distinct conformational changes within PDI are probably all related to its function.

The active site of PDI, and other thioredoxin-superfamily members, exists in two redox states, the reduced dithiol state and the oxidized disulfide state, as well as in transient mixed disulfides. The oxidized and reduced species are able to catalyze different reactions, oxidation of protein substrate dithiols, and isomerization of protein substrate disulfides, respectively. The different redox states of PDI must interact with different protein substrates, or the same protein substrate at different stages of the folding pathway. In addition, PDI must interact with Ero1 (see Section VIII) only when it is in the reduced state. Very little is known about conformational differences between reduced and oxidized PDI. For other superfamily members, such as thioredoxin or DsbA, small, but distinct conformational differences exist between the oxidized and reduced states (106, 123). These are centered mainly on the active-site region, as would be expected, but at least in DsbA, they are also transmitted to the farthest point in the protein. Preliminary NMR studies have revealed that the **a** domain of human PDI also undergoes small, but distinct, conformational changes related to changes in redox state (K. Korhonen, R.A. Williamson, L.W. Ruddock, and M.J. Howard, unpublished results). It is not known to which extent these conformational changes are transmitted to other domains of PDI, but it should be remembered that even small local changes in protein structure can result in significant changes in distant parts of the protein structure. One report indicated that changes in the redox state of PDI influence substrate binding, with the reduced state of PDI binding to the A-subunit of cholera toxin, and the oxidized state releasing it (299). However, other studies revealed no such redox-dependent bind–release cycle and implied that the GSSG used to oxidize PDI can compete with protein substrates for the binding site in PDI (188).

The second conformational change that occurs in PDI is linked to the paradox of the pK_a of the C-terminal active-site cysteine. For catalysis of oxidation of a substrate protein to occur, the pK_a of the C-terminal active-site Cys must be high, so as to prevent the reverse reaction from occurring (see Fig. 12). However, in one of the steps of reoxidation of PDI, this cysteine must be in the thiolate state (see Fig. 12B). This implies that a significant change in the pK_a of the C-terminal active-site Cys (Cys 56 and Cys 400) must occur during the catalytic cycle of PDI. The pK_a value of chemical moieties varies depending on their local environment (see Section III). In the NMR structure of the **a** domain of PDI, Cys 56 is partially buried, consistent with the higher than usual pK_a of its thiol group. To reduce the pK_a significantly, the solvent accessibility of the thiol must be increased, and preferably, at least one positively charged group should be moved into its local environment. This positively charged group was identified as being the side chain of arginine 120 (178). This amino acid is very highly conserved in the human PDI family and beyond, but it had no known function. In the NMR structure of the **a** domain (148, 150), the guanido group of the arginine 120 side chain was located >17 Å from the active site and made no contacts other than with the solvent. However, molecular dynamic simulations revealed that the side chain of arginine 120 was highly flexible and that it could swing into and out of the active site locale (see Fig. 12E) (178). The pK_a calculations showed a shift in the pK_a of Cys56 from 12.8 to 6.1 between the structures found in NMR model 1 to that obtained in the closest approach of the arginine 120 side chain to the active site in the molecular dynamic simulations. A significant proportion of this shift was directly due to the positive charge on the arginine side chain rather than to other conformational changes. Such a shift in pK_a would potentially resolve the requirements for Cys 56 during catalysis, if it were linked with the appropriate phase of the catalytic cycle. Mutation of arginine 120 resulted in a significant decrease in the ability of PDI to introduce disulfide bonds into protein substrates, with a loss of >98% of its activity when the positively charged arginine was substituted with a negatively charged aspartic acid (178).

Other human PDI-family members similarly saw a significant reduction in activity on mutation of the analogous arginine residue. In addition to being linked to the reoxidation cycle of PDI, the movement of arginine 120 is also potentially linked to the release of kinetically trapped mixed-disulfide species between PDI and substrate proteins during isomerization (see Fig. 12D). This dual function implies a balance between the kinetics of oxidation (which require a fast deprotonation of Cys 56) and the kinetics of isomerization (which require a more-stable mixed disulfide between PDI and the substrate protein to be formed). However, this has yet to be shown experimentally. The positively charged amino acid, usually an arginine, is conserved in many catalytically active PDI-family members (71).

The third conformational change reported to occur in human PDI is linked to the substrate-binding site in the **b**′

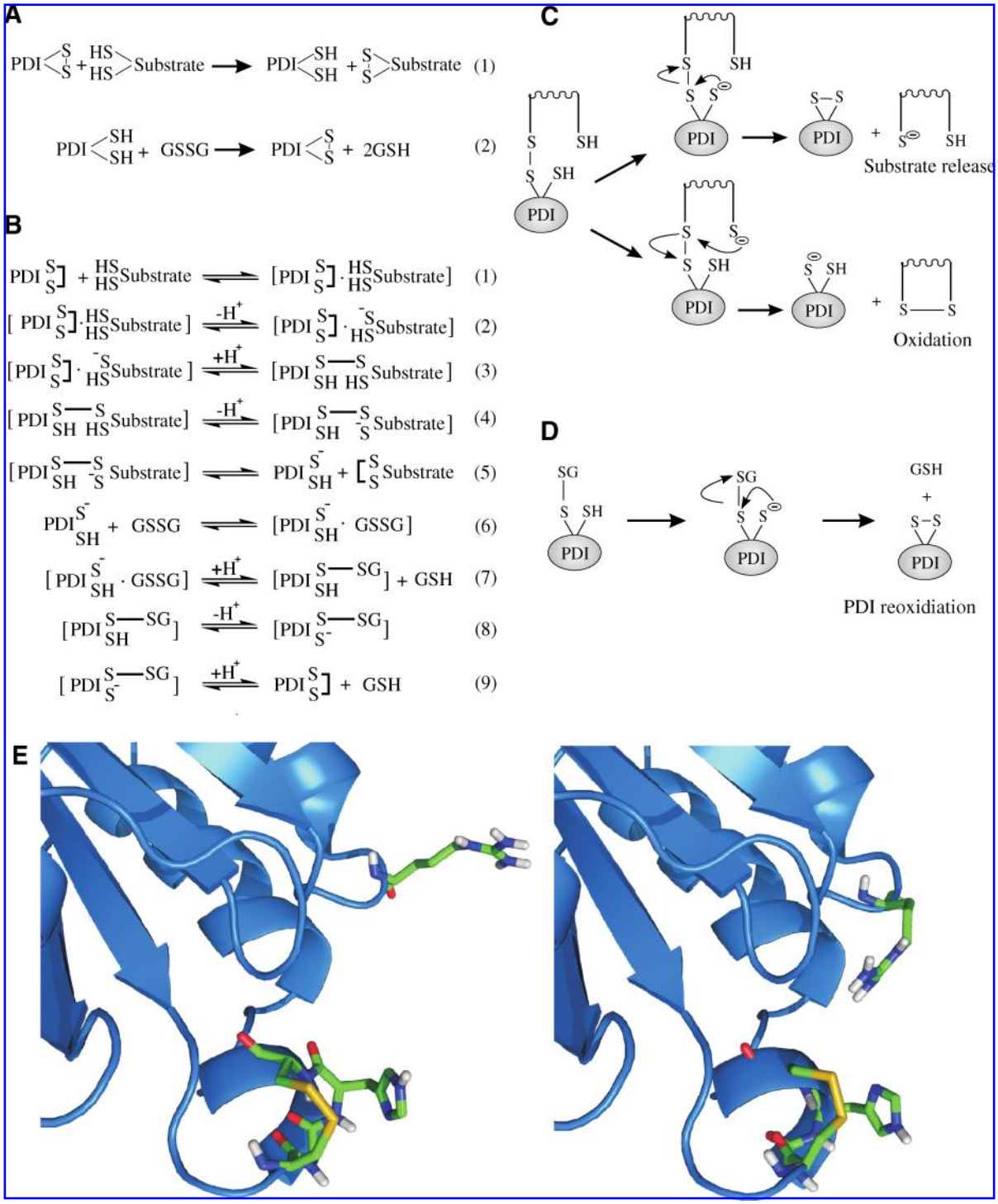

FIG. 12. The role of conformational exchange and modulation of the active-site cysteine pK_a values in the catalytic cycle of PDI. (**A, B**) Schematics of the redox cycle for the introduction of a disulfide bond into a substrate peptide or protein by PDI *in vitro* by using GSSG as the electron acceptor. (**A**) This shows the usual two-step reaction scheme. (**B**) This shows the minimal number of steps in the process, including the formation of the Michaelis-Menten complexes and proton abstraction. Because PDI is able to catalyze this reaction at pH values as low as pH 4.0, an efficient mechanism must exist for reducing the pK_a of substrate thiols or proton abstraction or both from these groups. The details of this mechanism are currently unknown. If the pK_a of the C-terminal active-site cysteine is high, the reverse of reaction 3 will be kinetically favored over reactions 4 and 5. However, for reaction 9 to proceed, a C-terminal active-site thiolate is needed. The pK_a of the C-terminal cysteine must be modulated during the catalytic cycle. (**C, D**) The change in the pK_a of the C-terminal active-site cysteine is linked to the "escape mechanism" releasing PDI from nonproductive mixed disulfides with substrate proteins and to oxidation of PDI by GSSG (or other oxidants including Ero1; see later). (**E**) Movement of the side chain of arginine 120 into the active-site locale. (*Left*) Structure of the NMR model structure of the **a** domain of human PDI with the lowest free energy; (*right*) structure from the MD simulation with closest approach of the arginine side chain to the active site (178). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

domain. The linker between the **b′** and **a′** domain is 19 amino acids in length and is the longest between the four domains of PDI (see Section V). This linker, known as the **x** region, contains a single tryptophan residue, Trp 364. Despite having only one tryptophan, the **b′x** domain construct gave a fluorescence spectrum with two tryptophan peaks, one in a hydrophilic environment, and the other in a hydrophobic environment (237). Biophysical and NMR analysis suggested that this was due to a dynamic conformational exchange in the **b′x** construct, an exchange that also occurred in a similar manner in full-length PDI (221). By using the tryptophan fluorescence spectra as a diagnostic, mutations were identified that seemed to lock the **b′x** construct into one of two conformers. Many of these mutations, including Ile272Ala, had been previously identified as inhibiting substrate binding by PDI (237). The subsequent crystal structure of the Ile272Ala mutant of human **b′x** (221) revealed a thioredoxin fold with a hydrophobic pocket, analogous to the substrate binding site identified in the structure of full-length Pdi1p (298), capped by the **x** region, in particular by Met 356, Leu 360, and Trp 364. The conclusions drawn from these studies were that the conformational change occurring in the **b′x** construct, and in full-length PDI, resulted from the **x** region moving from a conformer in which it capped the substrate binding site, to that observed in the crystal structure of Pdi1p, in which the substrate binding site was uncapped (see Fig. 13A). Such a change in conformation may be linked to the substrate bind–release cycle and may also modulate the specificity of substrate binding by PDI. The change in conformation of the **x** region between the "capped" and "uncapped" states would also lead to a significant movement of the **a′** with respect to the **b′** domain. This may be linked to a requirement to induce conformational changes in substrate proteins (see later) and may also be linked to our observed dramatic differences in the proteolytic stability of human PDI in the substrate-bound and substrate-free states (A. Pirneskoski and L.W. Ruddock, unpublished observations). A similar "capping" event of a hydrophobic pocket by a C-terminal extension to the **b′** domain has also been recently observed in the PDI-family member ERp44 (see Fig. 13B) (326) and a similar regulatory role in substrate interactions assigned.

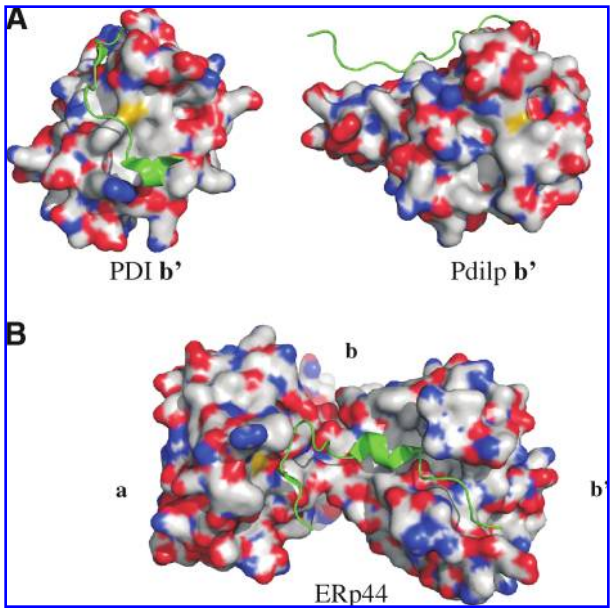

FIG. 13. Different conformers of the x region relative to the b′ domain. (**A**) The crystal structure of the Ile272Ala mutant of the **b′x** region from human PDI (221) on the left and the **b′x** region taken from the crystal structure of full-length Pdi1p (298) on the right. The x region is shown in green. (**B**) The C-terminal region of human ERp44, shown in green, caps a hydrophobic pocket in the second noncatalytic domain (326) and extends up to the active site in the catalytic domain. These capping events happen in the full-length proteins (221, 326) and are thought to be linked to the substrate bind–release cycle. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

One of the most important aspects of PDI function, rarely considered, is the ability of this enzyme to access buried disulfides and thiols in substrate proteins, and so the need to induce conformational change in the folding substrate. As discussed earlier, folding proteins are not in their most thermodynamically favored state, and it should be relatively easy to induce conformational change in what is essentially an unstable state. We recently identified a second conformational change in the catalytic domains of PDI, which contributes significantly to the ability of PDI to induce conformational change in a folding substrate (A.-R. Karala, M.F. Lensink, A.H. Juffer, and L.W. Ruddock, unpublished observations) and which we believe is conserved across most of the PDI family. However, clearly other factors are at work, and an intermolecular conformational change in the structure of PDI (*i.e.*, conformational change within one or more domains of PDI or an interdomain conformational change) would be a simple method for inducing conformational change in substrate proteins, especially if they were bound to all three substrate-binding sites in the **a**, **b′**, and **a′** domains (see Section V). We believe that this is linked to the conserved conformational change in the **x** region, which moves **a′** relative to the **abb′** fragment (see earlier). In contrast to this potential movement of **a′** relative to **abb′**, based on an alternative 3.7-Å resolution structure of Pdi1p, Tian and co-workers (297) hypothesized that the **a** domain of Pdi1p is able to adopt a very different orientation with respect to the **bb′a′c** fragment with a 22-Å dislocation of the center of gravity of the **a** domain between the two structures (see Fig. 14A). This result was surprising to the authors as they, and others, had previously speculated that the **a′** domain should be the more flexible because of the long connecting **x** region (298), whereas the **a-b** linker is only a few amino acids in length (see Section V). To confirm this result and to determine the physiological relevance, they then undertook additional experiments. First, they did protease digestions *in vitro* to confirm the greater flexibility of the **a** domain relative to **a′**. Unfortunately, the analysis of these digestions was indirect, using antibodies against tags found at the C- and N-terminus of the full-length protein, rather than direct, and these results may have been influenced by the presence of trypsin and chymotrypsin cleavage sites in the flexible **c**-region, resulting in the rapid loss of the C-terminal tag. Second, they introduced disulfide bonds into the proteins, designed to fix the conformation of the **a** and **a′** domains relative to the **bb′** fragment. These additional disulfide bonds reduced the activity of Pdi1p, with the largest effect being observed for the introduction of a

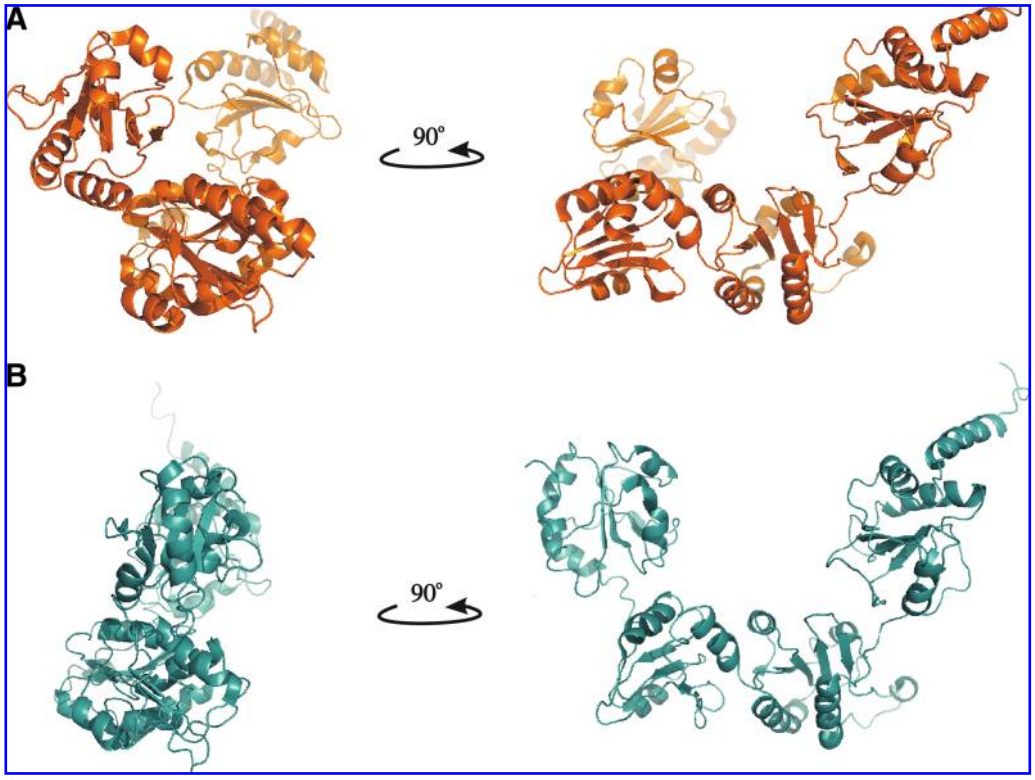

FIG. 14. The crystal structures of Pdi1p. (**A**) The "twisted U" structure reported to 2.4-Å (298) resolution. (**B**) The "boat" structure reported to 3.7 Å (297). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

disulfide that locked the conformation of the **a-**domain conformation. Surprisingly, these solvent-exposed disulfide bonds were stable in a buffer containing 1 m*M* GSH and 0.2 m*M* GSSG. Although these results are supportive of the authors' hypothesis, it should be noted that one of the residues chosen in the **a** domain to make the new structure-rigidifying disulfide bond, Glu 123, is in the same loop as Arg 126. This arginine is the residue analogous to Arg 120 in PDI whose motion into and out of the active-site locale modulates the pK_a of the C-terminal cysteine residue (see earlier). Furthermore, the reduction in activity seen in the Pdi1p mutant is comparable to that observed when the analogous arginine was mutated in the **a** domains of four human PDI-family members (178). The effect of the rigidifying mutation in the **a–b** interface in Pdi1p may result entirely from inhibiting the motion of the Arg 126 side chain during the catalytic cycle. Finally, it should be noted that Pdi1p is *N*-glycosylated, with four of the five potential *N*-glycan sites being in the **a** and **b** domains. The Pdi1p used for the crystallization trials was made in the cytoplasm of the gram-negative bacteria *Escherichia coli* and is therefore not glycosylated. In the original crystal structure (298), the *N*-glycan sites in the **a** and **b** domains are located near to, but not at the domain–domain interface. In contrast in the subsequent structure, with the **a** domain rotated by 123 degrees, the side chains of Asn 82, from the **a** domain, and Asn 174, from the **b** domain, are facing each other across the **a–b** interface and are separated by only 10.6 Å. It is unclear whether sufficient room exists in this structure for the *N*-glycans in this interface region. It is, therefore, unclear whether these results imply a further significant difference between PDI and Pdi1p, with respect to interdomain conformational flexibility, or whether they reflect different experimental methods.

Although *in vitro* analysis of PDI allows us to determine potential mechanisms of action of the protein, it must always be viewed as an artificial system, so correlations with the *in vivo* activities of PDI are essential. Before examining these *in vivo* activities, we must first examine in more detail the potential mechanisms of oxidation and reduction of PDI *in vivo* and the *in vivo* redox state of PDI.

VIII. Oxidation and Reduction of PDI *in vivo*

Over the past decade, a revolution has occurred in the perception of the physiologically relevant mechanisms for PDI oxidation *in vivo*. Prior to 1998, the consensus in the field was that the active sites of PDI were oxidized *in vivo* by reaction with GSSG (*i.e.*, by the same mechanisms by which PDI was oxidized *in vitro*). This viewpoint was overthrown by the discovery and subsequent characterization of the Ero1 subfamily of sulfhydryl oxidases. This change in viewpoint went so far that the GSSG present in the ER came to be regarded as a nonfunctional byproduct of the natural pathway for disulfide-bond formation and that any reactions of GSSG *in vivo* were either irrelevant or even possibly deleterious to the system. The actual physiologically relevant mechanisms for *in vivo* oxidation of PDI probably lie between these two extremes.

A. Ero1-based oxidation

The Ero1 family was first reported as a gene product in the yeast *S. cerevisiae* that was required for the formation of

disulfide bonds in the ER (84, 239). The yeast protein, Ero1p, is a highly glycosylated, cysteine-rich, membrane-associated enzyme that is essential for *S. cerevisiae* viability and for which homologues in a wide range of eukaryotic organisms were immediately identified. Overexpression of Ero1p gives increased resistance to the addition of the reductant DTT. It was subsequently reported that Ero1p is essential for the oxidation of Pdi1p and that Ero1p and an N-terminal active-site cysteine of Pdi1p form a mixed disulfide *in vivo*, implying that Ero1p directly oxidizes Pdi1p (85). Two distinct Ero1-family members, Ero1α and Ero1β, are found in mammals, and both are able to complement several phenotypic traits associated with the functional loss of Ero1p in *S. cerevisiae* (29, 230). It is still unclear why we need two Ero1-family members. However, Ero1α expression is induced by hypoxia (98) and ER stress (198), whereas Ero1β expression is induced by stress resulting from an accumulation of unfolded proteins in the ER (230). Ero1α was subsequently shown to form a mixed disulfide complex with wild-type PDI *in vivo* (21). Because this complex was observed only 1 h after the start of the pulse-chase, it was concluded that the Ero1α–PDI complexes were the intermediates in the oxidation of PDI by Ero1α (see Fig. 15) and not the alternate possibility that they represented Ero1α being a folding substrate for PDI. Both Ero1α and Ero1β were then shown to be involved in the oxidation of PDI, but not of the PDI-family member ERp57 (209). Ero1 family members have a large number of cysteine residues, and many of these were found to be essential for the folding and function of Ero1p (86) and Ero1α (21). These cysteines form a number of distinct disulfide-bonded states *in vivo*, states that were subsequently found to be regulatory (see later). The next big step forward was the publication by Tu and co-workers (302) that identified Ero1p as a FAD-binding protein and showed that Ero1p plus PDI and FAD allowed the effective *in vitro* reconstitution of a pathway for oxidative-folding protein by using RNaseA as a substrate. Mutagenesis studies of Ero1α and Ero1p (23, 272), aided in part by the crystal structure of Ero1p (104), revealed a complex pathway of intra- and intermolecular disulfide-bond transfer between the FAD cofactor and PDI or Pdi1p. Furthermore, a strong preference exists for Ero1p to oxidize the active site of the **a**′ domain of Pdi1p, and Ero1α to oxidize the **a**′ domain of PDI *in vitro* (169, 325) because of the asymmetry of the structure of the PDIs and of the location of this domain next to the **b**′ domain, which also is required for Ero1α–PDI interactions (325). Open questions still exist regarding the *in vivo* electron acceptor used by Ero1-family members, but *in vitro* molecular oxygen can be efficiently used, as can flavins and certain exogenous redox metalloproteins, such as cytochrome *c* and cytochrome *b5* (105, 303). The low K_m value of Ero1p for molecular oxygen implies that oxygen is likely to be at least one of the electron acceptors for the Ero1 family *in vivo*, except under anaerobic or severe hypoxic conditions. The use of molecular oxygen as an electron acceptor by Ero1 has one potentially lethal side effect: for each disulfide bond made, it makes one molecule of hydrogen peroxide (see Fig. 15A), as for other sulfhydryl oxidases (125). The production of potentially harmful reactive oxygen species (ROS), combined with the need for the reduction potential of the ER to be optimal for native disulfide-bond formation (see Section VI), implies that the activity of Ero1-family members must be highly regulated. Early studies implied that multiple distinct disulfide-bonded states of Ero1 exist (21) and that at least one of these probably represents the quiescent state. These regulatory disulfides were subsequently identified and character-

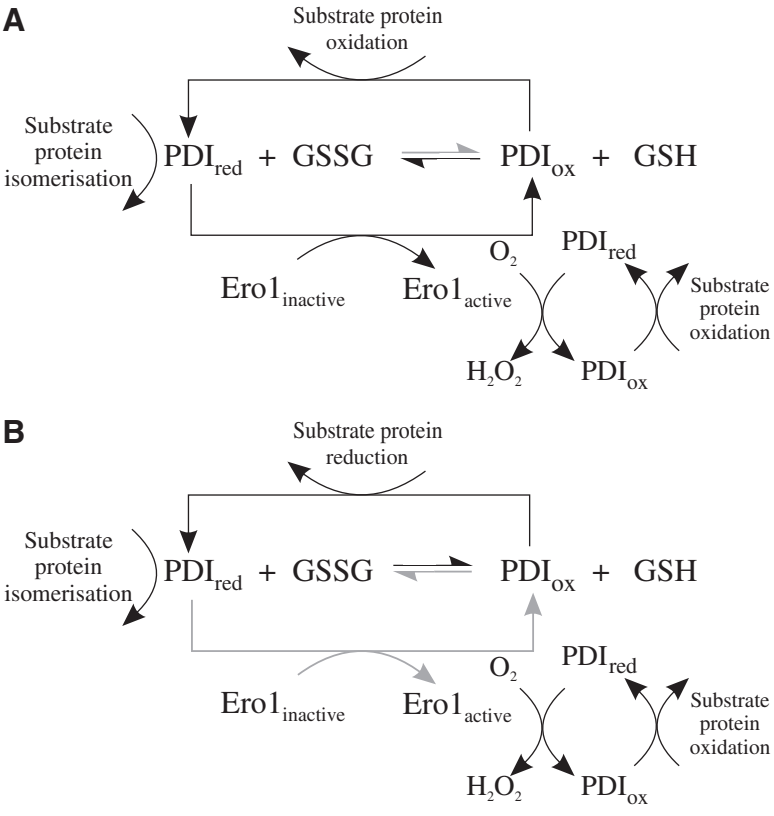

FIG. 15. Schematic representations for a role for the glutathione buffer in regulating Ero1 activity *via* a PDI family member. (**A**) If GSSG levels are low, or large amounts of substrate protein oxidation by PDI is occurring, then the PDI/glutathione redox equilibrium is pushed to the left. This results in (a) an increase in the level of reduced PDI that can be oxidized by ERo1 and help to keep Ero1 in the active state; and (b) the production of GSSG. (**B**) If GSSG levels are high, or substrate protein oxidation rates are low, *e.g.*, less PDI-substrates are being made. Then the PDI/glutathione redox equilibrium is pushed to the right. This results in (a) an increase in the level of oxidized PDI; (b) the production of GSH; and (c) the inactivation of Ero1. The system is self-regulatory to provide the optimal reduction potential for oxidative protein folding in the ER. The system will also work without glutathione, but the buffering capacity of the system is much lower; it will be subject to wilder oscillations, and it will be less adaptable to changes in the rate of production of disulfide bond–containing proteins.

ized for Ero1p (273) and human Ero1α (10, 13), with regulation being based on the same mechanism for both (*i.e.*, the formation of regulatory disulfide bonds), but the position of these being different. It is still unclear why human and yeast Ero1-family members have evolved different regulatory disulfides or what the consequences of these differences are for disulfide-bond formation in the ER.

B. GSSG-based oxidation

The primary redox buffer in the ER is thought to be based on the oxidized and reduced states of glutathione. Whereas the cytoplasm is a highly reducing environment with <1% of the glutathione present in the oxidized GSSG state, the ER is more oxidizing, with ~25% of the ER-resident glutathione thought to be present as GSSG (20, 130). *In vitro* GSSG is able to oxidize the active site of PDI efficiently (see Section VI), and for many years, it was thought to be the primary oxidant of PDI *in vivo*. However, whereas Ero1p is an essential gene product in yeast (84, 239), Gsh1p, the first enzyme in glutathione biosynthesis, is not (226). This led to the idea that GSSG may not be directly involved in native disulfide-bond formation in the ER, but rather that its production is competing with disulfide-bond formation in folding proteins (see ref. 33 for a recent review on the role of glutathione in disulfide-bond formation). The evidence for this comes from several indirect sources that are linked, not to the function of GSSG, but to the function of GSH. GSH is a reductant; *in vitro,* it is able to reduce disulfide bonds in folding proteins and the active-site disulfide in PDI, and it is essential for the isomerization reactions that many proteins require to reach the native disulfide-bonded state (see Section VI). A similar function would be expected *in vivo*, as the ER probably contains millimolar concentrations of GSH (20, 130). The first evidence for a role of GSH *in vivo* was reported from *S. cerevisiae*. Here the production of cellular GSSG was shown to be linked to Ero1p activity (50), most likely through the reduction of disulfides in folding proteins or in PDI-family members (*i.e.*, the direct oxidation of GSH by Ero1p is limited (303). Furthermore, in a screen for suppression of the growth defect of the *ero1-1* temperature-sensitive Ero1p mutant, from 21,000 transposon insertions, all nine positive mutants were linked to insertions in the gene for Gsh1p (50) (*i.e.*, inhibition of glutathione biosynthesis rescued the viability of a strain harboring a mutated Ero1p). Furthermore, the maturation of a widely used model protein for monitoring disulfide-bond formation, carboxypeptidase Y, showed a strong defect in the *ero1-1* mutant, but in the wild-type strain and in the *ero1-1 gsh1* double-mutant, maturation was complete by the first time point of the assay. These results were interpreted to mean that GSH acts as a competitor with protein thiols for Ero1p, with the GSSG produced being secreted from the ER. However, these results do not show that GSSG has no function in the ER during disulfide-bond formation, especially because the disulfide bond in GSSG may be transferred directly, or indirectly *via* a PDI-family member, to a folding protein. In mammalian systems, cytoplasmic GSH has been shown to limit disulfide-bond formation in the ER of HeLa cells; specifically, it was required to maintain PDI and Ero1α in their physiological redox states (214). Reducing the level of cellular glutathione in CHO cells resulted in acceleration of oxidative folding, after treatment with the reductant DTT, but not in the rate of native disulfide-bond formation (32). These results suggest that glutathione is directly linked to the reductive or isomerization pathway or both *in vivo*. Glutathione has also been shown to reduce directly the PDI-family member ERp57 *in vivo* (135), and presumably, PDI is reduced *via* the same direct mechanism, as well as indirectly *via* reduction–oxidation cycles with folding proteins. This is required to keep at least part of the population of PDI, or other PDI-family members, in the dithiol state required to catalyze thiol-disulfide isomerization reactions (see Sections VI and IX).

How do the *in vivo* GSSG data correlate with data from *in vitro* kinetics? The second-order rate constant for oxidation of the active site of the **a** domain of PDI by GSSG *in vitro* is $188\,M^{-1}s^{-1}$ (146). Although the absolute concentration of GSSG *in vivo* is not known, it has been estimated to be at least 2.5 m*M* (with data from ref. 20). This would imply a pseudo first-order rate constant for oxidation of PDI by GSSG at physiological conditions of at least 0.5 per s and a maximal half-time for oxidation of 1.5 s. In contrast, the rate of oxidation of PDI by Ero1α *in vitro* is relatively slow. The maximum turnover for Ero1α by using PDI as a substrate was once every 20 s (13). Given that PDI is in large molar excess over Ero1α *in vivo*, the half-time for oxidation of PDI by Ero1α would be measured in minutes. The *in vitro* data therefore suggest that the physiologically relevant oxidant for PDI *in vivo* is GSSG and not Ero1. How do we then reconcile these data? One issue clearly is that *in vitro* systems at best only approximate *in vivo* conditions. Furthermore, we do not currently know whether essential modulatory components are missing from the *in vitro* system, or even whether molecular oxygen is the terminal acceptor for Ero1 *in vivo* (see earlier). It is possible that *in vivo* Ero1-family members are able to oxidize PDI faster than GSSG can and that the physiological route for the oxidation of substrate proteins under normal conditions does not directly involve GSSG. However, when examining this system, several key facts should be remembered.

1. GSSG is not an oxidase or a net oxidant. The oxidation of the active site of PDI by GSSG is a net isomerization reaction; with the formation of the disulfide bond in PDI being accompanied by the reduction of GSSG to GSH. Thus, GSSG cannot be the net oxidant in the system for the production of disulfide bonds, but it can be an intermediary.
2. The oxidant for converting GSH to GSSG is the subject of much debate in the literature, but two widely acknowledged routes are the reduction of (nonnative) disulfide bonds in folding proteins and the reduction of the active site of PDI-family members. In both of these cases, the pathways can be traced back to Ero1, consistent with the published link between Ero1 activity and cellular GSSG levels (50).
3. Although the absolute concentration of GSSG and GSH in the ER lumen is not known, the *in vivo* ratio between these species and estimates of their concentration (20, 130) are consistent with the optimal concentrations of these species required for efficient native disulfide-bond formation *in vitro* (see Section VI). Furthermore, no reports exist of naturally occurring variations in this ratio with varying production of secreted disulfide-bonded proteins. This implies that the ratio is probably carefully regulated and does not solely arise with the production of GSSG as a byproduct of native disulfide-bond formation.

4. Ero1-family members are membrane-associated proteins. Although disulfide-bond formation occurs cotranslationally, we suppose that many of the reactions connected with native disulfide-bond formation of soluble proteins happen in the ER lumen. This would either require two pools of PDI, one associated with cotranslation disulfide-bond formation and one with subsequent formation of native disulfide bonds, or for PDI to cycle back between the substrate in the lumen and Ero1 on the membrane. Such a requirement for PDI cycling seems inefficient, especially given the very high intracellular protein concentrations, which reduce the rate of diffusion. It would be more efficient to use the glutathione redox buffer to change the redox state of PDI in the lumen as required.
5. *In vitro*, the rate-limiting steps associated with native disulfide-bond formation are late-stage events in substrates with a high degree of native-like secondary structure (see Section VI). If this is mirrored *in vivo*, and it is likely to be, then *in vivo* oxidative events could have their efficiency significantly reduced without an overall effect on the rates or yields of native disulfide-bond formation.

Given these arguments, it is possible that both GSSG and GSH are involved in the physiological oxidation, reduction, and isomerization events in native disulfide-bond formation. If so, this would primarily be *via* a PDI-family member, because the rates of their direct reactions with folding substrate proteins are low [*e.g.*, whereas the second-order rate constant for GSSG reacting with reduced PDI is $188\,M^{-1}s^{-1}$, the initial oxidation of reduced BPTI by GSSG is only $7.3\,M^{-1}s^{-1}$ (146)]. This difference is probably determined primarily by the 20-fold difference in the proportion of the thiolate state of the nucleophilic cysteines in PDI and an unfolded protein (see Section VI). In addition, glutathione could serve multiple other purposes in the ER: (a) It could act as a redox buffer, buffering the ER against both oxidizing and reducing agents; (b) It could act as a reservoir of both oxidizing and reducing potential, allowing the cells to react rapidly to changes in the levels of production of disulfide bond–containing proteins; (c) Via the oxidation and reduction of PDI family members, it could regulate the activity of Ero1 (see Fig. 15B). Under resting conditions, the activity of Ero1 and the reduction potential of the glutathione buffer would be intimately interlinked *via* PDI-family members. If the rate of production of secreted disulfide bond–containing proteins increased, the initial effects would be a net decrease in GSSG concentrations, which in turn would result in the activation of Ero1 (*via* PDI) until redox homeostasis was resumed. Similarly, if the rate of production of secreted disulfide bond–containing proteins decreased, the initial effects would be a transient net increase in GSSG concentrations, which would lead to the increased net inactivation of Ero1 (*via* PDI) until redox homeostasis was resumed. Although cells lacking glutathione would be able to survive and to produce disulfide-bonded proteins *via* Ero1–PDI alone, they would lack the ability to adapt smoothly and rapidly to changes in disulfide-bond protein production and to other changes in redox conditions. This would be especially important when considering the redox conditions in the microenvironment around the membrane-associated Ero1, which would oscillate more wildly than the bulk ER. The finding that a major fraction of the glutathione in the ER may be in mixed disulfides with proteins (20) supports the hypothesis of a direct role for GSSG in native disulfide-bond formation; however, this article described microsomal preparations, and the validity of the results for the ER have been questioned (65).

C. Peroxide-based oxidation

Other systems may also result in oxidation of PDI *in vivo*. *In vitro* sulfhydryl oxidases, including Ero1-family members, make one molecule of hydrogen peroxide per disulfide bond. Although it has yet to be shown that hydrogen peroxide is similarly generated during disulfide-bond formation *in vivo*, the assumption in the field is that it is produced. This peroxide generation has been widely viewed as a harmful byproduct, such that the formation of native disulfide bonds in folding proteins is thought to cause oxidative stress in cells (116, 304). However, peroxide is an oxidant that can lead to the formation of disulfide bonds from cysteine thiols *via* the formation of a sulphenic acid intermediate (see Fig. 16A). The exogenous addition of hydrogen peroxide to cells results in the widespread formation of both protein sulphenic acids (27, 34) and protein disulfides (48, 267). It is possible that any peroxide generated by Ero1 during disulfide-bond formation *in vivo* is used in the formation of disulfide bonds. Recently, *in vitro* data supporting this hypothesis were published (146), with Karala and co-workers showing that peroxide added directly to folding BPTI or generated *in situ* by the action of

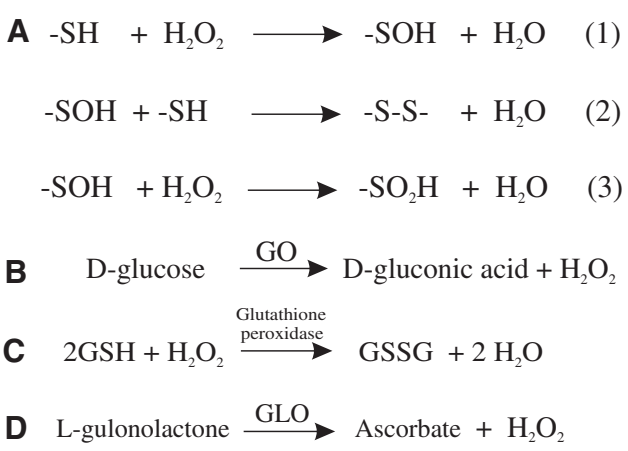

FIG. 16. Schematic representations of reactions involving peroxide. (**A**) The formation of disulfide bonds by using peroxide. The initial reaction is the reaction of peroxide and a cysteine thiol group to form a cysteine sulfenic acid intermediate. This species either can undergo another intermolecular reaction with peroxide to generate the nonproductive cysteine sulfinic acid species (reaction 3), or it can undergo an intra- or intermolecular reaction with another cysteine thiol to generate a disulfide bond (reaction 2). (**B**) The *in situ* formation of hydrogen peroxide by the action of glucose oxidase on glucose is similar to the formation of hydrogen peroxide by the action of Ero1 during disulfide-bond formation. (**C**) Catalysis of peroxide-mediated glutathione oxidation by glutathione peroxidases. (**D**) The formation of hydrogen peroxide by the action of GLO on L-gluconolactone in the formation of ascorbate (vitamin C) in the ER. It is not known whether the peroxide generated in this reaction is used productively.

glucose oxidase and glucose (see Fig. 16B), was sufficient to result in the efficient formation of a natively folded protein containing three disulfide bonds. At pH 7, peroxide-mediated oxidation was much faster than that mediated by a glutathione redox buffer. Furthermore, as long as the peroxide was not present in large excess, no detectable formation of dead-end cysteine sulphenic acid species and minimal side reactions, such as methionine oxidation, were seen. Peroxide was also able to oxidize the active site of PDI, or less efficiently to oxidize GSH to GSSG. It was also speculated that the peroxide generated may be able to oxidize the regulatory disulfides in Ero1, providing an efficient negative-regulation feedback mechanism. *In vivo* data supporting the notion that the peroxide generated during disulfide-bond formation may be involved in disulfide-bond formation is limited; however, two supporting pieces of evidence exist. First, two ER-resident human glutathione peroxidases have been reported as part of a study on KDEL variants for ER localization (252). Because glutathione peroxidases catalyze the peroxide-mediated oxidation of GSH (see Fig. 16C) their existence in the ER implies a functional role for peroxide in disulfide-bond formation, especially when combined with an observed *in vivo* interaction of these proteins with Ero1α (V.D. Nguyen, H.I. Alanen, and L.W. Ruddock, unpublished data). Second, in a landmark study by Malhotra and co-workers (197), it was shown that the overproduction of a protein containing eight disulfide bonds did not result in oxidative stress in cell culture or in mice, whereas the overproduction of a protein that misfolded in the ER did result in oxidative stress. Disulfide-bond formation in the ER *per se* does not result in oxidative stress *in vivo*. These results imply that very efficient cellular mechanisms must exist either for the disposal of peroxide generated by Ero1 during disulfide-bond formation *in vivo* or for productive utilization of this peroxide. It should also be remembered that other processes in the ER generate peroxide (for example, the last step of ascorbate biogenesis by L-gluconolactone oxidase (GLO) results in the formation of one molecule of hydrogen peroxide per ascorbate (see Fig. 16D) (184 for a review of ascorbate metabolism in mammals). It is unclear whether this peroxide is also used productively, but it should be noted that the plant homologues of mammalian GLO are found in the inner mitochondrial membrane and transfer electrons directly to cytochrome *c* rather than oxygen, and therefore, do not make hydrogen peroxide as a byproduct of the reaction (276, 347). It is possible that GLO or Ero1-family members or both have similar alternative electron acceptors and do not produce peroxide in significant quantities *in vivo*.

D. Other oxidation systems

Ascorbate also features in another possible route for PDI oxidation *in vivo*. In the 1960s and early 1970s, dehydroascorbate (DHA) was used as the net oxidant in disulfide bond formation *in vitro* (see refs. 100, 277, 283, and 317 as examples). DHA is one of the oxidized forms of ascorbate formed during its function as a cellular antioxidant (see Fig. 17A) (184). DHA is unstable in aqueous solutions, undergoing a hydrolysis reaction with a reported half time of ~100 min at 20°C, pH 7 (26). DHA is also able to react with thiols to form disulfide bonds and ascorbate (see Fig. 17B), although the uncatalyzed reaction with cellular thiols such as glutathione is

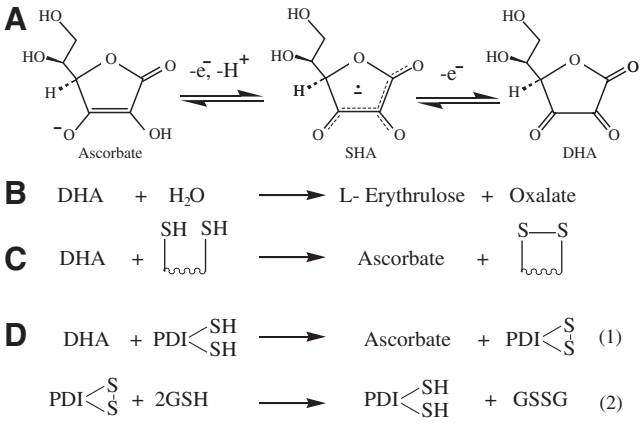

FIG. 17. Schematic representation of reactions involving DHA. (**A**) The oxidative states of ascorbate, including dehydroascorbate (DHA) and semidehydroascorbate (SHA). (**B**) DHA is unstable in aqueous buffers undergoing a hydrolysis reaction. (**C**) The reaction of thiols with DHA to form a disulfide bond. (**D**) Reaction scheme for the two-step process whereby PDI acts as a dehydroascorbate reductase *in vitro.*

thought to be slow (338). PDI and glutaredoxin (a thioredoxin-superfamily member whose primary function is thought to be the catalysis of deglutathionylation reactions) are regarded as dehydroascorbate reductases (76, 333, 341). The initial step in the reaction is the reduction of DHA to ascorbate, resulting in the concomitant oxidation of the active site of PDI (see Fig. 17C). *In vitro*, the oxidized PDI is reduced by GSH to form GSSG, but oxidized PDI can also introduce disulfide bonds into folding proteins. The potential function of DHA as an oxidant of PDI *in vitro* and *in vivo* has largely been overlooked for the past 40 years. One group has repeatedly proposed an *in vivo* role for DHA in disulfide-bond formation (for examples, see refs. 14, 15, 45–47, 219, and 289), but this has been largely ignored by the field, in part because of the lack of supporting data and the gaps in the model proposed [for example, the identity of the metalloenzyme required to oxidize ascorbate in the ER microsomal preparation (14)], but also in part probably owing to the timing of this work against the proven *in vivo* and *in vitro* role of Ero1 in PDI oxidation (see earlier). However, this area deserves further investigation and recognition.

Other potential oxidation systems have been mentioned in the literature. One for which some supporting experimental evidence exists is based on vitamin K. The term vitamin K covers a collection of related compounds (see Fig. 18A). One of these, vitamin K_3, otherwise known as menadione, is routinely used as a strong oxidant in the study of the effects of overoxidation on disulfide-bond formation. The dione structure of menadione is similar to that found in DHA, which also acts as an electron acceptor in disulfide-bond formation *in vitro* (see earlier). One of the physiological functions of vitamin K is to play a role in the biogenesis of γ-carboxyglutamate–containing proteins. This reaction is a two-step process (see Fig. 18B). The reduced form of vitamin K, vitamin K hydroquinone, is used by the vitamin K–dependent γ-carboxylase as a cofactor during γ-carboxylation. The vitamin K 2,3-epoxide formed from this reaction is then reduced back to vitamin K hydroquinone by the action of vitamin K 2,3-epoxide reductase (VKOR) (227, 255). *In vitro*, the electron acceptor in

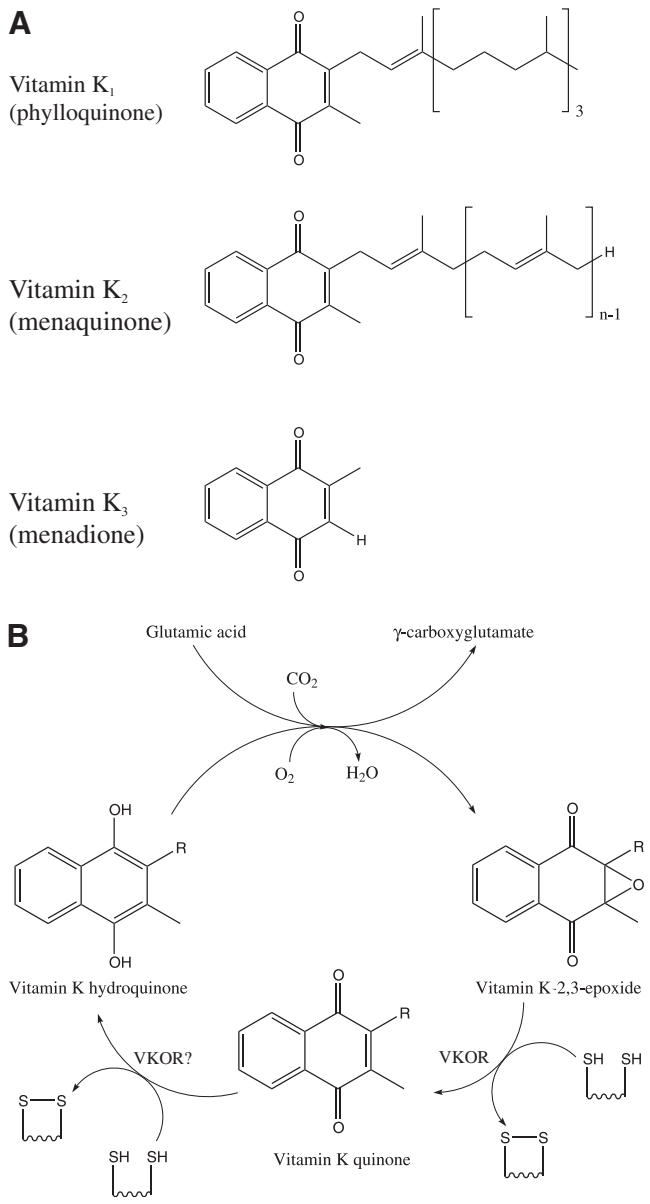

FIG. 18. The potential role of vitamin K in disulfide-bond formation. (**A**) The structures of vitamin K_1, K_2, and K_3. (**B**) Schematic representation of the redox cycle of γ-carboxylation connected with disulfide-bond formation. The upper part of the cycle is catalyzed by vitamin K–dependent γ-carboxylase, whereas the lower part of the cycle is catalyzed by vitamin K 2,3-epoxide reductase. *In vitro* DTT is able to act as the necessary electron donor, whereas *in vivo* PDI has been shown to be in a complex with VKOR and is therefore thought to be the electron acceptor (320). This would link γ-carboxylation with oxidative protein folding in the ER.

this process is DTT. It was proposed that *in vivo* thioredoxin played the role of electron acceptor (139), but it was subsequently shown that the thioredoxin–thioredoxin reductase combination was unable to act in this manner in intact microsomes (241). Recently, PDI was shown to be in a complex with VKOR (320), so γ-carboxylation is probably linked to disulfide-bond formation in the ER.

Other evidence for a role of vitamin K in disulfide-bond formation is forthcoming from prokaryotic systems. Very recently Dutton and co-workers (68) showed in a landmark article that a bacterial homologue of VKOR may functionally replace the transmembrane protein DsbB (which plays a role in disulfide-bond formation in the periplasm similar to that Ero1 plays in the ER). In addition, they found that most bacteria that lack a DsbB homologue, and that would be predicted to make disulfide bonds, have a VKOR homologue, and, in some cases, this VKOR homologue was found fused to a DsbA homologue. Although no evidence was presented that menaquinone, rather than another quinone, was the electron acceptor in this system, it strongly suggests a direct role for vitamin K in disulfide-bond formation in at least some prokaryotes.

Under what conditions could vitamin K play a major role in disulfide-bond formation in eukaryotes? Menaquinone (vitamin K_2) is synthesized by bacteria and used for anaerobic respiration (see ref. 306 for a review). For example, in *E. coli*, a shift occurs in the quinone intermediate used in respiration from ubiquinone under aerobic growth to menaquinone under anaerobic growth by using fumarate as the electron acceptor. It would, therefore, be tempting to speculate that vitamin K may play a role as a cofactor in a process for disulfide-bond formation under hypoxic or anoxic conditions or both. This has yet to be examined experimentally.

E. Reduction of PDI

Three possible sources exist for the formation of the dithiol state of the active site of PDI. The first is the catalysis of oxidation of protein substrates, during which the active site converts from the disulfide to the dithiol state. The second option is reduction of the active-site disulfide by GSH. *In vitro*, GSH is able to reduce the active sites of the isolated **a** and **a′** domains of PDI with a second-order rate constant of the order of $200\ M^{-1}s^{-1}$ at pH 7.4 (53). GSH also reduces the active site of the PDI-family member ERp57 *in vivo* (135) and acts as a "competitor" for oxidative protein folding (see earlier). The pK_a of glutathione is 8.75. The higher than average pK_a of the thiol in glutathione is probably a major factor in determining the kinetics of disulfide-bond formation in the ER. If glutathione had a typical pK_a value, the kinetics of reduction and isomerization of disulfide bonds in folding intermediates by glutathione would be much faster and would compete more with oxidation processes. The third possibility is the existence of an ER protein with similarity to the cytoplasmic enzyme thioredoxin reductase, which could use NAD(P)H to reduce the active site of PDI. This possible option has yet to be confirmed experimentally.

IX. The Redox State of PDI *in vivo*

The redox state of the active sites of PDI in the ER is another subject on which considerable differences of opinion are held. Some of these differences may be in the method or reagents used; some, due to real differences in the redox state of PDI in different cell types or under different conditions; but some are undoubtedly due to the technical challenges involved in obtaining an accurate picture of this highly dynamic system.

The active sites of PDI can exist in four potential redox states (see Fig. 19A); both active sites in the dithiol reduced state, both in the oxidized disulfide state, with the **a** domain active site in the oxidized state and the **a′** domain in the reduced and with the **a** domain in the reduced state and the **a′** in

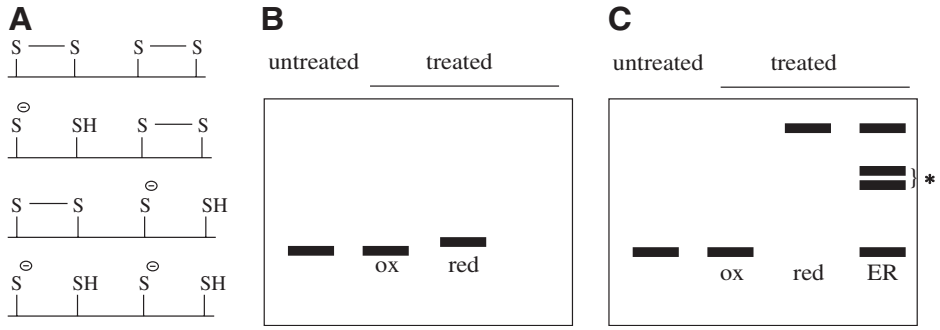

FIG. 19. Determination of the *in vivo* redox state of PDI. (**A**) Schematic representation of the four potential redox states of PDI *in vivo*. The two cysteine residues in the **b**′ domain have not been reported to exist in different redox states *in vivo*. Only the dithiol and disulfide redox states of the active sites are shown. Other states, such as mixed disulfides with glutathione or other proteins, or the cysteine sulfenic and sulfinic acid states of the active-site cysteines of PDI, also may exist. (**B, C**) Schematic representation of the observed shift in molecular weight by SDS-PAGE after the determination of the redox state of PDI by thiol reactive reagents. Ox, the oxidized state of PDI (*i.e.*, both active sites in the disulfide form; red, the reduced state of PDI (*i.e.*, both active sites in the dithiol form). (**B**) The reaction with 4-acetamido-4′maleimidylstilbene-2-2′-disulfonic acid. This has a molecular mass of 356 Da and gives a small shift in apparent molecular weight with SDS-PAGE (135). (**C**) Methoxy polyethylene glycol 5,000 maleimide. This has a molecular mass of 5 kDa, but results in much larger shifts in apparent molecular weight of PDI with SDS-PAGE and allows the resolution of the different intermediate redox states (*i.e.*, one active site in the oxidized form and one in the reduced) (8). The species marked *represent one active site in the dithiol state and the other in the disulfide state. Note that these schematics represent the idealized situation. In practice, the bands are often either very weak or poorly resolved. These issues are not helped by the heterogeneity of the molecular weights of the polyethylene glycol maleimide compounds used or by the potential for significant batch-to-batch variation.

the oxidized state. In addition, the N-terminal active-site cysteines in the **a** and **a**′ domains may be in mixed disulfides with Ero1α and β, GSSG, and substrate proteins.

The method for determining the redox state of the active sites and trapping mixed disulfides *in vivo* is essentially the same as that *in vitro*. A thiol-reactive reagent is added to the system and reacts with the active-site cysteines in the reduced or mixed disulfide states, but not in the disulfide state. To trap the thiol-disulfide state, *in vitro* reagents such as iodoacetamide, iodoacetic acid, and *N*-ethylmaleimide are often used, and these are also used for cell-based systems, although only *N*-ethylmaleimide is membrane permeable. Acid quenching is also frequently used, often in combination with subsequent neutralization and reaction with a chemical quencher. Because these reagents do not result in significant changes in molecular weight, other maleimides, such as 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid (for example, see 135) or methoxy polyethylene glycol 5,000 maleimide (for example, see 8), are used to allow rapid identification of the trapped dithiol/disulfide state of PDI with SDS-PAGE (see Fig. 19B and C) (for an overview, see 8).

Determining the redox state of PDI or trapping PDI-mixed disulfides *in vivo* by direct chemical trapping is extremely technically challenging. The first consideration is the ability to pass a thiol-reactive reagent across multiple cellular membranes into the ER and at high enough concentrations to quench the system rapidly. Although the exact concentration of reactive thiol groups in the ER is not known, and will vary depending on cell type and the amount of disulfide-containing proteins that it is producing, from a combination of the glutathione and thiol groups on proteins, the minimum estimate of ER thiol concentration must be on the order of 20 m*M*. Any thiol-reactive reagent must be present in excess of this. *In vitro* reagents, such as iodoacetamide, are often used at concentrations as high as 1 *M*, but even this is insufficient to prevent disulfide rearrangement (see Section VI). *In vivo* reagents are often used at much lower concentrations, typically 10–50 m*M*, including sometimes at concentrations below the concentration of free thiols in the system. This reduces the chance of obtaining physiologically relevant information.

The second consideration is the kinetics of the system. Accurately to determine the redox state of PDI *in vitro*, any quenching reaction must happen on a faster time scale than thiol-disulfide exchange. *In vitro*, and presumably *in vivo*, thiol-disulfide exchange reactions occur on the second time scale. For example, the nucleophilic attack of the N-terminal active-site cysteine of PDI on GSSG, with GSSG approaching the best estimates of physiological concentrations, has a half-time of $<$1.5 s (see Section VIII). Similarly, the nucleophilic attack by the C-terminal cysteine on the PDI–glutathione mixed disulfide has a half-time of $\sim$2.6 s *in vitro* at pH 7 (146). By comparison the half-time for the reaction of the N-terminal active-site cysteines (a highly reactive thiol group) with 20 m*M* iodoacetamide at physiological pH would be $\sim$3 s.

The third consideration is the accessibility of the C-terminal active-site cysteine of PDI. In the structure of the **a** domain of PDI, this is only partially solvent accessible (148, 150). This allows it to do its function (*i.e.*, to exist either in the free thiol state or in a disulfide with the N-terminal cysteine, but not to form mixed disulfides with other species). However, this inaccessibility, combined with the high pK_a of the C-terminal cysteine (see Section V), makes the C-terminal cysteine relatively unreactive with thiol-modifying reagents. Because the C-terminal cysteine in the PDI–glutathione mixed disulfide is not accessible to reaction with thiol-reactive reagents *in vitro*, even at concentrations of up to 1 *M* (see Section VI), this implies that at physiological concentrations of GSSG and physiological pH, the window of opportunity to trap, effectively and quantitatively, the reduced state of PDI is less than the half-time of the first step of oxidation of PDI by GSSG (*i.e.*, $<$1 s). Currently the kinetics of reduction of oxidized PDI by physiological concentrations of GSH is not known, but it, too, presumably occurs on the second or subsecond time scale. It is unclear what the trapping time of any system *in vivo* is, so the accuracy of determining the absolute redox state of PDI

in vivo is open to question, but certainly qualitative results can be obtained.

The most accurate systems are probably those based on acid quenching of thiol-disulfide exchange reactions followed by rapid processing, addition of a large excess of chemical quenching reagent, and neutralization of the pH to allow the quenching reaction to occur. In a recent study, Appenzeller-Herzog and Ellgaard (8) were able to show for the first time that all four possible thiol/disulfide redox states of PDI exist *in vivo*. Furthermore, these states in relative ratios are consistent with the expected ratio from the reduction potential of PDI and the possible reduction potential of the ER, as defined by glutathione (see Section IV).

In addition to the problems associated with determining the redox state of PDI *in vivo*, considerable confusion exists in the field regarding the significance of the *in vivo* redox state of PDI and how this relates to the function of the protein. For example, in a highly simplified scheme, PDI can be considered to be predominantly in the reduced state or predominantly in the oxidized state. The reduced state of PDI is linked to its ability to catalyze isomerization or disulfide reduction reactions, whereas the oxidized state is linked to its ability to introduce disulfide bonds into substrate proteins. It is, therefore, easy to fall into the trap of saying that if, for example, PDI exists predominantly in the reduced state, that the primary function of PDI *in vivo* is to act as an isomerase. This is, however, misleading without an understanding of the kinetic steps in the various processes that result in this steady-state measurement and especially without knowing which steps are rate limiting *in vivo* (see Fig. 20). Even if the *in vivo* redox state can be determined accurately, the existence of one predominant redox state does not directly, by itself, give information on function. However, vital information on the interconnectedness of *in vivo* pathways and function can be obtained from examining how the *in vivo* redox state of PDI changes as a function of manipulating the environment of the cell (for example, overexpressing an Ero1-family member or a substrate protein) (10).

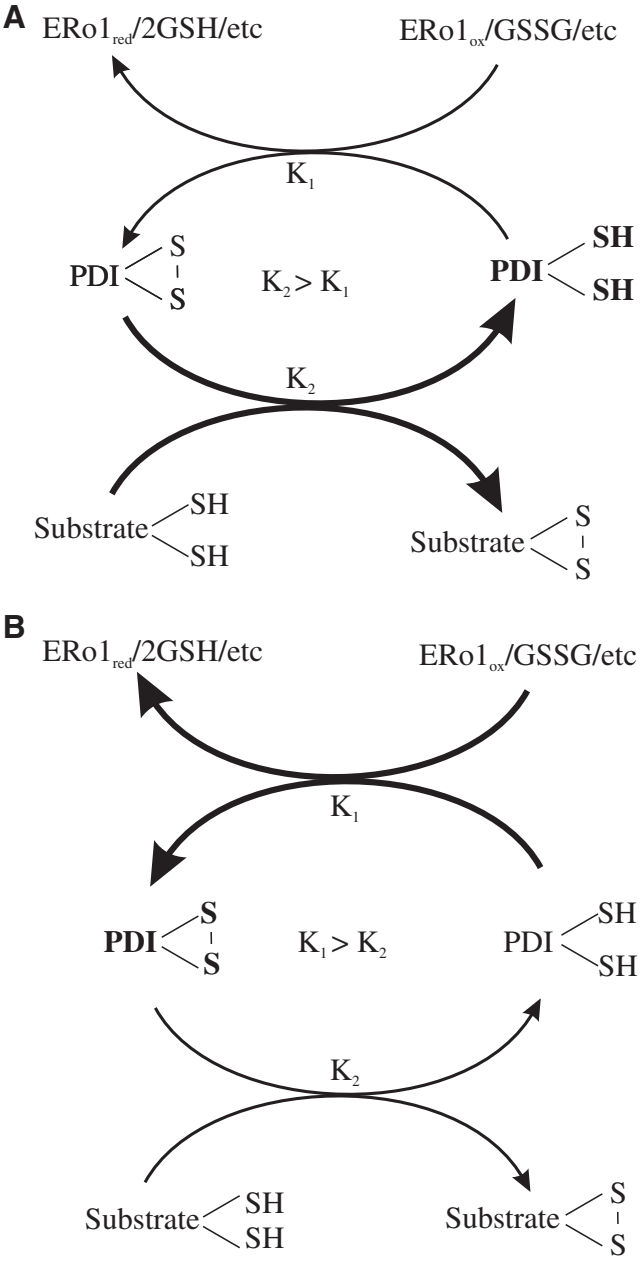

FIG. 20. Schematic representation of the potential significance of the *in vivo* redox state of PDI. The *in vivo* redox state of PDI is often assumed to be an equilibrium system, allowing the determination of relative reduction potentials and function. In practice, the steady state is probably not a thermodynamic equilibrium. For example, if PDI is predominantly in the reduced state, the state that is involved in isomerization reactions, it may be assumed that the primary function of PDI *in vivo* is isomerization. However, this overlooks the relative kinetics of the system, which is often unknown. Two different scenarios may be envisaged, in both of which, the primary function of PDI is the oxidation of substrate proteins. Either the rate of oxidation of substrates by PDI is faster than the rate of oxidation of PDI required to complete the catalytic cycle (**A**) or the rate of oxidation of PDI is faster (**B**). The scheme in (**A**) results in PDI being predominantly in the dithiol state—which is also the state required for the catalysis of isomerization reactions—whereas the scheme in (**B**) results in PDI being predominantly in the disulfide state. Although both scenarios are based on the same physiological function for PDI, they give different steady-state redox states for PDI *in vivo*.

X. *In vivo* Thiol-Disulfide Exchange Reactions of PDI

A direct role for PDI in catalyzing thiol-disulfide exchange reactions *in vivo* was elegantly shown in two landmark articles. First, it was shown that defective co-translational formation of disulfide bonds exist in γ-gliadin in PDI-deficient microsomes, a defect that could be reversed by the addition of purified PDI (28). Second, the formation of mixed disulfides between PDI, and the PDI-family member ERp57, with folding proteins was observed in living cells (213). Despite these studies, relatively little work has been done on determining the nature of the reactions that PDI catalyses *in vivo,* in part because of the complexity of mammalian systems and the lack of a viable PDI-knockout strain. In contrast, a considerable number of apparently contradictory reports exist on the essential function of Pdi1p, and an examination of these can be used to gain some idea of the *in vivo* function of PDI.

Pdi1p has been recognized as an essential gene product since it was first identified by multiple groups in 1991 (74, 175, 293). Although now a common consensus agrees that the essential function of Pdi1p is related to its function in native disulfide-bond formation, considerable disagreement concerns whether this represents its ability to oxidize protein substrates or its ability to isomerize protein substrates (35, 173, 174, 280, 340). The truth is that both are physiological functions and that trying to define the "essential" function can be misleading. However, because this forms a significant section of the literature, it should be examined.

Because Ero1p is an essential gene product (84, 239), which appears to be an inefficient electron acceptor from GSH (272) or from folding substrate proteins, any disruption of the ability of Pdi1p to interact with Ero1p, or to transfer the disulfide bond from Ero1p to either substrate proteins or glutathione, would be expected to be lethal. The ability to act as an oxidant, or, more specifically, to act as a transfer molecule between Ero1p and folding proteins, is almost certainly a physiological function of Pdi1p (and PDI). This can be used as an argument that it is the essential function of the protein. Similarly, *in vitro* at physiological pH, Pdi1p (and PDI) has significant catalytic activity as an oxidant; however, by far the biggest fold difference between the catalyzed and uncatalyzed rate is for isomerization reactions, with some *in vitro* uncatalyzed reactions having half-times measured in days (see Section VI). The isomerization of nonnative disulfide bonds is almost certainly a physiological function of Pdi1p (and PDI).

So how do we explain the results that show that either oxidation or isomerization are not essential *in vivo* functions of Pdi1p? These conclusions were reached on evidence derived from studies using different constructs to rescue the viability of a *PDI1*-null or disrupted strain. Three distinct classes of construct have been used, active-site mutants of Pdi1p, domain constructs of Pdi1p and PDI, and the use of other thioredoxin-superfamily members.

The initial work reported was based on truncations and mutations of Pdi1p (174). This showed that truncations of Pdi1p from the C-terminus down to the equivalent of an **a-b-b′** construct were able to rescue the viability of the *PDI1* deletion, whereas shorter constructs, equivalent to either **a** or **a-b** were not. Similarly, an N-terminal truncation of Pdi1p that expressed the equivalent of only the **a′** domain was unable to replace the essential function of the full-length protein (108). Because *in vitro* analysis of both Pdi1p (298) and PDI (56) have shown that the **b′** domain, in a linear combination with either the **a** or **a′** domains, is required for efficient isomerization (see Section VI), these *in vivo* results are consistent with the essential function of Pdi1p being isomerization. An identical conclusion can be reached by examining the active-site mutants presented in the same initial article; full-length Pdi1p with both active sites mutated to be similar to Eug1p, including the loss of the C-terminal cysteines, were able to rescue viability (174), as per Eug1p overexpression (291). This construct will lack the ability to act as an oxidant, but retains isomerase activity (see earlier). This conclusion also was reached by using mutants in Pdi1p in which only the N- or C-terminal active-site cysteines had been mutated (173). Mutant forms of thioredoxin, in which the active site had been modified to CGHC or CGPS, but not SGPS, were also able functionally to replace the essential function of Pdi1p, although the doubling time of the strains were significantly slower (35). This implies that an enzymic thiolate is all that is required, consistent with the essential function being isomerization and not oxidation. Similarly, although the **a** or **a′** domains of rat PDI or the **a** domain of Pdi1p was able to complement the essential function of Pdi1p when overexpressed from a strong promoter on a multicopy-number plasmid (280, 340), they were not able to when expression was from the *PDI1* promoter on a low-copy-number plasmid. Because the isolated **a** and **a′** domains of PDI are as active as the full-length protein in various oxidation assays (see earlier), this requirement for significant overexpression is not consistent with oxidation being the essential function. However, the overexpression requirement is consistent with the isolated domains having low intrinsic isomerase activity and requiring a higher concentration of enzyme to catalyze the essential activity (see Section VI). Although detailed studies on relative protein levels were not undertaken, from combining *in vitro* experimental data on the relative activities of the proteins with the presented *in vivo* data on viability, it can be estimated that as little as 10–20% of the intrinsic isomerase activity of endogenous Pdi1p may be required for viability. This does not imply that only 10–20% of the isomerase activity of Pdi1p is used under normal physiological growth, as clear evidence indicates that, in many of these rescued strains, native disulfide bond formation is compromised (173), and the growth rate can be more than fourfold slower (35). However, 10–20% activity represents the minimum for cell viability under standard laboratory conditions in rich media.

This analysis leaves us with another question. How are constructs such as Eug1p or CGPS thioredoxin able to support the viability of a *PDI1*-null or disrupted strain if the construct replacing Pdi1p lacks the C-terminal active-site cysteine required for the flow of electrons from folding proteins to Ero1p? Two answers to this question are possible. First, potential mechanisms exist by which a one-thiol thioredoxin-superfamily member can act as a catalyst of substrate protein dithiol-disulfide oxidation *in vivo* (see Fig. 21). However, these have not been demonstrated *in vitro*. Second, Pdi1p does not exist in isolation in the ER. For example, it is possible that under physiological *in vivo* conditions, Ero1p would be able to directly oxidize GSH to GSSG, although it does not do this

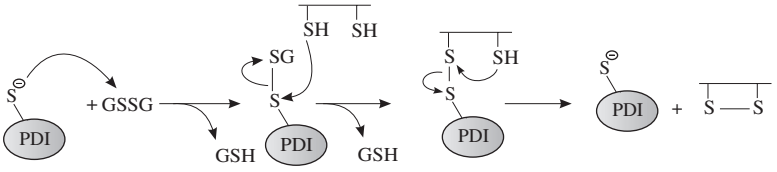

FIG. 21. Hypothetical schematic by which a mutant form of PDI with only the N-terminal cysteine can catalyze oxidation of a protein or peptide dithiol to a disulfide by using GSSG as the electron acceptor (*in vivo* GSSG could be replaced in this scheme by ERo1). It is currently unclear why C-terminal cysteine mutants of PDI are so inefficient at catalyzing this reaction.

efficiently *in vitro* (272). Pdi1p is also only one member of the yeast PDI family, and it has been shown that another family member, Mpd2p, is essential for Pdi1p with its C-terminal active-site cysteines mutated to replace endogenous Pdi1p functionally (225). Because a C-terminal active-site mutant of Mpd2p has been found to be in a mixed disulfide with Ero1p (85), the most likely explanation is that Ero1p is able to use Mpd2p as well as Pdi1p to transfer disulfide bonds to folding proteins.

Is PDI essential? Whereas reports of knockout cells and animals have been found for other ER-resident protein-folding catalysts and molecular chaperones (for example, 61, 96, 207), no such report exists for a knockout of PDI. Given the primary role of PDI, such an omission probably implies that such knockouts are not viable. Furthermore, PDI knockdown studies using RNAi show that efficient knockdown is linked to cytotoxic effects (231). Both of these suggest that PDI is an essential gene product, and, given the similarities between PDI and Pdi1p, it is likely that the physiological function is two-fold: to act as an isomerase and as a direct, or indirect, transfer molecule between Ero1α and folding proteins (*i.e.*, to act as a protein dithiol-sulfide oxidant).

To date, very few natural substrates for PDI have been reported (12, 64, 213, 257, 312), although the assumption from the promiscuity of the enzyme *in vitro* and its apparently essential nature *in vivo* is that a very significant number of proteins that fold in the ER will require PDI for disulfide-bond formation. A number of studies implicating PDI-family members in thiol-disulfide exchange reactions, especially outside the ER, are based heavily on the use of inhibitors. However, care must be taken in the interpretation of *in vivo* results based solely on the use of inhibitors; thiol-modifying agents will alter thiol-disulfide exchange reactions irrespective of whether PDI is catalyzing the reaction, and a strong argument can be made that bacitracin, the one "selective" inhibitor of PDI, is neither an effective inhibitor nor selective for PDI (110).

XI. Other PDI-Family Members

Although PDI was, for a long time, considered to be the sole enzymatic catalyst of thiol-disulfide exchange reactions in the ER, it has been clear for more than two decades that it is just part of an ever-growing family, a family named after the archetypal and most abundant member, the PDI family.

The physiological functions of the PDI-family members are far from well defined. For many of them, it is possible to state reactions that they can catalyze, but whether they actually catalyze these reactions under normal physiological conditions is unclear. What is clear is that different organisms have different families of PDI, some of which may be connected in function, such as human PDI and yeast Pdi1p, but many of which are unique to a subset of organisms. Because each organism has a unique set of PDIs, some focus must exist. In this review, the focus is on mammalian PDI-family members and, in particular, on human PDI-family members. However, it is worth saying a few words first about the PDI-family members found in *S. cerevisiae*, as they are among the best characterized as a grouping from a single organism.

Saccharomyces cerevisiae has four soluble PDI-family members, Pdi1p, Eug1p (291), Mpd1p (294), and Mpd2p (292), along with the transmembrane protein Eps1p (327), all of which are N-glycosylated, unlike PDI, which is not. Any of these family members can rescue the viability of a *PDI1*-null or disrupted strain when highly overexpressed. However, in a landmark study, Nørgaard and co-workers (225) found that the ability of different family members to rescue viability was, in part, dependent on the presence of low endogenous levels of one or more other family members. This implies that the different yeast PDI-family members are not functionally interchangeable.

Eug1p shares the same overall domain architecture as Pdi1p and PDI and shows 42.5% identity over the region **a-b-b′-a′**, but has active sites, WCLHS and WCIHS, that lack the C-terminal cysteine. This suggests that Eug1p may have isomerase activity but not the ability to catalyze protein dithiol-disulfide oxidation. However, wild-type Eug1p has little activity in the refolding of the yeast protein procarboxypeptidase Y (oxidation or isomerization) or in the insulin-reduction assay (224), raising questions as to its physiological role.

Mpd1p and Mpd2p are small, two-domain PDI-family members with active-site sequences WCGHC and CQHC, respectively. Mpd1p associates with Cne1p (153), the yeast homologue of calnexin (compare with ERp57 later) and is the only yeast PDI-family member that can rescue strains deleted of any of the other family members when placed under the control of the *PDI1* promoter (225). The crystal structure of Mpd1p was recently solved to 2.0-Å resolution (318). This structure revealed that Mpd1p comprises one catalytic and one noncatalytic domain, both with thioredoxin-like folds. The relative orientation of these domains is significantly different from that found in other multidomain PDI-family member structures solved to date. Relatively little is known about Mpd2p. However, the CQHA active-site mutant of Mpd2p forms a mixed disulfide with Ero1p (85). Furthermore, mutation of the active sites of Pdi1p to CGHS restricted the ability of the gene to rescue a *PDI1*-null strain to systems in which Mpd2p was present (225). These two results imply that, in this strain, Mpd2p acts as the direct or indirect transfer molecule between Ero1p and folding proteins when Pdi1p is unable to act in this way (see Section X).

Eps1p is a large transmembrane PDI-family member. It has been implicated in ER quality control (327, 328) and may play a role in presentation of misfolded proteins to the degradation machinery. However, the precise role of Eps1p in this process is unclear, and it should be noted that homologues of Eps1p can be found only in Saccharomycetales.

Whereas *S. cerevisiae* has only five PDI-family members, humans currently have 20 defined PDI-family members. These are defined by similarity to PDI and localization in the ER rather than by physiological function. All of the human PDI-family members therefore contain at least one domain that is similar to one of the four domains of PDI (see Fig. 22). Most contain at least one catalytic domain, but several have either no catalytic domain or have lost one or both of the active-site cysteines in **a**-like domains. Detailed studies on most of the human PDI-family members have not yet been performed, and for many, we currently have a collection of observations rather than detailed coherent proposals for their unique physiological functions. Because they all share similarity to at least one domain of PDI, they also often share broadly similar catalytic abilities. For example, recombinantly expressed and purified human PDI, ERp57, PDIp, ERp72, and P5 all show a remarkably similar ability to catalyze the formation of a disulfide bond in a peptide substrate *in vitro* by

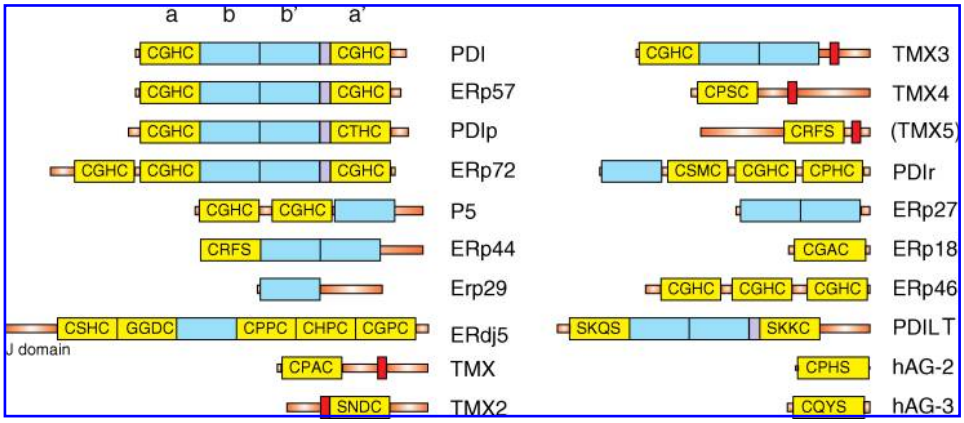

FIG. 22. The human PDI family (adapted from 71). Catalytic domains that probably have a thioredoxin-like fold are shown in yellow; noncatalytic domains that probably have a thioredoxin-like fold are shown in blue; transmembrane regions are shown in red. The active-site sequence is written in each catalytic domain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

using GSSG as the net electron acceptor (4). Given the similarities in reduction potential (as measured or as predicted from sequence) and *in vitro* activities, it is likely that all of the human PDI-family members with a WCGHC active site (*e.g.*, PDI, ERp57, PDIp, ERp72, P5, ERp46, and TMX3) will be able to catalyze the same thiol-disulfide exchange reactions as PDI *in vitro*, but differences may be found in their relative kinetics for oxidation, reduction, and isomerization. However, being distinct proteins with unique features and differing tissue distribution, all PDI family members should have distinct physiological functions, although these may partially overlap. It is likely that the physiological functions of the WCHGC active-site PDI-family subgroup will be determined not by differences in thiol-disulfide chemistry but also by their interactions with other proteins (*e.g.*, substrate specificities, interactions with Ero1, and other ER-resident chaperones). In some cases, we know that specialization has occurred, for example, in the substrate-binding **b**′ domains of ERp57 and PDIp (see later), but in many cases, the molecular characterization is far from complete, or raises as many questions as it answers.

As per PDI, many PDI-family members have been implicated in a wide range of physiological functions, but here we concentrate on those related to protein folding and in particular to the catalysis of thiol-disulfide exchange. We start the examination of other human PDI-family members with the two that show the same architecture as PDI (*i.e.*, **a-b-b′-a′-c**, ERp57, and PDIp).

A. ERp57

ERp57 (122), (also known as ERp60, ERp61, GRP57, GRP58, HIP-70, and Q-2) was initially identified as a phosphoinositide-specific phospholipase C (22). Despite this inauspicious start, ERp57 rapidly became one of the most-reported human PDI-family members. It is very similar in length and domain architecture to PDI, with the most striking difference being that whereas the **c** region of PDI is highly acidic, that of ERp57 contains multiple lysine residues. In addition, ERp57 has a structural disulfide bond in the **a** domain (90), a bond found in the same position in many other PDI-family members, including Pdi1p, in which it destabilizes the **a** domain (337). The active-site residues of ERp57, WCGHC, are the same as those of PDI, and *in vitro* titrations against glutathione show that the catalytic domains have reduction potentials of −167 and −156 mV, respectively, for the **a** and **a**′ domain (90), similar to those of PDI (see Section V). Although ERp57 can functionally replace DsbA in the periplasm of *E. coli* (90), it, unlike PDI or ERp72, cannot functionally replace the essential function of Pdi1p (108), nor can it replace PDI as the β-subunit of P4H (238). Both of these findings suggest that ERp57 and PDI have different functions *in vivo*.

ERp57 has been assigned an even greater range of functions outside the ER than has PDI itself. These fall outside of the range of this review, but again, it is unclear how ERp57 moves to other cellular compartments, although it is the only PDI-family member that has a C-terminal motif that is inefficient at ER retrieval (252). In addition, these functions are often assigned to ERp57 based on general thiol-reactive reagents or from the use of the peptide "PDI-specific inhibitor" bacitracin at concentrations of up to 7 mg/ml (see 110 for a discussion on bacitracin as a PDI inhibitor).

ERp57 is involved in MHC class I folding and forms part of the MHC I peptide-loading complex along with calreticulin, TAP, and tapasin (232), although the exact function of ERp57 in this complex is the subject of significant discussion in the literature. Tapasin is a potential substrate for ERp57, and it forms a very stable mixed-disulfide complex with ERp57 (233). Very recently, the crystal structure of ERp57 and tapasin, linked by a disulfide, has been solved to 2.6-Å resolution (66). The structure shows extensive contacts between the two molecules, which the authors suggest accounts for the inactivation of the escape pathway (see earlier). Given the supposed specificity of the ERp57–tapasin complex, it is surprising that all of the contact residues in ERp57 are conserved in PDI and ERp72.

ERp57 is most associated with the oxidative folding of glycoproteins (see ref. 258 for a review of the quality control cycle for glycoprotein folding). The involvement of ERp57 in the folding of glycoproteins was first demonstrated by chemical cross-linking of ERp57 to nascent glycoproteins in microsomes (229) and further substantiated in a subsequent study in living cells (213). It was subsequently shown that ERp57 interacts with the ER lectins calnexin and calreticulin (228), and detailed analysis of the ERp57 engagement with viral glycoproteins indicated that ERp57 acts co-translationally with these lectins (212). Both calreticulin and calnexin contain a remarkable armlike domain called the P-domain. ERp57 binds to the P-domain with a K_d of 26 μM (166) and 10 μM (91) for calnexin and calreticulin P-domains, respectively. The ERp57 interaction site has been mapped with NMR to near the tip of this highly acidic and proline-rich arm (91). By a combination of a cross-comparison with PDI and mutagenesis

studies, a binding site in the **b′** domain of ERp57 was identified as the lectin P-domain interaction site (264). This result implied that the **b′** domains of PDI and ERp57 have become specialized in function relating to direct and indirect substrate recognition. Subsequently, the interaction site was mapped out with NMR and found to be predominantly in the **b′** domain of ERp57, although a single lysine in the **b** domain also contributes to the interaction (166).

As is the case for other ER housekeeping enzymes, the deletion of ERp57 caused embryonic lethality in mice (96), suggesting the importance of this enzyme in the early development and differentiation of embryonic cells. In contrast, ERp57-deficient cell lines are viable and show no gross cellular defects. Furthermore, examination of protein-folding pathways in these cells did not show a global folding perturbation, with only a few proteins showing significant changes in folding and with ERp72 being recruited to assist in the folding of orphan ERp57 substrates (279). ERp57 has been shown to associate by mixed disulfide bonds with proteins that traverse the secretory pathway. Examples include ER-resident proteins, plasma surface-membrane proteins, secretory hormones, and endocytosed viral proteins. One intriguing example was shown in a study to dissect the stepwise entry and uncoating program of Simian Virus 40, a DNA virus that enters the cell through the endocytic vascular system (268). In this study, it was shown that ERp57 functions in a calreticulin/calnexin-independent manner, to isomerize disulfide bonds in the viral capsid, which allows viral access to the cytoplasm. In another elegant study, Jessop and co-workers (136) trapped ERp57-substrate mixed disulfides by using a C-terminal active-site cysteine mutant and thereby identified a range of ERp57 substrates *in vivo*. These included integrins, laminin, collagen, clusterin, and others. Although this technique does not trap all possible substrates (see ref. 109 for discussion), it does offer a unique insight into the possible range of substrates that a PDI-family member may have. In a follow-up study, this substrate specificity of ERp57 was shown to be determined primarily by its interaction with calnexin and calreticulin (137).

B. PDIp

Human PDIp is one of the few PDI-family members that show very-specific tissue distribution. Its name derives from the fact that it was initially reported to be expressed only in the acinar cells of the pancreas (62, 63), although current database analysis implies expression in both the pancreas and the brain, with potentially lower levels also found in organs adjacent to the pancreas, such as the stomach and bladder. PDIp shares the same domain architecture as PDI, and the **a-b-b′-a′** fragment has 49.5% identity with PDI. PDIp does have a number of unique features. For example, the **a′** domain has a unique WCTHC active-site motif, the **c** region is much less acidic than that of PDI, and an additional acidic N-terminal extension occurs before the **a** domain.

The unusual tissue distribution of PDIp led to the speculation that it evolved to fold a subset of pancreatic enzymes that PDI was unable to (*i.e.*, that it would have a different range of substrate specificities). In microsomes, PDIp is able to bind peptides derived from a range of proteins including prolactin, procecropin, glycophorin C, pro-α-factor (72, 319), and *in vitro,* to peptides from pancreatic digestive enzymes, including pancreatic lipase, pancreatic α-amylase, cholesterol esterase, colipase, pancreatic trypsin inhibitor, and somatostatin (159). This binding can be inhibited by a wide variety of estrogens and xeno-estrogens (159) and other hydroxyaryl-containing compounds (155), which partially mimic the natural tyrosine and tryptophan recognition motifs used by PDIp to bind peptides (259).

The physiological function of PDIp is still unclear, but two potential pathological roles have been reported. First, PDIp was the only one of eight PDI-family members tested to be upregulated in a cellular experimental model for Parkinson disease, and it was found in Lewy bodies from the brains of postmortem human Parkinson disease subjects (39). It is unclear whether this association is a net effect of the disease state or contributes in some way to it. Second, in an animal model for type 1 diabetes with a loss of function of autoimmune regulator, the major targets of autoimmune destruction were acinar cells, and this was linked to the production of an autoantibody against PDIp (222).

C. ERp72

ERp72, also known as CaBP2, was one of the first PDI-family members reported (203), but it currently remains the poor cousin of ERp57, with which it shares considerable sequence homology. The domain organization and active-site chemistry suggest that it can catalyze thiol-disulfide exchange reactions similar to those of PDI. ERp72 has been shown to have such activity *in vitro* (1, 167, 192, 263), and it can complement lethality of yeast deficient in Pdi1p *in vivo* (108). The sequence homology between ERp72 and ERp57 extends to the **b′** domain (71) and, in particular, to residues implicated in ERp57-calnexin/calreticulin interactions (see earlier). Although no direct interaction between ERp72 and calnexin or calreticulin has been reported under normal physiological conditions, ERp72 at least partially functionally replaces ERp57 in knockout cell lines (279). In addition, ERp72 has been reported to be in several complexes, which include calreticulin or calnexin but not ERp57 (183, 290, 311)

ERp72 forms a complex with PDI, P5, ERdj3, BiP, CypB, HSP40, GRP94, GRP170, UDP-glucosyltransferase, and SDF2-L1, both in the presence (where the complex also includes ERdj3) and in the absence of an immunoglobulin heavy chain (208). ERp72 has also been reported in association with a number of wild-type or mutant proteins including thrombospondin (170), thyroglobulin (206), and matrilin-3 (41), and presumably, this is linked to its ability to catalyze thiol-disulfide exchange. Reducing the levels of ERp72 by siRNA enhanced retrotranslocation of cholera toxin (81) and boosted SV40 infectivity (268), the former by an unknown mechanism, and the latter, probably linked to a lowered capacity to reduce disulfide bonds.

D. P5

P5, also known as ERp5 and CaBP1, is, like ERp72, one of the less well studied and known PDI-family members despite being the third one identified, after PDI and ERp72 (192). Like ERp72, its active-site chemistry in the two catalytic domains implies that P5 should be able to catalyze thiol-disulfide exchange reactions, and indeed, *in vitro*, it can (4, 152, 167, 192, 263). P5 is the smallest of the family members with both catalytic and noncatalytic **a**- and **b**-like domains. P5 has been

reported to be in the same complex as ERp72 (see earlier). P5 is upregulated by XBP-1 as part of the unfolded protein response (179), suggesting a physiological role in protein folding. However, the best known reports for P5 function are outside the ER and include participation in a complex with PDI, ERp72, and BiP, which occludes the cytosolic face of the translocation protein Sec61p (286), a role in shedding tumor-associated ligands and tumor metastasis (107, 138, 142) and a role in platelet function (140). It is unclear how a protein that contains a classic KDEL ER-retrieval signal (94) escapes the ER.

E. ERp44

Human ERp44 was first reported in 2002 (6). It is 408 amino acids in length, with homologues being found across metazoans. The gene is localized to chromosome 9, and transcription is under an ER stress-response element (343), $CCAACN_9CCCAG$, which is similar to that of PDI, $CCAACN_9CCCCG$. The protein is highly expressed in secretory tissues and during B-cell and adipocyte differentiation (329). Although the protein has an efficient ER-retention motif (252), the endogenous protein is localized mainly to the ER-Golgi intermediate compartment and to the *cis*-Golgi, as visualized by immunofluorescence (7, 329). However, overexpressed ERp44 largely localizes to the ER.

The crystal structure of ERp44 was recently solved to 2.6-Å resolution (326). The three domains (**a-b-b′**) form a V-shaped molecule, with most of the features seen in other PDI-family members (*e.g.*, thioredoxin fold, *cis*-proline) being conserved. However, the single active-site sequence is unusual, with the WCRFS motif lacking the C-terminal active-site cysteine. Also lacking is the buried salt bridge under the active site implicated in modulating the function of the C-terminal cysteine (see Section V). The crystal structure of ERp44 reveals that the C-terminus of the protein loops back to occlude the putative peptide-binding site in the **b′** domain and partially to shield the active site in the **a** domain. The occlusion of the **b′** domain is similar to that seen in PDI (see Section VII). Although the protein was recombinantly expressed in the cytoplasm of *E. coli*, the structure shows a structural disulfide bond in the third domain, whose formation is probably catalyzed by air oxidation, and two other cysteine side chains are juxtaposed in the second domain and probably form a structural disulfide bond in the appropriate redox environment.

ERp44 can be immunoprecipitated with a number of proteins that reside in the ER, including Ero1α and β (5, 6) and inositol 1,4,5-triphosphate receptor type I (119), and also with proteins that mature and oligomerize throughout the secretory pathway, including adiponectin (329), SUMF1/FGE (83, 199), and IgM (7). Some of these complexes can be isolated without and with reducing agents, implying that at least some of the interactions between ERp44 and other proteins are stable and are not limited only to the formation of a mixed disulfide with the unique CRFS active site. The data currently available are at times contradictory, but the simplest interpretation for the role of ERp44 is that it plays a role in either a late-stage, thiol-dependent, quality-control system or trafficking or a role in late-stage oligomerization reactions or both. The interaction of ERp44 with inositol 1,4,5-triphosphate receptor type I plays a role in the regulation of cellular calcium homeostasis (119), although further characterization of this system is required.

F. ERp29

ERp29, also known as ERp28, is a two-domain protein that lacks a catalytic **a** domain (59, 77). It is widely expressed (59) and reported to be highly expressed on ER stress (211), although no special stress-promoter elements could be identified (265). The crystal structure of human ERp29 has recently been solved to 2.9-Å resolution (16), and it, and the 1.9-Å crystal structure of the orthologue Wind protein from *Drosophila* (195), show that ERp29 is composed of two domains. The N-terminal domain is **b-**domain like and adopts a similar thioredoxin-like fold, whereas the C-terminal domain is an all α-helical fold. Wind protein is involved in the correct targeting of a type II Golgi-resident transmembrane protein called Pipe (271) and, based on the noncatalytic **b**-like domain, it is likely that ERp29 is either a specialist molecular chaperone, or is an "escort" molecule. Based in part on the crystal structures, two substrate-binding sites were identified in ERp29 and Wind, one in each domain (16, 18, 185, 195). The substrate specificity of both proteins is also similar, with both binding to peptides with the motif -F/Y-F/Y- or -F/Y-X-F/Y- (16, 18), although ERp29 is also able to bind to peptides in which the aromatic amino acids are farther apart (16).

Rat ERp29 has been purified from a natural source, and, consistent with it lacking an **a** domain, ERp29 does not show any ability to act as an oxidant or reductant of protein disulfide bonds (128). In addition, rat ERp29 was shown to lack all classic chaperone activities toward thermally or chemically denatured proteins (128), consistent with the narrow substrate specificity of the Wind homologue (see earlier). Two functions have been assigned to ERp29 to date, a role in thyroglobulin processing and a role in the entry of polyomavirus into cells. Homodimerization of ERp29 is required for both functions (247).

The role of ERp29 in thyroglobulin processing was first postulated by the identification of ERp29 as part of a folding complex that also included the molecular chaperones BiP and GRP94 (266). Overexpression of ERp29 increases thyroglobulin secretion, whereas RNAi-mediated silencing of ERp29 led to the attenuation of thyroglobulin export (19). What is surprising about the role of ERp29 is that it appears to be secreted into the media with thyroglobulin. The authors concluded that ERp29 functions as an escort "chaperone" that accompanies thyroglobulin all the way through the secretory pathway, although how a KEEL-containing protein can escape the ER-retrieval machinery is currently unknown.

Apart from the assigned "escort" role of ERp29, a second distinct function has been discovered related to protein conformational remodeling before "retrotranslocation," a function that is exploited by polyomaviruses. Polyomaviruses, such as SV40, are non-enveloped viruses that require direct interaction between viral coat proteins and the biological membrane to be internalized across the ER membrane to initiate infection (300). ERp29 is reported to alter the conformation of the VP1 major structural-coat protein, exposing the C-terminal arm of V1 and triggering viral membrane binding (196). The altered conformation of VP1 exposes the internal-coat protein VP2 and triggers perforation of the membrane (246). The C-terminal domain of ERp29 is responsible for this function (248). Surprisingly, bacterially expressed ERp29 is unable to have the same effect (196), and therefore, either an unknown posttranslational modification of ERp29 is required,

or ERp29 may work in concert with one of the other six proteins in the active ERp29-enriched fractions. As yet, no reports exist of a similar function for ERp29 on potential endogenous substrates, either secreted or ER-resident proteins.

G. ERdj5

The gene for ERdj5 (49), also known as JPDI (127), is located on human chromosome 2 and encodes for a 793-amino-acid protein. It is unique among human PDI-family members in many ways. It has five recognized domains (see later), four of which are **a-**domain like. These four have unusual active sites, GCHSC, WCPPC, WCHPC, and WCGPC, with three of these being similar to thioredoxin in having C*XP*C. From studies on the effect of the *XX* residues on reduction potential (see Section IV), these active sites would be expected to have a reductase function. Consistent with this, the cited redox equilibrium constant of ERdj5 with GSH/GSSG is 190 m*M*, equivalent to a reduction potential of −220 mV, the most reducing equilibrium constant reported for a PDI-family member (307). However, it is unclear which of the 11 potential active-site or structural disulfides or both (see later) this relates to, and it should be noted that this reduction potential is the same as that of disulfide bonds in nonstructured proteins (see Section IV). Each of the catalytic domains of ERdj5 also has a pair of conserved cysteines located in the same position as the thiols that form the structural disulfide in ERp57 and yeast PDI-family members, suggesting that they too have the same structural disulfide. The fifth reported domain of ERdj5 is a DnaJ domain, which modulates the activity of the HSP70 molecular chaperone BiP. By having an orphan J domain, ERdj5 is classified as a type III DnaJ.

Although ERdj5 is currently recognized as having five domains, an undefined stretch of 219 amino acids remains between the first and second catalytic domains; this is approximately the size of two thioredoxin-like domains. This region contains six cysteine residues, which are conserved between the human, mouse, and *Caenorhabditis elegans* proteins (49). The position and spacing of four of these are equivalent to the structural disulfides found in the catalytic domains of ERdj5 and other PDI-family members. Probably two more thioredoxin-like domains exist in ERdj5, bringing the total number of domains to seven, six of which are thioredoxin like. The second of these domains contains no other cysteine residues, and so should be considered as **b** or **b**′ like. The first domain is more interesting. In addition to the "structural disulfide," it contains two other conserved cysteines. The C-terminal of these additional cysteine residues aligns with the C-terminal cysteine of the active site of an **a**-like domain, whereas the N-terminal additional cysteine would lie in the loop preceding α2 of an **a** domain thioredoxin fold, as the spacing is C*XXXXX*C rather than C*XX*C. By alignment, this first additional putative thioredoxin-like domain gives an internal hit with the third acknowledged catalytic domain of ERdj5, as well as showing 25.7% identity (>74 amino acids) with the **a** domain of ERp57, including the active site and structural disulfide. Furthermore, a BLAST search by using this domain gives a hit with the **a** domain of yeast Eug1p. It is therefore likely that the uncharacterized region of ERdj5 contains a catalytic domain with a highly unusual active-site motif. This may be relevant, given the *in vivo* function or ERdj5.

ERdj5 is among the many genes upregulated in response to overexpression of a disease-causing and folding-incompetent mutant of surfactant protein C and among the few genes that has three unfolded protein response elements in its promoter region (67). This is consistent with induction of expression of ERdj5 by stress-promoting compounds such as thapsigargin, DTT, tunicamycin, or fenretinide (40, 49). Recently, two groups independently published details of the role of ERdj5 in the degradation of misfolded ER proteins (67, 307). This activity required both the J domain and the redox activity of the catalytic domains, and so, ERdj5 provides the missing link that exhibits the prerequisite reductase activity for ER-associated degradation. However, it still remains to be elucidated whether GSH provides the direct source of electrons required for reduction, or whether an ER-resident protein similar to the cytoplasmic thioredoxin reductase reduces ERdj5 directly.

H. The transmembrane PDI-family members

Whereas *S. cerevisiae* has only one transmembrane PDI-family member, humans probably have five, all shorter in length than Eps1p. To date, three of these have been published, TMX (202), TMX2 (205), and TMX3 (111), with TMX4 appearing in reviews (9, 71). The ER localization of TMX2, TMX4, and TMX5 has not yet been reported in the literature, but all three have putative type I transmembrane protein ER-localization signals. TMX2 is unusual, in that the active site in the **a**-like domain is WSNDC, and the probable membrane topology has this domain on the cytoplasmic side of the ER membrane; however, no experimental work has been reported on this protein. TMX (202) also has an unusual active-site motif, WCPAC, in its **a**-like domain, but this domain is in the ER lumen (201), has oxidoreductase activity (201), and this may help relieve ER stress caused by brefeldin A (202). TMX3 is the best characterized of the transmembrane human PDI-family members to date. Unlike PDI, it is not upregulated by the unfolded protein response (111), but it does have the ability to act as a protein dithiol-disulfide oxidant, and the recombinant luminal portion of the protein has reduction potential similar to that of PDI (111). The rate constant for the reaction of the recombinant luminal fragment of TMX3 with GSSG, fitting to a single pseudo first-order rate constant, was 433 $M^{-1}s^{-1}$ (112), comparable to that reported for the **a** domain of PDI (see Section VIII). TMX3 has the domain structure **a-b-b**′, and the kinetics of TMX3 oxidation, along with the stability of the catalytic domain, is influenced by the noncatalytic domains (112). From these results, it is likely that the *in vivo* activity of TMX3 is to act either to introduce disulfide bonds into folding proteins, or to isomerize them, but the proteins it acts on are as yet undefined.

I. Other PDI-family members

PDIr was one of the first PDI-family members identified more than a decade ago (115), and yet the physiological function of the protein is still unclear. Like PDI, PDIr is a four-domain protein, but with the three C-terminal domains being **a** like. The second catalytic domain has an active-site motif like that of PDI, whereas the third has WCPHC, similar to that of the periplasmic oxidant DsbA, and the first has the unique sequence WCSMC. These catalytic domains are linked by regions of 17 to 19 amino acids in length, which show some homology with each other, including a high percentage of

regular structure-destabilizing proline residues. The role of the active-site cysteines of PDIr in "isomerization" (with the coupled insulin-reduction assay) has been examined, although it should be noted that wild-type PDIr has only ~2% of the activity of PDI in this assay (126). The physiological function of PDIr is unknown. However, it is an androgen (270) and Hox (171)-regulated gene.

ERp27 is, like ERp29, a small human PDI-family member that lacks a catalytic domain. It can bind to peptides and nonnative proteins *via* its **b**′-like domain and interactions with ERp57 *via* the same mechanism with which ERp57 interacts with calnexin and calreticulin (2); however, the physiological significance of this is unclear at the moment.

ERp18, also known as ERp19, ERp16, and hLTP19, was originally reported independently by two groups in 2003 (3, 160). It is the smallest of the human PDI-family members, comprising a single, slightly extended, catalytic domain. The active site of ERp18 is unusual, having a CGAC motif, with a reduction potential of −165 mV (134) and being able to catalyze thiol-disulfide exchange reactions, although with relatively slow kinetics (3, 134). The kinetics of catalysis of peptide oxidation by ERp18, by using GSSG as the electron acceptor, is unusual, in that the rate was limited by oxidation of the peptide and not subsequent oxidation of the active site by GSSG to complete the catalytic cycle (3). The physiological function is still unknown.

ERp46 (160), also known as endoPDI (287), was reported independently by two groups in 2003. ERp46 also has a unique domain architecture with three catalytic domains linked by long spacers, 22 and 30 amino acids, respectively, with the linker between the first and second domains being very rich in proline and glutamic acid. Mouse ERp46 can functionally replace Pdi1p (160) and has been reported to play a role in protecting cells against hypoxia (287). ERp46, along with the PDI-family members ERp57 and ERp29, was downregulated in fructose-fed (insulin-resistant) hamsters, whereas PDI itself was upregulated (215), implying differential regulation and function.

Like PDIp, PDILT (309) is unusual in that it shows a highly specific tissue distribution, being expressed primarily in the testes after puberty and being the first PDI-family member to show developmental control (310). Whereas it has the same overall domain architecture as PDI, PDILT has the unusual active-site motifs SSKQS and WSKKC. PDILT forms mixed disulfide complexes with a variety of ER proteins, including Ero1α (309), and forms a complex with calmegin (310), the testis-specific homologue of calnexin that is required for sperm fertility (131). Again the physiological significance of this interaction is unclear at the moment.

hAG-2 and hAG-3 are the latest members added to the PDI family (236), with both having recently been shown to be located in the ER (252). Both are small single-domain proteins that show sequence homology with ERp18, although they have very different active-site motifs, WCGAC (ERp18), ECPHS (hAG-2), and DCQYS (hAG-3). Despite this, the crystal structure of human hAG-3 (M. Salin, V.D. Nguyen, R. Wierenga, and L.W. Ruddock, unpublished observations) shows a high degree of structural homology with human ERp18. hAG-2 and hAG-3 have been suggested to be molecular markers or potential therapeutic targets for hormone-responsive breast tumor (78), or both, but few clues exist as to their physiological function.

XII. Conclusions

These are exciting times in the PDI field. After years with limited structural information to guide studies, we now have the publication of six structures in the past year, increasing the number of structures of full-length human PDI-family members fourfold. Some details from these new structures could have been predicted beforehand, but many are new, including new concepts relating to the bind–release cycle of PDI and novel orientations of thioredoxin folds. We have the information available to make significant progress in structure–function relations, in determining the mechanisms of action of this remarkable enzyme, and perhaps in using this information to increase the efficiency and yields of *in vitro* refolding or cell factories or both for the production of therapeutic or industrially relevant proteins. However, the clear need exists for a greater degree of correlation between *in vitro* and *in vivo* results and a wider acknowledgment that both may be prone to artifact. We also need new, preferably physiological substrates, to assay activity, as well as to use existing assays in a more-appropriate and quantitative manner, including greater consideration of mimicking physiologically relevant conditions. We need to distinguish whether observed changes in the "catalytic activity" are due to changes in K_m or changes in k_{cat} (or due to significant changes in structure). We must acknowledge that even the simplest oxidation reaction that PDI catalyzes has four distinct covalent steps, which we need to differentiate. We must consider more carefully issues relating to heterogeneity in substrates and substrate specificities and affinities for different proteins and for different intermediates of the same protein. PDI was the first protein-folding catalyst to be reported. Much has been learned about it, but much more is still unknown.

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Abbreviations Used

BPTI = bovine pancreatic trypsin inhibitor
DHA = dehydroascorbate
DTT = dithiothreitol
ER = endoplasmic reticulum
GLO = gluconolactone oxidase
GSBP = glycosylation-site binding protein
GSH = reduced glutathione
GSSG = oxidized glutathione
NEM = *N*-ethyl maleimide
PDI = protein disulfide isomerase
RNase = ribonuclease
ROS = reactive oxygen species
VKOR = vitamin K 2,3-epoxide reductase

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