THE ROLE OF PROTEIN DISULFIDE ISOMERASE IN THE CELL

by

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I know my corn plants intimately, and I find it a great pleasure to know them

Barbara McClintock
A Feeling for the Organism

To

Jane e José Rubens

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Protein disulfide isomerase (PDI; EC 5.3.4.1), an abundant protein of the endoplasmic reticulum, catalyzes *in vitro* disulfide bond reduction, isomerization, and sulfhydryl group oxidation using the active site: CGHC. *In vivo* PDI is part of different protein processing complexes. Null PDI mutants of *S. cerevisiae* are inviable, but the essential *in vivo* function of PDI is unclear.

A yeast expression system which allows for the study of both the *in vitro* and *in vivo* properties of PDI has been developed. Correctly processed recombinant rat PDI, with catalytic activity identical to that of PDI isolated from bovine liver, was expressed and secreted into culture medium by *S. cerevisiae*.

To evaluate the ability of mammalian PDI to complement a *pdi* null mutation, *pdi1Δ S. cerevisiae* cells were transformed with rat PDI constructs directed by the full pre-pro α-factor leader sequence or the pre-α-factor leader sequence, with either KDEL– the mammalian ER retention signal, or HDEL– the yeast equivalent at the *C*-terminus. In tetrad analysis studies, only the constructs containing the pre-pro leader sequence and the HDEL retention signal complemented. About half of the nonviable spores proceeded through a few cell divisions

while the rest did not germinate. In plasmid shuffling experiments all constructs were able to complement, albeit with different efficiencies. These results demonstrates that for vegetative growth basal levels of PDI are sufficient while for efficient spore germination high levels of PDI are required.

To evaluate the role of each cysteine in the functions of PDI, mutants were created where serines replace active site cysteines. Haploid *pdi1Δ S. cerevisiae* can be complemented with either a wild-type or a mutant rat *pdi* gene coding for [CGHS]PDI but not with a gene coding for [SGHC]PDI. *In vitro*, [CGHS]PDI is an efficient catalyst for the shuffling of existing disulfide bonds but not for disulfide bond reduction or sulfhydryl group oxidation. [SGHC]PDI catalyzes none of these. These results indicate that *in vivo* protein folding pathways contain nonnative disulfide bonded intermediates, and that the essential role of PDI is to unscramble these intermediates.

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DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
ER	endoplasmic reticulum
5'FOA	5' fluoro-orotic acid
FPLC	Fast protein liquid chromatography
KAc	potassium acetate
M_r	relative molecular mass
MTP	microsomal triglyceride transfer protein
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
poly(C)	poly(cytidylic acid)
RNase A	bovine pancreatic ribonuclease A
rPDI	rat protein disulfide isomerase
SDS	sodium dodecyl sulfate
sRNase A	scrambled ribonuclease A
Trx	thioredoxin
yPDI	yeast protein disulfide isomerase

Chapter I

Introduction

1. Protein Folding

The first report of protein refolding *in vitro* dates to half a century ago when Anson and Mirsky (1945) demonstrated that alkali-denatured hemoglobin spontaneously recovers its biological properties when the pH is returned to neutrality. Anfinsen and colleagues followed these studies by demonstrating that the amino acid sequence of a protein guides its folding (Anfinsen, 1973). Working with highly purified bovine pancreatic ribonuclease A (RNase A), which contains four disulfide bonds, these researchers showed that reduced and denatured RNase A refolds spontaneously when denaturant is removed. This result established that the native tertiary structure of proteins is defined by the amino acid sequence (Anfinsen, 1973).

Similar results have been obtained with several other small single domain polypeptides and a few larger, more complex proteins. Studies have shown that even though the renaturation procedure is rarely perfect (reported recoveries of biological activity range between a few and 90%), it does yield the native molecule provided that artifacts such as aggregation and precipitation of the unfolded protein, autolysis, or chemical damage do not occur. These intriguing results have lead to what has become known as the 'protein folding problem', namely, how is the information contained in the amino acid sequence translated into the native conformation?

The first theory used to explain protein folding was the thermodynamic hypothesis, which suggests that the most stable state of a polypeptide is that in which the Gibbs free energy of the system is the lowest (Epstein et al., 1963). The polypeptide chain would explore many conformations by rotations about bonds until it reaches its thermodynamically most stable state. Thus, the native conformation is determined by the amino acid sequence in a given environment through the totality of intramolecular, protein—solute, and protein—

solvent interactions. This theory was supported by studies of the reversible transitions between the native and denatured states of proteins. These studies suggested that no partially or incorrectly folded intermediates exist between the denatured and native state, and led to the 'two-state model', which hypothesizes that a protein can exist in one of two states: the polymorphous unfolded state or the unique well defined, native conformation (Anfinsen, 1973).

In 1968, Levinthal (Levinthal, 1968) showed that the random search mechanism on which the thermodynamic hypothesis was based would lead to unacceptably slow protein folding. Since folding of proteins *in vivo* usually takes place in seconds or less, it was concluded that protein folding cannot occur by a random search process. This insight led to the idea of kinetic pathways for protein folding. The several models proposed for such pathways, have two features in common: the existence of partially structured folding intermediates and the possibility that the folding pathway may yield a native conformation that is not necessarily the most stable state (Goldberg, 1985; Baldwin & Eisenberg, 1987).

Studies of protein refolding *in vitro* with small single domain proteins that unfold and refold in the absence of any other factors suggest that refolding may start by one of the following means: i) rapidly reversible formation of local secondary structure – folding starts independently and more or less simultaneously, in many regions of the polypeptide chain by the formation of short stretches of secondary structures like α-helices or β-sheets; ii) collapse of hydrophobic regions to form the interior of the molecule; iii) formation of covalent interactions such as disulfide bonds that stabilize the polypeptide. This initial step is followed by the formation of domains through specific interactions. Such domains, already folded in a native-like conformation, then assemble to yield the folded polypeptide. Monomer conformation is then refined, followed by assembly of monomers and final bond rotations to yield the native protein (Goldberg, 1985; Baldwin & Eisenberg, 1987).

Still, differences exist between the rates observed in protein folding *in vitro* and in protein folding *in vivo*. *In vitro* protein folding studies have shown that although individual domains of a nascent polypeptide may fold very rapidly, the whole protein acquires its native structure comparatively slowly—in seconds or minutes. In contrast, *in vivo* proteins fold in seconds or less. *In vitro* a significant percent of a folding protein becomes misfolded or aggregated, while *in vivo* 95% of newly synthesized polypeptides attain their native three-dimensional structures (Goldberg, 1985). Exceptions to the high efficiency of protein folding *in vivo* are mutant proteins with compromised native structure, proteins synthesized at elevated temperatures, and proteins produced in heterologous hosts (Wittrup, 1995).

It is now evident that accessory proteins assist in the *in vivo* folding process. Two classes of accessory proteins have been described— molecular chaperones and enzymes. Molecular chaperones are proteins that are thought to prevent improper interactions leading to aggregation and events that the divert the protein from a productive folding pathway. Chaperones inhibit misfolding, but do not catalyze folding. Enzymes have been identified for two rate-limiting steps in protein folding: the *cis-trans* isomerization of peptide bonds and the formation of native disulfide bonds. Clear evidence for prolyl isomerases as folding catalysts *in vivo* is still lacking; however, the phenotype of mutant organisms has confirmed that native disulfide bond formation is assisted *in vivo* (Gething & Sambrook, 1992; Craig et al., 1993).

1.1 Molecular Chaperones

Molecular chaperones are proteins that bind to and stabilize the non-native conformation of other proteins, facilitating their correct folding by releasing them in a controlled manner. [For reviews see: Craig (1993); Hartl (1994); Gething (1992)]. Chaperones are widely distributed in both prokaryotes and eukaryotes, where they are found in different cellular compartments and organelles. Chaperones were originally thought to

have a specialized role in dealing with heat-induced damage to proteins. It is now known that the majority of the family members are expressed constitutively and abundantly in the absence of any stress, and thus chaperone proteins have a more generalized housekeeping role. Genetic studies show that many of these proteins are essential for cell viability under normal conditions of growth (Gething & Sambrook, 1992).

A common property shared by molecular chaperones is the ability to recognize and temporarily stabilize structural elements such as hydrophobic surfaces exposed in unfolded or partially folded proteins during polypeptide folding, assembly, and disassembly, and to prevent aggregation by maintaining these elements in a state competent for subsequent folding. Thus, chaperones ensure that polypeptides will fold or be transported properly. Chaperones interact with different polypeptide chains without exhibiting a significant sequence preference, covalently modifying their substrates, or being part of the finished product.

The need for chaperones is easily envisioned in light of the protein folding process in a cell. The formation of stable tertiary structure requires the presence of a complete polypeptide or at least a complete protein domain. Proteins emerge from ribosomes as unfolded chains with their amino terminus first and are unable to fold stably until a complete domain has been synthesized. In the unfolded state, polypeptides could potentially interact with each other through their hydrophobic surfaces forming aggregates even at relatively low concentrations (Hartl et al., 1994).

Molecular chaperones act by one of two mechanisms. They block nonproductive protein – protein interactions by shielding hydrophobic surfaces so as to avoid aggregation of an incomplete polypeptide chain, and they mediate the folding of proteins to their native state by sequestering a complete but unfolded protein to prevent aggregation or to allow the folding of domains or assembly of oligomers to proceed (Craig et al., 1993). In addition, it

has been shown that different chaperone families can act together in a sequential pathway of assisted folding (Georgopoulos, 1992; Gething & Sambrook, 1992).

There are at least three major unrelated chaperone families. Each family is composed of two or more members that work together in particular cellular compartments.

1) Stress-70 chaperones are found in all major cellular compartments of eukaryotes and every bacterium examined to date. Stress-70 chaperones have been implicated in the stabilization or generation of unfolded protein precursors before assembly in the cytosol, translocation into organelles including the ER and mitochondria, stabilization of newly translocated polypeptides before folding and assembly, rearrangement of protein oligomers, dissolution of protein aggregates, and degradation of rapidly turned-over cytosolic proteins. The release of bound proteins follows ATP hydrolysis.

2) Chaperonin-60s are found in prokaryotic and eukaryotic cells, and bind to unfolded proteins preventing aggregation both *in vivo* and *in vitro*. In eukaryotes, chaperonin-60s aid in the folding and assembly of polypeptides translocated into organelles. The release of bound proteins is coupled to ATP hydrolysis.

3) Stress-90 chaperones bind unfolded polypeptides and stabilize them in an inactive or unassembled state.

1.2 Enzymes

1.2.1 Peptidyl prolyl cis-trans *isomerase*

The unfolded state of a polypeptide chain can be divided into two substates: one in which every prolyl peptide bond is in the same *cis*-or *trans*-isomerization state as in the native protein and another in which at least one prolyl peptide bond is the wrong isomer. Brandts (Brandts et al., 1975) suggested that unfolded polypeptide chains with all the proper prolyl

peptide bond isomers would refold rapidly, while for those with an incorrect isomer the *cis–trans* isomerization of proline would be rate limiting. Thus, even though locally folded intermediates are rapidly formed before the prolyl peptide bonds acquire their native state, prolyl peptide bond isomerization appears to be essential in controlling the overall rate of refolding, at least for small globular proteins (Brandts et al., 1975).

Peptidyl prolyl *cis – trans* isomerases (PPIases) are proteins that are thought to catalyze the *cis – trans* isomerization of the peptidyl prolyl bonds in peptides and proteins during protein folding. PPIases are abundant and widely distributed in prokaryotes and eukaryotes, where they are found in different tissues, cellular compartments and organelles. In addition, secreted PPIases have also been isolated (Gething & Sambrook, 1992).

There are three families of PPIases. Two of these families have been known for almost a decade and were named after clinically important immunosuppresive drugs that inhibit their activity. Cyclophilins bind to cyclosporin A, while FK506 binding proteins (FKBPs) bind FK506 and rapamycin. The third family, named PpiC, was only identified recently (Rudd et al., 1995). The three families are structurally unrelated. Although the cyclophilins are mostly β-barrel structures and the FKBPs are largely β-sheets, PpiCs are predicted to be mostly α-helical (Rudd et al., 1995).

The hypothesis that PPIases catalyze protein folding via prolyl bond isomerization is based mostly on *in vitro* studies with artificial peptide substrates. *In vivo* evidence is based on studies done with the *ninA* gene of *Drosophila melanogaster*. This eye-specific PPIase of the cyclophilin family is localized to the ER, and is required for proper trafficking and folding or stability of a class of rhodopsins (Gething & Sambrook, 1992; Craig et al., 1993).

The *in vivo* roles and physiological substrates of enzymes with PPIase activity is still unclear. PPIases are thought to act *in vivo* as conformases, catalyzing the initial folding or/and rearrangement of protein structure. The mechanism by which this catalysis occurs is unknown. Cyclophilins and FKBPs display dramatic differences in substrate specificity.

Mechanistic studies suggest that both catalyze the interconversion of *cis* and *trans* rotamers of peptide substrate by non-covalent stabilization of a twisted amide transition state (Gething & Sambrook, 1992). In addition, genetic studies with yeast show that cyclophilins and FKBP gene products are not essential for cell viability under normal conditions of growth (Gething & Sambrook, 1992).

1.2.2 Protein Disulfide Isomerase

The rest of this introduction will summarize results of studies done with the protein disulfide isomerase (PDI) family and other oxidoreductases and their role in the catalysis of disulfide bond formation and isomerization.

1.3 Disulfide bond formation and isomerization in proteins

Disulfide bonds are important structural features of many proteins because they decrease the conformational entropy of the polypeptide chain. In a folded protein, disulfide bonds are ordinarily stabilizing and non-reactive. In contrast, the role of disulfide bond formation during the process of protein folding is not as clear. There are two current views regarding the role of disulfide bonds in protein folding. The first, pioneered by Creighton and collaborators, states that disulfide bonds do not dictate folding of the polypeptide chain. Folding is instead determined by total energy considerations. Disulfide bonds are formed only when the protein conformation is favorable (Creighton, 1988). The alternative view, pioneered by Kim and Baldwin, proposes that disulfide bond formation should be regarded as a structure-determining step that locks a region of a protein into a particular folded state (Kim & Baldwin, 1990). Both views agree however that the pairing of cysteines to form disulfide bonds is approximately random during the early stages of protein refolding. As folding proceeds, acquisition of secondary structure and disulfide bond formation become mutually cooperative processes. Thus, a particular bond may allow a folded structure to be

formed stably and thus will be retained preferentially. Conversely, the acquisition of elements of secondary structure will energetically favor the formation of certain disulfide bonds (Creighton, 1988; Kim & Baldwin, 1990).

Both scenarios predict that non-native disulfide bonds may be formed transiently during protein folding. This transience is particularly important during protein refolding *in vitro* because all the cysteine residues in a polypeptide chain are available simultaneously for disulfide bond formation. Studies of the mechanism of the uncatalyzed, oxidative folding of small proteins has led to the conclusion that the specific disulfide bonds observed in the native structure do not form in an ordered sequential fashion (Creighton, 1978; Weissman & Kim, 1991). Non-native disulfide bond formation invariably precedes the regain of native structure and function. These non-native disulfides must be rearranged by thiol/disulfide exchange to form the native disulfides. However, it is not certain whether the presence of non-native disulfide bonds is a common occurrence during protein folding *in vivo* where protein disulfide isomerase and/or other oxidoreductases and thiol/disulfide redox couples are present to assist disulfide rearrangements (Segal et al., 1992).

The majority of proteins containing disulfide bonds are transmembrane or secretory proteins that are either transported along the exocytic pathway of eukaryotic cells or exported directly through the plasma membrane of prokaryotic cells (Wittrup, 1995). Folding of these proteins *in vivo* can begin when only a portion of the nascent polypeptide chain has been translocated across the lipid bilayer from its site of synthesis to the cytoplasm, and it has been well documented that native disulfide bonds can be formed before a polypeptide is released from the ribosome. This situation could decrease the likelihood of formation of non-native disulfide bonds during folding of proteins such as immunoglobulin light chain or serum albumin in which the cysteine residues of native disulfide bonds are either adjacent or close to one another in the amino acid sequence. However, the probability of non-native disulfide bond formation may be increased when the proper cysteine partners are separated in the

polypeptide chain by many residues, including other cysteine residues. This separation is the case for many disulfide–bonded proteins (Segal et al., 1992).

Protein folding may involve both the formation of new disulfide bonds and the intramolecular isomerization of existing disulfide bonds. The need for oxidation of dithiol bonds *in vivo* is clear since proteins are synthesized in the reduced form (Bardwell & Beckwith, 1993). The isomerase activity may be required for both the normal protein folding pathway and the rescue of proteins that have become misfolded or aggregated. The formation of a new disulfide involves the transfer of a disulfide bond from a source external to the protein through the formation of a mixed disulfide. Unless there is an oxidant present, disulfides will not form spontaneously between the sulfhydryl side chains of cysteines. Thus, cells must provide a redox environment. This environment exists in the ER of eukaryotes and in the periplasmic space of prokaryotes. In eukaryotes, reduced glutathione (GSH) and oxidized glutathione (GSSG) are thought to be the major electron donor and acceptor in the ER. Disulfide bond isomerization, on the other hand, is an intramolecular reaction and in principle does not require an external electron acceptor (Wittrup, 1995).

2. Protein Disulfide Isomerase

2.1 Discovery

The search for an enzymic catalyst of protein disulfide formation began more than 30 years ago. At that time, an enzyme capable of accelerating the conversion of reduced bovine pancreatic RNase A to the catalytically active form was discovered in microsomal preparations from rat liver (Goldberger et al., 1963) and chicken and pigeon pancreas (Venetianer & Straub, 1963). This enzyme, eventually named protein disulfide isomerase, was initially shown to catalyze the air oxidation of reduced proteins (Goldberger et al., 1964). It was later discovered that the rate of disappearance of sulfhydryl groups in reduced RNase A was independent of the presence of the enzyme, but a high rate of reactivation of the reduced RNase A was achieved only in the presence of the enzyme (Givol et al., 1964). These results implied that the activating enzyme was not catalyzing the oxidation of sulfhydryl groups. Rather, air oxidation involved uncatalyzed chemical oxidation to form non-native disulfide–linked products followed by the enzyme–catalyzed isomerization of non-native disulfide bonds (Givol et al., 1964). The observation that the rat liver extract could greatly enhance the rate of reactivation of both reduced RNase A and reduced lysozyme pointed to the possibility that the enzyme was a general and nonspecific catalyst for disulfide bond interchange in proteins containing disulfide bonds (Goldberger et al., 1964).

Although PDI was first detected and studied in liver and pancreas, surveys of mammalian tissues indicate the enzyme is ubiquitous. The concentration of PDI is highest in liver, pancreas, lymphoid, and connective tissues, which are all active in the synthesis and secretion of disulfide–bonded proteins. Indeed a rough correlation exists in tissues between the levels of PDI and the extent of synthesis of disulfide bonded proteins (Freedman, 1984; Bassuk & Berg, 1989). More precise correlations have been established through the study of tissues and cell lines specialized in the synthesis of a single class of disulfide–bonded

proteins and have lead to a clear association between PDI activity and the synthesis of a diverse range of disulfide–bonded proteins (Roth & Koshland, 1981; Myllyla et al., 1983). The localization of this enzyme in microsomes and the catalytic activity ascribed to PDI are consistent with the idea that the rearrangements of disulfide bonds from non-native to native occurs *in vivo* as an integral part of secretory protein biosynthesis.

2.2 Cloning of mammalian PDI

The primary structure of the 2.4–kb cDNA encoding rat PDI was determined by sequence analysis of overlapping clones derived from libraries and primer extensions (Edman et al., 1985). The rat mRNA contains 40 bases of 5′ untranslated sequence and two 3′ polyadenylation sites. An open reading frame of 1524 bases encodes nascent PDI. Northern blot analysis revealed a major species of PDI mRNA at 2800 bases and a minor component of 2000 bases. The enzyme is synthesized as a 528 amino acid residue precursor that contains a 19 residue signal peptide. The mature enzyme contains 509 amino acid residues, resulting in a M_r of 56,800 (Edman et al., 1985; Gilbert et al., 1991) . The protein translated in rat liver contains an *N*-terminal signal peptide and two pairs of homologous regions: amino acid residues 9 – 90 (region a) and 353 – 431 (region a'), and amino acid residues 153 – 244 (region b) and 256 – 343 (region b'). Regions a and a' each contain an active site with the sequence WCGHCK, which is homologous to the active site in *Escherichia coli* thioredoxin (Holmgren, 1968). The *C*-terminus of PDI ends with the sequence KDEL, which has been implicated as the signal for retention of a protein in the endoplasmic reticulum (ER) of mammalian cells (Munro & Pelham, 1987). cDNAs made from mRNAs encoding PDI have also been isolated and sequenced from human (Pihlajaniemi et al., 1987), rat (Edman et al., 1985), mouse (Gong et al., 1988), and bovine (Yamauchi et al., 1987) tissues. The similarities among these sequences is about 95% at the DNA level and from 80 to 97% at the protein level.

2.3 Cellular Functions of PDI

PDI has been shown to be a component of several unrelated cellular complexes. These complexes are the β subunit of prolyl-4-hydroxylase, and the microsomal triglyceride transfer protein. The PDI in these complexes is the product of a single gene (Pihlajaniemi et al., 1987). PDI has also been shown to be a thyroid hormone binding protein and has been postulated to be involved in the ER quality control of eukaryotic cells by acting as a chaperone. These functions of PDI are discussed below.

2.3.1 *β subunit of prolyl 4-hydroxylase*

The cDNA coding for the β subunit of human prolyl-4-hydroxylase was isolated from an expression library using antibodies generated against the holoenzyme. Sequencing of the cDNA and of the *N*-terminus, and tryptic peptides, showed that the β subunit of human prolyl 4-hydroxylase exhibits 94% amino acid similarity to human PDI (Koivu & Myllyla, 1987). Southern blot analysis of human genomic DNA with a cDNA probe for the hydroxylase β subunit indicates that only one gene contains these sequences (Pihlajaniemi et al., 1987). The β subunit isolated from the prolyl hydroxylase tetramer has the same isomerase activity *in vitro* as does PDI isolated separately, and the β subunit has one half of that activity when present in the intact tetramer (Koivu & Myllyla, 1987). Further, the apparent molecular mass and antibody probing of both PDI and the β subunit isolated from human placenta and chick embryos show that these two proteins are identical (Koivu & Myllyla, 1987).

Prolyl 4-hydroxylase catalyzes the post-translational hydroxylation of proline residues within pro-α-chains of procollagen (Pihlajaniemi et al., 1987). The substrates for this reaction are the nascent procollagen peptide, α-ketoglutarate, and O_2, and the enzyme requires ascorbic acid and Fe^{+2} as cofactors. This enzyme has been solubilized and isolated

from tendon cartilage, has a M_r of 250,000, and consists of 2 different types of enzymically inactive monomers with M_r of 64,000 (α) and 60,000 (β) which form a tetramer ($\alpha_2\beta_2$). The enzyme is found in cells in two forms—active tetramers and an inactive form that corresponds to the β subunit. The β subunit is synthesized in great excess, and a free pool of this subunit, distinct from the intact enzyme, is present in most cells. No function for this excess material was apparent. The α subunit appears to be used immediately to form active hydroxylase whereas the β subunit is synthesized in excess and enters a precursor pool of monomers before being incorporated into the tetramer (Pihlajaniemi et al., 1987).

Active–site mutants of PDIs that lack PDI activity can combine with the α subunit to form tetramers that have full prolyl 4-hydroxylase activity (Vuori et al., 1992c). PDI activity is, therefore, not required for tetramer assembly or for the prolyl 4-hydroxylase activity of the tetramer. Since the α subunit alone has no hydroxylase activity, a portion of the PDI subunit may be critical for keeping the α subunit in a catalytically active, non-aggregated form. Alternatively the β subunit may be required to retain the enzyme tetramer within the lumen of the ER since deletion of the KDEL sequence leads to secretion of considerable amounts of both free β subunit and the $\alpha_2\beta_2$ tetramer (Vuori et al., 1992c). Mutation of the KDEL sequence to the HDEL leads to little or no secretion. Prolyl 4-hydroxylase that lacks the β subunit has been isolated in species of green algae (Haggren & Kolodrubetz, 1988; Kaska et al., 1988) indicating that the β subunit plays no direct role in the catalytic mechanism of prolyl hydroxylation (Kaska et al., 1987; Kaska et al., 1990).

2.3.2 *Thyroid hormone binding protein.*

The thyroid hormone binding protein (T3BP) was identified by affinity labeling with a radioiodinated bromoacetyl derivative of the T3 hormone (Cheng et al., 1987). T3BP was originally isolated as a mobile plasma membrane protein that could be labeled in whole cells with a rhodamine T3 derivative. Subsequent studies of membrane preparations from rat and

human tumor cells using bromoacteyl T3 preferentially labeled an acidic protein of M_r 55,000, which in contrast to the original description of T3BP, was found in the ER but not in the plasma membrane. Antibodies against this protein were used to screen the bovine cDNA library. The sequence of triiodo-l thyronine binding protein (T3BP) from human and bovine sources showed homology with PDI (Cheng et al., 1987; Yamauchi et al., 1987). T3BP was purified and found both on cell surfaces and in the ER. The physiological importance of T3 binding to PDI is at present unclear and the fraction of PDI that is present on the cell surface as opposed to the ER is an unresolved question. This result raises the possibility that PDI has a role in binding thyroid hormones and mediating cellular responses to these hormones (Bassuk & Berg, 1989).

2.3.3 *58–kDa subunit of microsomal triglyceride transfer protein*

Microsomal triglyceride transfer protein (MTP) catalyzes membrane transfer of triglycerides, cholesteryl esters and phosphatidyl choline between membranes during the synthesis of lipoproteins (Wetterau et al., 1991a; Wetterau et al., 1991b). MTP is composed of two proteins having apparent M_r of 58,000 and 88,000. The 58–kDa component of MTP has been identified as PDI by amino terminal sequence analysis, peptide mapping experiments, immunochemical characterization, and a disulfide isomerase activity following the dissociation of the subunits (Wetterau et al., 1990). Isolated PDI has no lipid transfer activity, implying that the 88–kDa subunit is the lipid transfer protein or that the complex with PDI confers transfer activity (Wunderlich & Glockshuber, 1993a). Transfer activity is lost when PDI is dissociated from the enzyme complex (Wetterau et al., 1991b).

The role of PDI in the transfer protein complex is not known (Gordon et al., 1995). PDI appears to be necessary to maintain the structural integrity of the transfer protein but may play a more integral role in the lipid transfer protein. It may contribute directly to the active site of the transfer protein, play a role on the folding of the transfer protein, or maintain it in

an active conformation (Kaska et al., 1990). The lack of evidence for the existence of an MTP system in yeast and studies with human patients, who live despite lacking MTP, suggest that MTP is not necessary for eukaryotic cell viability (Laboissière et al., 1995a).

2.3.4 *Peptide binding*

A peptide binding site in PDI was initially identified using an affinity probe containing the N-glycosylation acceptor sequence Asn-Xaa-Ser/Thr (Noiva et al., 1991b). The site has also been demonstrated to bind other tri and tetra peptides of varying sequence. The location of the peptide binding site in the primary sequence has been identified as a region between residues 451 and 476 (Noiva et al., 1991b). This peptide binding site is contained within a region close to the C terminus of PDI, which has a very high percentage of acidic residues with 13 of the C-terminal 20 residues being either glutamic or aspartic acids.

The specificity of PDI for polypeptide substrates has been investigated using peptides of assorted lengths and composition (Morjana & Gilbert, 1991). The peptides were used as competitive inhibitors of PDI activity as measured by glutathione-insulin transhydrogenase activity. When the length of the peptide was increased, the dissociation constant K_i decreased. This result suggests that PDI preferentially binds to longer peptides and that a large portion of the peptide interacts with PDI (Morjana & Gilbert, 1991). In addition, these experiments showed that cysteine–containing peptides bind 4 – 8 fold tighter than do peptides of the same length lacking cysteine and that performic acid oxidation of cysteine containing peptides increased the K_i by 5– fold. These results illustrate a clear preference for cysteine residues or negative charge. No other correlation of inhibitory activity was found with charge, hydrophobicity, or sequence of the peptide, suggesting that PDI principally recognizes only the peptide mainchain. Recognition of the side chains of the individual amino acid residues is limited to the Cys side chain (Morjana & Gilbert, 1991). One might argue

that although the substrate binding site of PDI might bind a short peptide region, longer peptides have a greater affinity because the effective concentration of peptide sites capable of binding PDI is higher. The lack of substrate sequence specificity would also be important for disulfide formation and/or isomerization because no sequence specificity has ever been noted for disulfide bridging. Finally the ability of PDI to associate with other proteins non specifically may help explain why PDI is found as a subunit of several enzyme complexes (Bardwell et al., 1993).

2.3.5 *ER quality control or the chaperone/anti-chaperone function of PDI*

Several researchers have hypothesized that PDI is a molecular chaperone that binds to misfolded proteins or to the nascent polypeptide undergoing folding in the ER (LaMantia & Lennarz, 1993; Noiva et al., 1993; Puig & Gilbert, 1994b; Puig & Gilbert, 1994a; Wang & Tsou, 1994). This hypothesis is supported by three observations — stoichiometric levels of PDI:polypeptide exist in the lumen of the ER; PDI levels in the ER are inducible as are the levels of other molecular chaperones; and, as discussed above, PDI interacts with a broad group of peptides with no discernible amino acid sequence or composition specificity.

Recent work has suggested that PDI has a chaperone activity. PDI has been found to prevent the *in vitro* aggregation of T4 lysozyme. The refolding of lysozyme is dependent on the concentration of PDI, the concentration of lysozyme, and the order of addition of the compounds to initiate folding (Puig & Gilbert, 1994b). If PDI is present at a large molar excess concentration when denatured lysozyme is diluted to initiate folding, PDI demonstrates a chaperone–like activity that prevents aggregate formation and promotes correct folding. At high lysozyme concentrations and substoichiometric PDI concentrations, an 'anti-chaperone' activity is ascribed to PDI. This anti-chaperone activity involves PDI–enhanced formation of lysozyme aggregates during folding (Puig & Gilbert, 1994b). Although the chaperone activity of PDI requires the presence of the thiol/disulfide sites, the

anti-chaperone activity appears to be independent of the presence of dithiols. In addition, blocking the PDI peptide binding site affects chaperone but not the anti-chaperone activity (Puig et al., 1994). This result implies that a portion of the protein other than either the active site or the peptide binding site is responsible for the chaperone activity.

PDI has been shown to associate stably with mutants of lysozyme (Otsu et al., 1994) and collagen (Chessler & Byers, 1992). In addition, a chaperone effect of PDI was demonstrated for GAPDH, a cytoplasmic protein that lacks disulfides (Cai et al., 1994). These results have led investigators to propose that PDI may be involved in an ER quality control system. However the oxidoreductases, PDI and DsbA have been shown to be truly acting as isomerases (Wunderlich & Glockshuber, 1993a; Lilie et al., 1994) as described below.

Looking at the effect of PDI on the refolding of the oxidized Fab fragment of the murine monoclonal antibody MAK33 (Fab) showed that under oxidizing conditions both the kinetics and yield of the reaction were not affected by PDI (Lilie et al., 1994). However, it has previously been shown that members of the protein binding family of chaperones do influence the refolding process of oxidized Fab (Schmidt & Buchner, 1992; Wiech et al., 1992). Thus, at least in this system PDI does not function as a chaperone. However, under appropriate redox conditions PDI is able to function as an 'isomerase' breaking disulfide bonds of refolding oxidized Fab molecules. Similar experiments were performed using reduced Fab fragments which demonstrated that the reactivation of the fragments is dominated by the formation of the intradomain disulfide bonds. PDI influences this process, resulting in higher yields of reactivation with a dependence on the redox conditions used. Alkylated PDI was used as a control since it cannot participate in redox reactions. Alkylated PDI has no effect on the refolding of reduced Fab, strongly suggesting that the observed effect of PDI is due to its isomerase activity (Lilie et al., 1994).

Reduced but not oxidized glutathione, when co-expressed with DsbA, leads to an increase in the yield of native RBI, an α-amylase/trypsin inhibitor from ragi. This result points out the importance of the reduction of incorrect disulfides during oxidative protein folding *in vivo*. The amount of insoluble RBI generated is independent of DsbA co-expression and the presence of thiols in the medium. Improved folding mainly prevents the inhibitor from being proteolytically degraded but does not decrease the overall amount of aggregated RBI significantly. Thus, DsbA, in this system, does not act as an 'aggregation inhibitor' in a chaperone-like way (Wunderlich & Glockshuber, 1993a).

Finally, two functions were ascribed to PDI in error. Even though it has some affinity for thyroid hormones and has been studied as a thyroid-hormone binding protein, it is not involved in thyroid hormone metabolism as a 5'-monodeiodinase (Boado et al., 1988; Schoenmakers et al., 1989). Similarly, PDI has an affinity for peptides, including glycosylatable peptides, but is not a glycosylation site binding protein responsible for core glycosylation of nascent chains in the ER (Geetha-Habib et al., 1988; Noiva et al., 1991a).

2.4 The active site

The mechanism by which PDI catalyzes disulfide bond rearrangement is not yet understood. Studies suggest that the enzymatic activity of PDI relies on specific active–site cysteine residues. PDI was shown to be active in its reduced form; however, carbamoylmethylation or carboxymethylation of the sulfhydryl groups of the enzyme caused irreversible inactivation (Fuchs et al., 1967; Hawkins & Freedman, 1991). The enzyme can be partially inactivated by carboxymethylation of a fraction of the sulfhydryl groups and the essential catalytic group has been found to have a pK_a of 6.7 (Hawkins & Freedman, 1991). In addition, PDI is inhibited by arsenite or Cd^{+2}, both of which are diagnostic inhibitors of

enzymes with active site dithiol groups (Ramakrishna Kurup et al., 1966; Hillson & Freedman, 1980). These results suggest that PDI activity absolutely requires cysteine residues for activity. The sequence WCGHCK appears twice in the amino acid sequence of PDI, and alkylation of these four cysteines residues inactivates the disulfide isomerase activity. These consensus regions have been defined as the active sites of PDI (De Lorenzo et al., 1966).

Several studies have determined the roles of various residues in the consensus sequence. Vuori and co-workers mutated the sequence WCGHCK to WSGHCK in one or both of the active sites of PDI (Vuori et al., 1992a). The PDI activity of polypeptides containing a single modified active site was about 50% of that of the wild–type enzyme; whereas, the polypeptide with both sequences modified had no isomerase activity. This result suggests that both WCGHCK sequences act as catalytic sites for the isomerase activity and that the two sites operate independently of one another (Vuori et al., 1992a). Mutations were also made on the second cysteine of the active sites. The resulting proteins were shown to be incapable of catalyzing the formation of disulfide bonds (LaMantia & Lennarz, 1993).

The conserved lysine residue adjacent to the second cysteine in both active–site sequences WCGHCK was originally believed to participate in the catalytic mechanism by stabilizing the second cysteine in the active thiolate form. Yet, mutagenesis of this lysine to an arginine residue resulted in only a modest loss in PDI activity (Lu et al., 1992). Mutagenesis of the histidine residue to a proline within the PDI active–site sequence, thus creating an active site similar to that of thioredoxin, caused an almost complete loss in oxidative activity (Lu et al., 1992). Thioredoxin has proline instead of histidine in its active–site sequence and is 40-fold less active than PDI in assays (Hawkins et al., 1991). Mutation of the active–site proline to histidine in thioredoxin generates a mutant thioredoxin with redox character more similar to that of PDI (Krause et al., 1991), and that has a 10-fold greater activity in assays of disulfide formation (Lundström-Ljung et al., 1992). These site-directed

mutagenesis experiments suggest that the two cysteine and the histidine residues are most directly involved in the reactions catalyzed by PDI.

2.5 The thiol disulfide oxidoreductase family

The presence of two thioredoxin-like active–sites (CXXC) places PDI in a large family of proteins that contains sequence similarity to the active–site of thioredoxin. This group of proteins is more generally known as the thioredoxin family and includes such proteins as thioredoxin and glutaredoxin, which also belong to the thiol-disulfide oxidoreductase family. Members of the thioredoxin family with defined function other than oxidoreductase activity include a developmentally–regulated Trypanosome gene product (Hsu et al., 1989), gonadotropic hormones (Boniface & Reichert, 1990), the eukaryotic protein ERp72 (Akiyama et al., 1992) and the yeast protein Eug1p (Tachibana & Stevens, 1992) whose function has not yet been defined. Some proteins containing the thioredoxin-like active sites have been tested for protein folding activity but have been found to be much less active than PDI (Boniface & Reichert, 1990). For example, while thioredoxin has both a protein reductase and isomerase activity (Holmgren, 1985), a putative phosphatidyl inositol phospolipase C protein containing two CGHC sequences does not have *in vitro* disulfide isomerase activity (Crooke & Bennett, 1989). This suggests that the presence of a CGHC active site does not portend a functional equivalence to PDI.

3. Other important members of the PDI family and oxidoreductases

3.1 Yeast PDI

Yeast PDI was initially isolated as part of the yeast genome sequencing project (Scherens et al., 1991). Subsequently, three groups independently cloned the gene based on an assumed homology to mammalian PDI (Farquhar et al., 1991; LaMantia et al., 1991; Tachikawa et al., 1991). Yeast PDI is a 522-amino acid protein that contains the *C*-terminal HDEL sequence — the mammalian ER retention signal. Yeast PDI also contains 5 consensus glycosylation sites, each of which appears to be modified in the protein found in wild-type cells. The yeast PDI amino acid sequence shares 29% identity and 44% similarity with mammalian PDI. Most significantly, the cloning of the gene for yeast PDI enabled Scherens et al. (Scherens et al., 1991) to demonstrate that PDI is essential for cell viability.

3.2 Eug1p

While examining the role of disulfide bond formation in protein transport and protein sorting in the yeast secretory pathway, Tachibana and Stevens (Tachibana & Stevens, 1992) cloned, by hybridization with a PDI active site sequence probe, the gene *EUG1* (ER protein that is unnecessary for growth). Eug1p is a soluble ER resident protein of 517 amino acids that shares 21.3% identity to rat PDI and 43% identity to yeast PDI. Eug1p synthesis is greatly induced in response to the accumulation of proteins in the ER. It has 5 sites for *N*-linked glycosylation and is a 65- to 67- kDa glycoprotein. Interestingly, EUG1 contains only one cysteine residue per active site—the active site sequences being WCLHSQ and WCIHSK (Tachibana & Stevens, 1992).

EUG1 expression is controlled by an unfolded protein response element that is responsible for induction of the genes that contain them in response to the presence of unfolded proteins in the ER. While yeast PDI is naturally 10 – fold more abundant than

Eug1p, overproduction of Eug1p allows growth in the absence of PDI (Tachibana & Stevens, 1992).

Absence or overproduction of Eug1p does not affect the rate at which carboxypeptidase Y (CPY) moves through the secretory pathway, although altered quantities of Eug1p cause a slight defect in the sorting of soluble vacuolar proteins. On the other hand, PDI depletion causes yeast cells to accumulate the ER form of CPY, indicating that transport of this soluble vacuolar glycoprotein is slowed in cells without PDI. Overproduction of Eug1p does not restore CPY movement of *pdi1Δ S. cerevisiae* cells. The accumulation of the ER form of CPY in PDI–depleted cells is consistent with the model that PDI catalyzes folding in the ER by acting as a protein disulfide isomerase (Tachibana & Stevens, 1992).

3.3 Thioredoxin

Thioredoxin (Trx) is a low molecular mass, ubiquitous dithiol protein that functions as an efficient protein disulfide reductant. [For reviews see: Eklund (1991); Holmgren (1985); Pigiet (1988); Pigiet (1986); Holmgren (1979)]. The redox-active site of Trx has the amino acid sequence CGPC, which is in a solvent exposed active center. The protein exists either in a reduced, dithiol form [Trx-$(SH)_2$], or in an oxidized form [Trx-S_2] where the cysteine residues form an intramolecular disulfide bridge.

Trx participates in redox reactions through the reversible oxidation of its active site dithiol to a disulfide. Concomitant with the oxidation of Trx is the reduction of a disulfide in a substrate protein. Trx-S_2 is reduced to Trx-$(SH)_2$ by NADPH and the flavoprotein, thioredoxin reductase (TR). Depending on the chemical environment, thioredoxin can catalyze reduction of a protein disulfide bond or oxidation of a dithiol to a disulfide.

E. coli Trx was isolated as a hydrogen donor for ribonucleotide reductase, an essential enzyme in the synthesis of deoxyribonucleotides from the corresponding ribonucleotides and NADPH. Trx can also serve as a reducing agent for ribonucleotide

reductase from *E. coli* and other organisms. In addition, *E. coli* thioredoxin-$(SH)_2$ is an essential subunit of phage T_7 DNA polymerase. Trx is also required for the assembly of the filamentous viruses F1 and M13. Thioredoxin has been identified in a variety of functional roles in different biological systems involving regulation of protein activity by thiol redox control. Thioredoxin interacts with a broad range of proteins which are involved in either electron transport in substrate reduction or regulation of activity via thiol-redox control.

3.3.1 *Structure of* E. coli *thioredoxin*

The three–dimensional structure of Trx-S_2 has been determined by X-ray diffraction analysis to a resolution of 2.8 Å (Holmgren et al., 1975), and recently refined to a resolution of 1.68 Å (Katti et al., 1990). Trx is a highly structured molecule with 90% of its residues involved in secondary structural elements. The active–site disulfide formed by residues 32 and 35 is located at the *N*-terminus of an α-helix. The 14-member disulfide ring composed of residues CGPC is located on the surface of the protein but points toward the interior of the protein. Cys32 is exposed to solvent, whereas Cys35 is recessed and interacts with residues in other parts of thioredoxin (Holmgren, 1985).

Although the amino acid sequences of Trxs from different species show varying identity with that of the *E. coli* Trx, the derived three–dimensional structures are predicted to be similar. Most of the differences in sequence result in substitutions of amino acid residues at the surface of Trx, and these substitutions should affect only the local structure of the molecule (Eklund et al., 1991).

Thioredoxin-$(SH)_2$ has been studied by 2D NMR and the secondary structure has been constructed by distance geometry and molecular dynamics methods (Eklund et al., 1991). The secondary structure and global fold are very similar to that of Trx-S_2. The main chains of both are essentially identical. Structural alterations are minor and localized to a hydrophobic surface with the exception of changes in the dihedral angles in the ring

containing the disulfide bond, notably a rotation of the side chain of Cys35. The side chain of Cys32 is tilted towards the solution in the reduced form to accomodate the increase in sulfur–sulfur distance upon formation of two sulfhydryl groups. The active–site helix protrudes from the rest of the molecule but the sulfur atoms of the disulfide bridge are located on the inside of the helix and are covered by it (Eklund et al., 1991).

3.4 DsbA

A mutation (*dsbA*) that renders *E. coli* severely defective in disulfide bond formation has been described (Bardwell et al., 1991; Kamitani et al., 1992). DsbA is a soluble periplasmic protein which appears to assist in protein folding by facilitating disulfide bond formation (Bardwell et al., 1991). Proteins that do not contain disulfide bonds apparently fold normally in *dsbA* mutants. DsbA is a 208-amino acid polypeptide directed to the periplasmic space by a cleavable 19 amino acid signal sequence at the *N*-terminus of the protein and is present at approximately 4 to 8 μM in the cell (Akiyama et al., 1992). The proposed active site of DsbA contains the sequence CPHC. This pair of cysteine residues is mostly oxidized, at least after purification (Akiyama et al., 1992). Purified DsbA is capable of reducing the disulfide bonds of insulin. *dsbA* is not an essential gene, although loss of *dsbA* has many phenotypic effects ascribed to the loss of disulfide formation in secreted proteins. The proteins affected range from *E. coli* proteins such as alkaline phosphatase (PhoA) and OmpA, to the cloned eukaryotic proteins urokinase and tissue plasminogen activator (Bardwell et al., 1991). In *dsbA* mutant cells, pulse-labeled β-lactamase, PhoA, and OmpA are secreted but largely lack disulfide bonds. These disulfide deficient proteins may represent *in vivo* folding intermediates, since they are protease sensitive and chase slowly into stable oxidized forms. The non-essentiality of DsbA in *E. coli* may be explained by the lack of a disulfide-bonded periplasmic protein that is essential for growth or by another mechanism of disulfide formation, either due to air oxidation or to the existence of additional

dsbA-like factors in the cell that are sufficient for supporting cell growth (Kamitani et al., 1992).

3.4.1 *Structure of* E. coli *DsbA*

The structure of DsbA has been determined by X-ray diffraction analysis to a resolution of 2 Å (Martin et al., 1993). Despite low primary sequence similarity, the structure of DsbA resembles closely the structure of *E. coli* Trx. The C-alpha atoms of 71 residues in the DsbA thioredoxin-like domain can be lined up with the corresponding residues in Trx with a root mean square difference in position of only 1.8 Å. The presence of a compactly folded helical domain that forms a cap over the Trx-like active site of DsbA is an important difference. The redox-active disulfide bond, which is responsible for the oxidation of substrates, is at the interface between the helical domain and the Trx domain and is surrounded by grooves and exposed hydrophobic side chains. These structural features suggest that DsbA might act by binding to partially folded polypeptide chains before oxidation of cysteine residues. The active–site disulfide is placed in the center of a relatively extensive hydrophobic protein surface, which could provide stabilizing interactions for partially folded substrates (Martin et al., 1993).

DsbA is a strong oxidizing agent, and catalyzes the formation of disulfide bonds in other proteins (Wunderlich et al., 1993a). It has been shown that the higher redox potential of DsbA relative to *E. coli* thioredoxin, due to the strained conformation of oxidized DsbA (Wunderlich et al., 1993a). This strain is not apparent in the structures of oxidized and reduced protein. The structure of DsbA resembles that of most enzymes with the active site at the interface between domains. This interface provides for a more complex and extensive binding surface than that in thioredoxin and may allow for the accommodation of a wide range of substrates through domain movements. (Martin et al., 1993).

Mutants of DsbA were made where either one or both active – site cysteines were changed to an alanine residue and their ability to refold hirudin in the presence of oxidized glutathione (GSSG) was examined at pH 4.0 or 8.7 (Wunderlich et al., 1995). C30A and C30A/C33A DsbA were inactive but both wild-type and C33A catalyzed hirudin refolding about two fold. C33A DsbA catalyzed the reaction at significantly lower levels at pH 4.0 than at pH 8.7. Since GSSG does not react significantly with reduced hirudin, an initially formed mixed disulfide with glutathione must be the oxidant in hirudin refolding catalyzed by the mutant enzyme (Wunderlich et al., 1995).

Similar studies were done in which one or both cysteine residues of DsbA were replaced by serine, and the ability to reduce insulin was assayed (Zapun et al., 1994). C33S DsbA exhibits some activity in the insulin reduction assay, but the C30S DsbA and double mutant enzyme were ineffective. Replacing C33 or C30 with a serine residue decreases the stability by 1.1 and 1.8 kcal/mol, respectively. This instability suggests that the thiol groups are involved in interactions that stabilize the folded conformation, which would cause any disulfide bonds, either inter- or intramolecular that involve these groups, to be unstable. Removing the thiol group, either by mutation or by forming a disulfide bond (either intramolecular or as a mixed disulfide) would therefore be energetically unfavorable in the folded state of DsbA (Zapun et al., 1994).

3.4.2 *Oxidoreductases from other organisms*

A periplasmic protein (TcpG/DsbA) that shares overall homology with DsbA from *E. coli* has been isolated from *Vibrio cholerae* (Peek & Taylor, 1992; Yu et al., 1992) Comparison of the amino acid sequence of *V. cholerae* DsbA and *E. coli* DsbA reveals 63% similarity and 40% identity. Neither protein shares significant similarity with other oxidoreductases beyond the region surrounding the active site, suggesting that *E. coli* and *V. cholerae* DsbA form a distinct class of enzymes involved in the formation of disulfide bonds

within the periplasm (Peek & Taylor, 1992). *E coli* DsbA complements the *dsbA* mutants of *V. cholera*, demonstrating the functional equivalence of the proteins (Yu et al., 1992). This conservation of primary structure and function suggests the presence of a unique class of bacterial periplasmic proteins necessary for the functional maturation of secreted proteins containing disulfide bonds (Peek & Taylor, 1992).

Similarly, a protein that contains the active–site sequence CPHC has been isolated from *Haemophilus influenza* (Tomb, 1992). This protein, Por, is required for the correct assembly and/or folding of one or more disulfide-containing cell envelope proteins involved either in competence development or in the DNA binding and uptake machinery. *H. influenza* Por complements an *E. coli* strain deficient in the production of DsbA and is thus a functional homologue of DsbA (Tomb, 1992).

A protein, HelX, necessary for the biogenesis of c-type cytochrome was cloned from *Rhodobacter capsulatum* (Beckman & Kranz, 1993). The predicted amino acid sequence of the *helX* gene product shows overall homology to bacterial thioredoxins and shares active-site homology with PDI. However, the function of HelX is not the same as that of DsbA. Alkaline phosphatase itself requires disulfide bonds, presumably for proper folding. Yet, high levels of alkaline phosphatase activity were observed in both the *R. capsulatum* wild-type and *helX* strains. The simplest function envisioned for HelX is to retain cysteine residues of apocytochrome C in a reduced state prior to heme ligation. Another possible function would be to keep heme or some other biosynthetic component reduced. Thus, HelX is a representative of a different class of periplasmic disulfide oxidoreductase (Beckman & Kranz, 1993).

3.5 DsbB

If DsbA and PDI are to act catalytically, the active site dithiol/disulfide must be regenerated. An integral membrane protein, DsbB, which is also necessary for disulfide bond

formation has been implicated in the reoxidation of DsbA (Bardwell et al., 1993; Dailey & Berg, 1993). DsbB, which contains 4 cysteine residues, may act by transferring disulfide bonds directly to DsbA, thus reoxidizing DsbA and thereby regenerating DsbA's ability to donate its disulfide bond to target proteins. The source of these oxidizing equivalents is unknown, but a possibility is that the electrons generated by disulfide bond formation are coupled through DsbB to the electron transport chain (Bardwell et al., 1993; Bardwell, 1994). *dsbA* and *dsbB* double mutants show defects identical to a *dsbA* single mutant. Cysteine, oxidized glutathione (Bardwell et al., 1993) and cystine (Dailey & Berg, 1993) can substitute for *dsbB* mutants but only inefficiently substitute for DsbA. DsbA likely oxidizes disulfide bonds directly, while DsbB regenerates DsbA. This result is supported by the observation that in strains lacking DsbB, DsbA is in the fully reduced state. In addition, overproduction of DsbA suppresses a *dsbB* mutant, but the reverse is not true. This paradox is explained if an increased amount of DsbA decreases the need for recycling of DsbA to the point that disulfide-containing small molecules, such as cystine, are sufficient for recycling DsbA (Bardwell, 1994).

3.5 DsbC

An additional gene involved in disulfide bond formation in prokaryotes has been identified. By looking for a gene able to complement an *E. coli* dsbA mutation (Shevchik et al., 1994) or by looking for DTT-sensitive mutants (Missiakas et al., 1994) the gene *dsbC* was identified and characterized in *E. coli* (Missiakas et al., 1994) and *Erwinia chrysanthemi* (Shevchik et al., 1994).

DsbC is a dimer of identical 24–kDa periplasmic monomers with a CGYC active site. The CGYC motif within each monomer forms an unstable and reactive disulfide bond (Zapun et al., 1995). The low stability of the DsbC disulfide bond implies that, as is the case with DsbA, it destabilizes the folded conformation of DsbC.

The presence of DsbC could explain the residual disulfide isomerase activity existing in *dsbA* mutants. Reoxidation of DsbC does not seem to occur through DsbB. DsbC is able to complement a *dsbA* mutation. Overexpression of DsbC can restore a wild type phenotype in cells lacking DsbA and/or DsbB, reciprocally, overexpression of DsbA can complement the lack of DsbC (Missiakas et al., 1994; Shevchik et al., 1994). No complementation was observed when the *dsbB* gene was present on a multicopy plasmid (Missiakas et al., 1994). The *dsbC* mutant has no phenotype probably because the effect of the absence of *dsbC* is masked by the presence of DsbA . The phenotype of double *dsbA dsbC* mutant is stronger than any of the single mutant suggesting that DsbA and DsbC are probably part of two parallel pathways of disulfide bond formation (Missiakas et al., 1994; Shevchik et al., 1994).

4. Catalysis *in vitro* by PDI: reactions and proposed mechanisms

PDI has been shown to catalyze *in vitro* the reduction and isomerization of protein disulfide bonds and the oxidation of protein dithiols. The reaction products are dependent on the substrate and on the reduction potential of the entire system (Freedman, 1984). Thus, with a reduced protein substrate in the presence of a mild oxidant, PDI catalyzes the formation of native disulfide bonds. This catalysis has been observed for a wide range of small proteins and also for multi-domain proteins such as serum albumin and multichain proteins such as Ig and procollagen. With an oxidized protein substrate containing non-native bonds, PDI catalyzes rearrangement of the disulfide bonds. In mildly reducing conditions, the rearrangement will be a true isomerization of protein disulfide bonds to yield the protein product with native disulfide bonds. Isomerization assays classically use scrambled RNase A as a substrate. However, PDI has been shown to catalyze the isomerization of Fab fragments, immunoglobulin chains, and small peptides. Finally, with an oxidized protein substrate in strongly reducing conditions, the overall effect of catalysis by PDI is to reduce protein disulfide bonds.

4.1 Disulfide bond formation and isomerization

The oxidation of free sulfhydryl groups by PDI may involve either the direct transfer of oxidizing equivalents by PDI (Fig. 1A) (Lyles & Gilbert, 1991b) or protein oxidation via oxidized glutathione (Wittrup, 1995). Direct transfer of oxidizing equivalents requires the presence of a disulfide bond in the PDI molecule. To catalyze the formation of disulfide bonds, oxidized PDI would first bind to a reduced protein, bringing a very reactive disulfide next to the free cysteines of the protein substrates. This disulfide is attacked by one of the free cysteines forming a mixed disulfide. As the appropriate free cysteine approaches this very reactive mixed disulfide, a second attack occurs, and the disulfide bond is then

transferred to the protein that is in the process of folding. PDI then undergoes an intramolecular rearrangement to yield a disulfide bond and reduced PDI, which is then reoxidized to continue the cycle (Creighton et al., 1980; Bardwell et al., 1993). *In vitro*, optimal PDI activity is obtained in an RNase A refolding assay under redox conditions that would reduce the active–site disulfide of PDI (Lyles & Gilbert, 1991a). Similarly under ER redox conditions (Hwang et al., 1992), the PDI active site is thought to be reduced (Wittrup, 1995).

Alternatively, oxidation may occur via a mechanism that involves oxidized glutathione (Fig. 1A - bottom) (Wittrup, 1995). This model allows for PDI to be present in the reduced state. Oxidized glutathione binds to the enzyme creating an enzyme–glutathione mixed disulfide intermediate that is attacked by the protein substrate, resulting ultimately in the formation of the disulfide bond in the substrate, the net release of 2 equivalents of GSH and the regeneration of a reduced PDI molecule.

Rearrangement of disulfide bonds could occur via thiol–disulfide interchange reactions involving transient breakage of one of the proteins disulfide by the enzyme, followed by formation of the correct disulfide bond (Fig. 1B) (Creighton et al., 1980).

4.2 Mechanism of catalysis by PDI

Attempts have been made to dissect the mechanism of catalysis by PDI but the results have been inconclusive.

Examining PDI catalysis of thiol/disulfide exchange between glutathione and defined peptide disulfide substrates, PDI showed no evidence for saturation behavior and only one of the two disulfides of PDI participates in turnover, at least for the reactions involving the model peptide examined (Gilbert, 1989). The kinetics are entirely consistent with the generally assumed mechanisms of disulfide reduction that involve the formation of a mixed disulfide intermediate with the substrate. Thus it is currently believed that the mechanism for

PDI catalysis involves the ordered addition of substrate and the formation of a ternary complex rather than a double displacement or a ping-pong mechanism (Gilbert, 1989; Morjana & Gilbert, 1991).

Mechanistic studies done with PDI and the S-sulfonated derivatives of insulin and RNase A show that the native forms can be formed independent of the presence of each other (Yu & Tsou, 1992). Since it had been shown previously that PDI forms disulfide linked intermediates with its substrates, these results suggest that once the substrate is bound to the enzyme through disulfide exchange, it probably remains bound to the isomerase molecule for further exchange until eventually the native protein is formed and released from the isomerase. It also suggests that the final formation of disulfide bonds and the folding to produce the native conformation of proteins are independent of the presence of other proteins, as would be required during biosynthesis of disulfide containing proteins (Yu & Tsou, 1992).

4.3 Catalysis by the CXXC motif

Evidence that PDI does in fact catalyze protein folding within the ER comes from studies using dog pancreas microsomes (Bulleid & Freedman, 1988). These microsomes were depleted of their lumenal contents, including PDI, and then tested for their ability to synthesize mature, native γ-gliadin, a plant storage protein. These lumenally–depleted microsomes were functional in translating γ-gliadin and translocating it into the lumen but were unable to fold it properly into its final stable form. The addition of oxidizing equivalents (i.e., GSSG) to the lumenally depleted microsomes did not restore folding capability, but repletion with PDI did (Bulleid & Freedman, 1988).

Studies by Creighton of the *in vitro* refolding of bovine pancreatic trypsin inhibitor (BPTI) indicated that significant quantities of BPTI molecules containing non-native disulfide bonds formed during refolding and that these molecules are obligate intermediates in the

folding pathway (Creighton, 1978). Creighton and collaborators looked at the effect of PDI on the refolding pathway of BPTI. PDI was shown to catalyze selectively the thiol/disulfide exchange processes. PDI did not change the nature of the disulfide-containing intermediates during the oxidative folding of BPTI (Creighton et al., 1980). PDI, at glutathione concentrations considerably lower than those thought to exist in the ER, was shown to catalyze the oxidative folding of BPTI approximately 40-fold relative to the uncatalyzed reaction (Creighton et al., 1993).

Using more modern techniques, Kim and colleagues have reexamined the refolding of BPTI *in vitro* (Weissman & Kim, 1991). Although there is no evidence for highly populated non-native intermediates, PDI is still found to rescue kinetically trapped folding intermediates (Weissman & Kim, 1993). The N' and N* intermediates on the BPTI folding pathway appear native by many criteria. However, they only contain 2 of the 3 disulfide bonds found in the native structure. Addition of PDI dramatically accelerates the isomerization reactions required for the completion of disulfide bond formation. Thus, PDI is able to gain access to buried thiols in a protein that has substantial tertiary structure.

Similar studies have been performed with human gonadotropic hormone. The *in vitro* folding of the human chorionic gonadotropin β subunit (hCG-β) is indistinguishable from the intracellular folding pathway. Comparison of *in vitro* and *in vivo* hCG-β folding and hCG subunit assembly in the presence of PDI, shows that PDI increases the rate of *in vitro* folding by 3- to 5- fold and *in vitro* assembly by 60-fold without changing the order of disulfide bond formation from that of the intracellular folding and assembly pathway. Catalysis of disulfide bond formation proceeds via a PDI–substrate disulfide-linked intermediate since a PDI–hCGβ complex was observed on nonreducing SDS-PAGE (Huth et al., 1993).

PDI is required for immunoglobulin A and immunoglobulin M biosynthesis (Della Corte & Parkhouse, 1973). The need for PDI in this particular system was reinforced when it

was shown that levels of PDI in lymphoid tissue varied with immunoglobulin secretory activity. Specifically, enzyme levels and Ig synthesis increase in parallel. The correlation indicated that the enzyme plays a critical role in the formation of intramolecular bonds common to all immunoglobulin molecules. Even though PDI was able to catalyze the formation of the interchain bond required for monomer IgM assembly, it did not catalyze the formation of the intermolecular bonds required for pentamer assembly (Roth & Pierce, 1987).

Protein folding studies using DsbA *in vitro* have shown mixed results. As an oxidant, DsbA is effective when present in a large molar excess over the reduced substrates RNase A and PhoA in the presence of GSSG (Akiyama et al., 1992). Oxidation of PhoA always occurs abruptly within a short time window, and higher concentrations of DsbA shorten the lag period preceding this conversion.

DsbA also catalyzes the oxidative folding of hirudin, a thrombin inhibitor with 3 disulfide bonds. At pH 8.7, DsbA decreases the half–time of refolding by 2- to 3- fold without changing the relative distribution of species. The reaction starts with a rapid initial oxidation reaction that is complete within mixing time. However, the native state is only reached after an hour. The catalytic efficiency of DsbA was more obvious at acidic pH where no spontaneous folding can be detected in its absence (Wunderlich et al., 1993b).

Acting as an isomerase, a large molar excess of DsbA converted scrambled RNase A to the active enzyme in the presence of GSH (Akiyama et al., 1992) or DTT (Yu et al., 1993). Conditions for oxidative folding (i.e.GSSG) did not allow DsbA stimulated activation of the scrambled RNase A. Periplasmic fluid preparations containing an increased level of DsbA can also isomerize scrambled RNase A (Yu et al., 1993).

In addition, DsbA has isomerase activity in the refolding of misfolded insulin-like growth factor I (IGF-I) (Joly & Swartz, 1994). DsbA breaks and reforms two disulfide bonds in a reaction that is only catalyzed by the reduced form and where this activity is

catalytic and not stoichiometric. The oxidized form of DsbA stimulates the oxidative folding of completely reduced IGF-I. Thus, the reduced form of the protein is a disulfide isomerase while the oxidized protein can assist formation of disulfide bonds in reduced substrates under physiological conditions (Joly & Swartz, 1994).

Studies done with BPTI show that DsbA is able to catalyze the oxidative folding of reduced BPTI, but that it has no disulfide isomerase activity in the rearrangement of incorrectly folded BPTI (Zapun et al., 1993). Studies of the effect of DsbA on the refolding processes of BPTI and α-lactalbumin showed that stoichiometric amounts of oxidized DsbA donate disulfide bonds to reduced BPTI and α-lactalbumin. DsbA, however, did not efficiently catalyze the isomerization of intramolecular disulfide bond rearrangements in BPTI or α-lactalbumin (Zapun & Creighton, 1994).

DsbC shows disulfide oxidoreductase activity *in vitro*. Oxidized DsbC rapidly transfered its disulfide bonds to reduced BPTI and to a peptide with only two cysteine residues (Zapun et al., 1995). DsbC is also active as an isomerase in the refolding of BPTI and α-lactalbumin. Disruption of the *dsbC* gene inhibits disulfide bond formation most in proteins with multiple disulfide bonds. The rate observed *in vitro* for correct disulfide formation in reduced BPTI using DsbA as an oxidant and DsbC as a catalyst of disulfide rearrangement is comparable to the rate observed with recombinant BPTI in the periplasm of *E. coli* (Zapun et al., 1995). Thus, the greatest functional difference between DsbA and DsbC is that the latter has a much greater ability to catalyze the rearrangement of protein disulfide bonds. This, together with the observation that the phenotypes of *dsbA dsbC* double mutants are stronger that than either *dsbA* or *dsbC* single mutants suggests independent pathways of oxidoreduction (Missiakas et al., 1994).

4.4 Physical and biochemical properties important to catalysis by the CXXC motif

Two biochemical properties have been recognized as important for catalysis by the CXXC motif: the pK_a of the first cysteine in the CXXC motif and the redox potential of both the disulfide bond of the catalyst and of the whole system.

4.4.1 pK_a

The pK_a of a thiol is a measure of the relative stability of its protonated and unprotonated forms (Chivers et al., 1995). The pK_a determines not only the extent of thiol ionization at any given pH, but also the intrinsic chemical reactivity of the sulfur atom, even when it is part of a disulfide bond (Bardwell, 1994). The pK_a's of protein thiols are usually around 8.6. However, the pK_a's of members of the PDI family and other oxidoreductases have been shown to lie significantly below this value (Table 1.2).

4.4.2 *Reduction potential of the disulfide bond of the catalyst*

The reduction potential of oxidoreductases is shown in Table 1.3. The disulfide bond in Trx has a reduction potential of -270 mV and is not readily reduced by GSH ($E^{o'}$ = -250 mV). The low reduction potential of Trx contributes to its low activity in the oxidation and rearrangement of RNase A in a GSH/GSSG redox buffer (Krause et al., 1991). The reduction potential of PDI (Hawkins & Freedman, 1991; Lyles & Gilbert, 1991a) is -180 mV indicating that the disulfides of PDI are vastly superior oxidants compared to thioredoxin. When the proline residue in the active site of Trx is replaced by a histidine to mimic PDI, the reduction potential is increased to -235 mV (Krause et al., 1991), making the mutant thioredoxin a 10-fold better oxidizing agent than wild type Trx (Lundström-Ljung et al., 1992).

DsbA has a reduction potential of -0.089 mV (Wunderlich et al., 1993a). Thus DsbA is a stronger oxidant than Trx and a weaker oxidant than eukaryotic PDI's (Wunderlich & Glockshuber, 1993b). The disulfide bond in DsbA is quite reactive, and its presence destabilizes the structure of the protein. Reduced DsbA is 5.4 kcal/mol more stable than oxidized DsbA. This supports a suggested mechanism for catalysis of dithiol oxidation by DsbA in which the potential energy of the tense conformation in the oxidized protein is used to shift the redox equilibria between DsbA and dithiols towards reduced DsbA and disulfides (Wunderlich et al., 1993a; Zapun et al., 1993).

4.4.3 *Reduction potential of the system*

The redox state of the reaction environment is important for disulfide isomerase activity. Changes in the concentration of GSH and GSSG resulted in changes in both the PDI catalyzed and uncatalyzed refolding of reduced, denatured RNase A. The PDI–catalyzed folding of RNase A is optimum when [GSH] = 1mM and [GSSG] = 0.2mM (Lyles & Gilbert, 1991a). The ratio of the PDI–catalyzed to uncatalyzed reaction rate of RNase A folding increases as $[GSH]^2/[GSSG]$ until it reaches a constant at $[GSH]^2/[GSSG]$ = 1mM suggesting that the active–site cysteines of PDI must be in a reduced form for catalyzing the rate-limiting step of RNase A folding.

Despite the amount of information that has accumulated about protein disulfide isomerase and other oxidoreductases, basic questions still remain to be answered. We have attempted to address some of these by creating a heterologous expression system of the cDNA for rat PDI that allows us to do both *in vitro* and *in vivo* work with the protein. Chapter II describes the system that was constructed and the purification procedure to obtain recombinant rat PDI. The ability of mammalian PDI to complement a *pdi1Δ S. cerevisiae* strain during germination and vegetative growth was explored using this system and is

described in Chapter III. Finally, active–site mutants were made to evaluate the reason why PDI is essential to viability in *S. cerevisiae* and the results are described in Chapter IV. These studies provide further insight into the role that PDI and the CXXC motif play during assisted protein folding.

Table 1.1 Active–site sequences of members of the PDI family and other oxidoreductases

Protein	Sequence	Reference
Rat PDI	FYAPWCGHCKALAP	(Edman et al., 1985)
Rat PDI	FYAPWCGHCKQLAP	(Edman et al., 1985)
Trypanosome gene product	FYVDTCGYCQMLAP	(Hsu et al., 1989)
I-PIPLC	FYAPWCGHCKALAP	(Bennett et al., 1988)
Yeast PDI	FFAPWCGHCKNMAP	(Scherens et al., 1991)
Yeast PDI	YYAPWCGHCKRLAP	(Scherens et al., 1991)
Yeast EUG1	FFAPWCLHSQILRP	(Tachibana & Stevens, 1992)
Yeast EUG1	YYATWCIHSKRFAP	(Tachibana & Stevens, 1992)
Thioredoxin	FWAEWCGPCKMIAP	(Holmgren, 1968)
E. coli DsbA	FFSFFCPHCYAFEM	(Bardwell et al., 1991)
E. coli DsbB	MLLKPCVLCIYERC	(Bardwell et al., 1993)
E. coli DsbC	FTDITCGYCHKLHE	(Shevchik et al., 1994)
V. cholerae DsbA	FFSFYCPHCNTFEP	(Peek & Taylor, 1992)
H. influenza Por	FFSFYCPHCYAFEM	(Tomb, 1992)
R. capsulatum HelX	FWASWCAPCRVEHP	(Beckman & Kranz, 1993)

Table 1.2 pK_a's of oxidoreductases

Protein	pK_a	Reference
thioredoxin	6.7	(Holmgren, 1985)
mammalian PDI	6.7	(Freedman et al., 1988)
DsbA	3.5	(Nelson & Creighton, 1994)

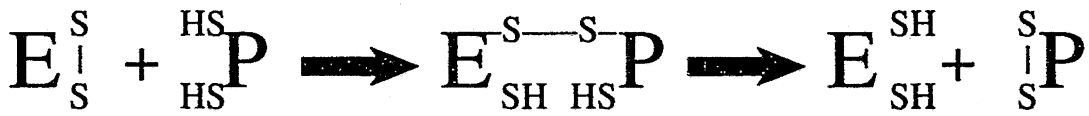
Table 1.3 Reduction potentials of oxidoreductases

Protein	Redox potential	Reference
thioredoxin	-270 mV	(Moore et al., 1964)
CGHC thioredoxin	-235 mV	(Krause et al., 1991)
rat PDI	-180 mV	(Lundström & Holmgren, 1993)
DsbA	- 089 mV	(Wunderlich et al., 1993a)

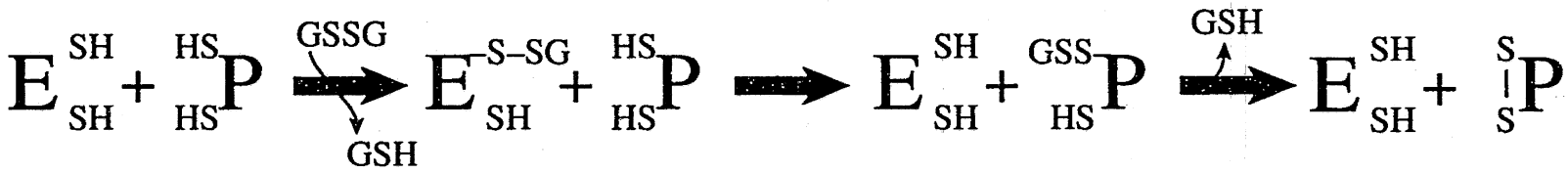
Figure 1.1 Reactions catalyzed by Protein Disulfide Isomerase. (A) Oxidation. (B) Isomerization.

A) Oxidation

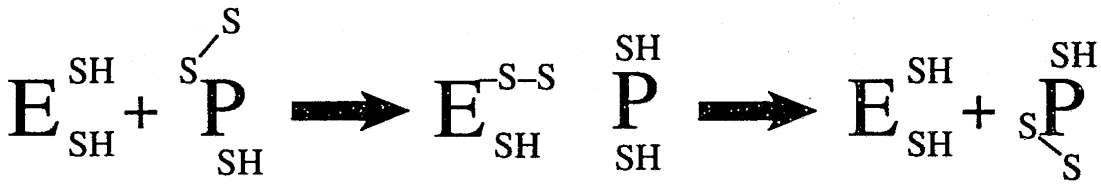
Direct



Glutathione-mediated



B) Isomerization



Chapter II

Production of Rat Protein Disulfide Isomerase in *Saccharomyces cerevisiae*

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ABSTRACT

Protein disulfide isomerase (PDI) is an abundant protein of the endoplasmic reticulum that catalyzes the oxidation of protein sulfhydryl groups, and the isomerization and reduction of protein disulfide bonds. *S. cerevisiae* cells lacking PDI are inviable. PDI is a component of many different protein processing complexes, and the actual activity of PDI that is required for cell viability is unclear. A cDNA that codes for rat PDI fused to the α-factor pre-pro segment was expressed in a protease-deficient strain of *S. cerevisiae* under the control of an ADH2–GAPDH hybrid promoter. The cells processed the resulting protein and secreted it into the medium as a monomer, despite having a KDEL or HDEL sequence at its C-terminus. The typical yield of isolated protein was 2 mg per L of culture. The catalytic activity of the PDI from *S. cerevisiae* was indistinguishable from that of PDI isolated from bovine liver. This expression system is unique in allowing the same plasmid to be used both to complement *pdi1Δ S. cerevisiae* and to produce PDI for detailed *in vitro* analyses. Correlations of the *in vivo* behavior and *in vitro* properties of PDI are likely to reveal structure – function relationships of biological importance.

INTRODUCTION

Protein disulfide isomerase (PDI, EC 5.3.4.1) is present at millimolar levels in the endoplasmic reticulum of eukaryotic cells. This protein is an enzyme that catalyzes the oxidation of protein sulfhydryl groups, and the isomerization and reduction of protein disulfide bonds (Bassuk & Berg, 1989; Freedman, 1989; Noiva & Lennarz, 1992). The active site of PDI can exist in either a reduced or oxidized state, or as a mixed disulfide with a substrate. The products of reactions catalyzed by PDI depend on the reduction potential of the solution.

PDI also appears to play other roles in cell biology. It has been identified as the β subunit of prolyl hydroxylase (Koivu & Myllyla, 1987), as a thyroid hormone binding protein (Cheng et al., 1987; Koivu & Myllyla, 1987; Yamauchi et al., 1987), and as a component of the microsomal triglyceride transfer protein complex (Wetterau et al., 1990). Although PDI is known to catalyze disulfide bond isomerization *in vitro* (Weissman & Kim, 1993), its role *in vivo* is not known (LaMantia & Lennarz, 1993). PDI has, however, been shown to increase the heterologous production of other proteins in *Saccharomyces cerevisiae* (Robinson et al., 1994).

The sequence of the cDNA that codes for rat PDI has been determined (Edman et al., 1985). Within its 527 residues, the protein translated in rat liver contains an N-terminal signal peptide and two pairs of homologous regions: amino acid residues 9–90 (region a) and 353–431 (region a'), and amino acid residues 153–244 (region b) and 256-343 (region b'). Regions a and a' each contain an active site with the sequence WCGHCK, and these sites have been shown to act independently of one another (Vuori et al., 1992a). The C-terminus of PDI ends with the sequence KDEL, which has been implicated as the signal for retention of a soluble protein in the endoplasmic reticulum of mammalian cells (Munro & Pelham, 1987).

The sequence of the cDNA that codes for PDI from *S. cerevisiae* has also been determined (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991). The encoded sequence of 522 amino acid residues is 30% identical to that of rat PDI. The amino acid sequence contains an N-terminal signal peptide, two putative active sites, four putative N-glycosylation sites, and a C-terminus ending with HDEL, the *S. cerevisiae* homolog of KDEL (Pelham et al., 1988; Scherens et al., 1991). *S. cerevisiae* mutants in which the PDI gene has been deleted are inviable (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991).

We are interested in revealing the role of PDI *in vivo*, and in correlating that role with properties of PDI determined *in vitro*. Here, we describe the expression in *S. cerevisiae* of the cDNA that codes for rat PDI. In our expression system, PDI is synthesized as a hybrid with the 85-residue yeast α-factor pre-pro segment. The hybrid protein is processed efficiently by endogenous *S. cerevisiae* proteases, and active PDI is secreted into the medium.

RESULTS

Production of Rat PDI in S. cerevisiae

The cDNA sequence that codes for rat PDI was inserted in a plasmid between ADH2–GAPDH hybrid promoter and GAPDH terminator sequences. The resulting plasmid, pMAL3.1 (Fig. 2.1), was used to transform the protease-deficient *S. cerevisiae* strain BJ2168. The expression of the PDI cDNA became derepressed as the concentration of fermentable carbon sources in the medium decreased. The PDI produced was secreted into the culture medium.

Purification of Rat PDI from S. cerevisiae *Medium*

PDI had been purified from bovine liver and other animal tissues by using heat treatment, ammonium sulfate precipitation, and cation exchange chromatography steps (Lambert & Freedman, 1983). These steps were not effective in purifying PDI from *S. cerevisiae* medium (data not shown). Accordingly, we developed a new purification scheme. A key method in this scheme was tangential flow concentration of the growth medium. We grew our cells in medium that had been filtered to remove components of $M_r > 5000$. After cell growth, we concentrated the medium to remove components of $M_r < 5000$ (Fig. 2.2, lane 1). We then used gel filtration chromatography to remove components of M_r <30,000. Concentrated medium was loaded directly onto a Pharmacia HiLoad 26/60 G-75 gel filtration column. The fractions containing PDI were approximately 15% pure as assessed by SDS-PAGE (Fig. 2.2, lane 2) and specific activity (Table 2.1). Since PDI is an acidic protein [p*I* = 4.2 (Lambert & Freedman, 1983)], we used anion exchange chromatography as the final purification step. The solution of PDI obtained after gel filtration chromatography was concentrated and loaded onto a Mono-Q anion exchange column, and PDI was eluted with a NaCl gradient. The fractions containing PDI were >95% pure as assessed by SDS-PAGE (Fig. 2.2, lane 3). The yield of isolated PDI was typically 2 mg per L of culture medium.

During gel filtration chromatography, PDI eluted at 120 – 125 mL—beyond the void volume of the column. Experiments using M_r standards showed that PDI is produced as a monomer (data not shown). This finding is in contradiction to reports that PDI is a dimer (Luz et al., 1993), but agrees with results from Gilbert (Morjana & Gilbert, 1991) and Kim (Weissman & Kim, 1993) who have also characterized the M_r of PDI by using gel filtration chromatography.

Processing of Rat PDI in S. cerevisiae

The location of the PDI produced by various plasmids was analyzed by immunoblotting. Polyclonal antibodies raised against bovine PDI recognize rat PDI but not endogenous *S. cerevisiae* PDI (data not shown). The medium from *S. cerevisiae* cultures was concentrated and probed with polyclonal antibodies against bovine PDI. Only those cells carrying plasmids that code for PDI fused to the α-factor pre-pro segment directed the secretion of PDI into the medium (Fig. 2.3A), and the presence of KDEL (lane 1) or HDEL (lane 2) did not alter dramatically the amount of protein secreted. This secreted protein had M_r 55,000, which is that expected for mature rat PDI. Amino acid sequence analysis of the rat PDI produced from pMAL3.1 showed that the nine N-terminal residues of the protein were identical to those predicted from the cDNA sequence. Thus, the fusion protein was processed correctly by the secretory system of *S. cerevisiae*.

To evaluate how much protein was being retained inside the cells, total protein was isolated from *S. cerevisiae* cells and probed by immunoblotting with polyclonal antibodies raised against bovine PDI. Extract from cells carrying plasmids that code for PDI fused to the α-factor pre segment contained one reacting protein of M_r 60,000 (Fig 2.3B, lanes 3 and 4), which is equivalent to that of rat PDI plus the pre segment. Extract from cells carrying plasmids that code for PDI fused to the α-factor pre-pro segment contained two reacting proteins of M_r 74,000 and 77,000 (Fig. 2.3B, lanes 1 and 2). These proteins had an M_r greater than that of rat PDI plus the α-factor pre-pro segment. Control samples transformed with the parental vector pWL, showed no PDI bands (Fig. 2.3B, lane 6).

The extra molecular mass in the PDI fused to the α-factor pre-pro segment suggests that this protein was glycosylated. Rat PDI does not contains an Asn – X – Ser/Thr sequence, which is the consensus site for N-glycosylation. In contrast, endogenous *S. cerevisiae* PDI contains 4 such sites, and it is glycosylated extensively (Scherens et al., 1991). The α-factor pro segment also contains two N-glycosylation sites. Immunoblotting after endoglycosidase

H treatment was used to determine if the fusion protein was indeed glycosylated in the pro segment. Treatment with endoglycosidase H produced a single product of M_r 65,000 (Fig. 2.4, lanes 3 and 5), which is equivalent to that predicted for rat PDI fused to the α-factor pre-pro segment.

Enzymatic Activity of Rat PDI from S. cerevisiae

The specific activity of recombinant PDI in the GSH-dependent reduction of insulin was 0.34 μmol/min/mg at pH 7.2 in the presence of GSH (0.5 mM). The specific activity of the rat PDI from *S. cerevisiae* was indistinguishable from that of the enzyme isolated from bovine liver (Table 2.1).

The specific activity of recombinant PDI in the isomerization of scrambled RNase A was 0.64 nmol RNase A reactivated/min/mg at pH 7.6 in the presence of GSH (1 mM) and GSSG (0.2 mM). Under these conditions, the enzyme isolated from bovine liver reactivated 0.68 nmol RNase A/min/mg.

DISCUSSION

Protein disulfide isomerase is an endoplasmic reticulum enzyme that has been implicated in the catalysis of protein disulfide bond formation *in vivo* (Bulleid & Freedman, 1988). Null PDI mutants of *S. cerevisiae* cell are inviable. Nonetheless, since PDI is a component of many different protein processing complexes *in vivo*, the actual activity of PDI that is required for cell viability is unclear (LaMantia & Lennarz, 1993). We anticipate that *in vivo* and *in vitro* comparisons of wild-type and mutant PDI's will resolve this dilemma.

Several systems have been developed for the heterologous production of PDI. Four groups have reported the production of human, rat, or murine PDI in *E. coli* (Gilbert et al.,

1991; Haugejorden et al., 1992; Vuori et al., 1992a; De Sutter et al., 1994), with yields as high as 30 mg/L. Of these groups, two reported that the expression of a PDI cDNA in *E. coli* resulted in PDI molecules that were truncated because of the initiation of protein synthesis from internal start codons (Haugejorden et al., 1992; De Sutter et al., 1994). PDI has also been produced in *Spodoptera frugiperda* (Vuori et al., 1992b) and *Bacillus brevis* (Tojo et al., 1994), with yields of 10 mg/L and 5 mg/L, respectively. In the former system, the aim was the study of the formation of the tetramer of prolyl-4-hydroxylase in which PDI is the β subunit. Finally, a functional expression cassette for human PDI has been integrated into the *LYS2* locus of an *S. cerevisiae* chromosome (Luz et al., 1993).

We have constructed a vector that directs the expression of rat PDI under the control of an ADH2–GAPDH hybrid promoter. In our vector, the endogenous signal sequence for the rat PDI has been replaced with the α-factor pre-pro segment. This sequence is effective in directing the secretion of PDI into the medium (Fig. 2.3, lane 1). We have also constructed a vector in which the rat PDI KDEL retention sequence was mutated to the *S. cerevisiae* HDEL retention sequence. Still, protein was secreted into the medium (Fig. 2.3, lane 2). Apparently, the α-factor pre-pro segment overrides the retention provided by the HDEL sequence. Alternatively, the retention system may become saturated so that excess protein is secreted into the medium (Pelham et al., 1988).

To determine the basis for the targeting of PDI *in vivo*, we deleted the 66 residues of the pro region α-factor pre-pro segment, leaving only the 19 residues of the pre region. This deletion should direct the protein into the endoplasmic reticulum, but not signal it to be secreted. Our results show that PDI is indeed retained within the cell.

Other heterologous expression systems can produce PDI at levels higher than does ours. Nevertheless, our system results in the synthesis of non-glycosylated, properly processed, full-length protein, which is easily purified from the culture medium in a enzymatically active, monomeric form. In addition, we have recently demonstrated that our plasmid

pMAL3.1 complements *pdi1Δ S. cerevisiae* cells (Chapter 4), which are otherwise inviable. Thus, our system allows for the meaningful study of wild-type and mutant PDI's both *in vivo* and *in vitro*. We believe that this system will enable us to reveal biologically relevant structure – function relationships in PDI.

Figure 2.1 Map of plasmid pMAL3.1, which directs the expression of rat PDI in *S. cerevisiae*. The plasmid is designed to facilitate the genetic manipulations required in protein engineering (delCardayré et al., 1995).

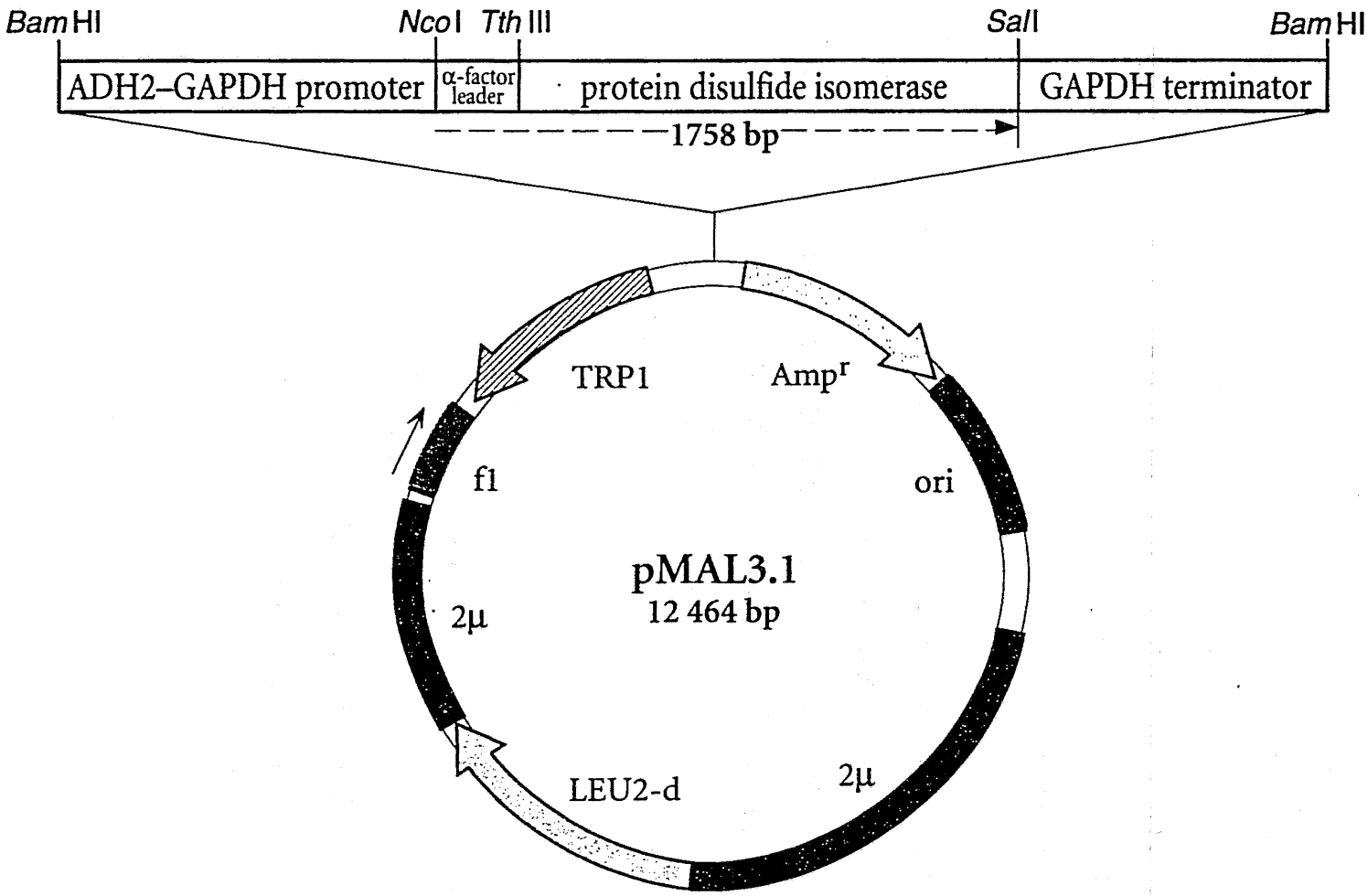

BamHI
NcoI
TthIII
SalI
BamHI
ADH2–GAPDH promoter
α-factor leader
protein disulfide isomerase
GAPDH terminator
1758 bp
TRP1
Amp^r
f1
ori
pMAL3.1
12 464 bp
2μ
LEU2-d
2μ

Figure 2.2 SDS-polyacrylamide gel showing the purification of PDI from *S. cerevisiae*. Recombinant PDI was produced in protease-deficient *S. cerevisiae* strain BJ2168. Concentrated proteins were separated by electrophoresis under reducing conditions after the following purification steps. Lane 1, crude extract from culture medium; lane 2, gel filtration chromatography; lane 3, anion exchange chromatography; lane M, unstained molecular mass markers (in kDa).

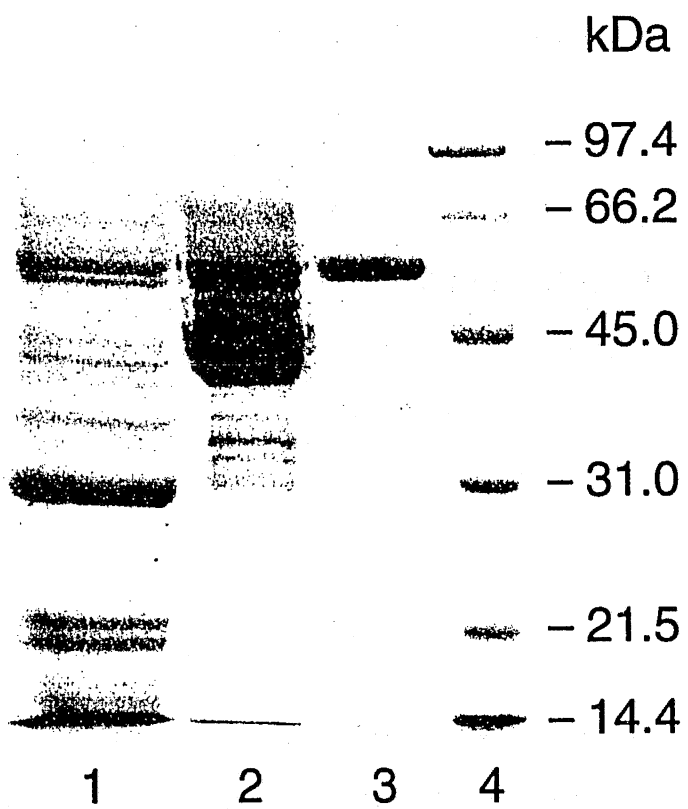

kDa
97.4
66.2
45.0
31.0
21.5
14.4
1
2
3
4

Figure 2.3 Immunoblot of secreted (A) and intracellular (B) proteins from *S. cerevisiae* strain BJ2168 probed with antibodies against bovine PDI. Lane 1, proteins from cells carrying plasmid pMAL3.1 (pre-pro α-factor~PDI~KDEL); lane 2, proteins from cells carrying plasmid pMAL5.1 (pre-pro α-factor~PDI~HDEL); lane 3, proteins from cells carrying plasmid pMAL 6.1 (pre α-factor ~PDI~KDEL); lane 4, proteins from cells carrying plasmid pMAL 7.1 (pre α-factor~PDI~HDEL); lane 5, empty; lane 6, proteins from cells carrying plasmid pWL(parental vector).

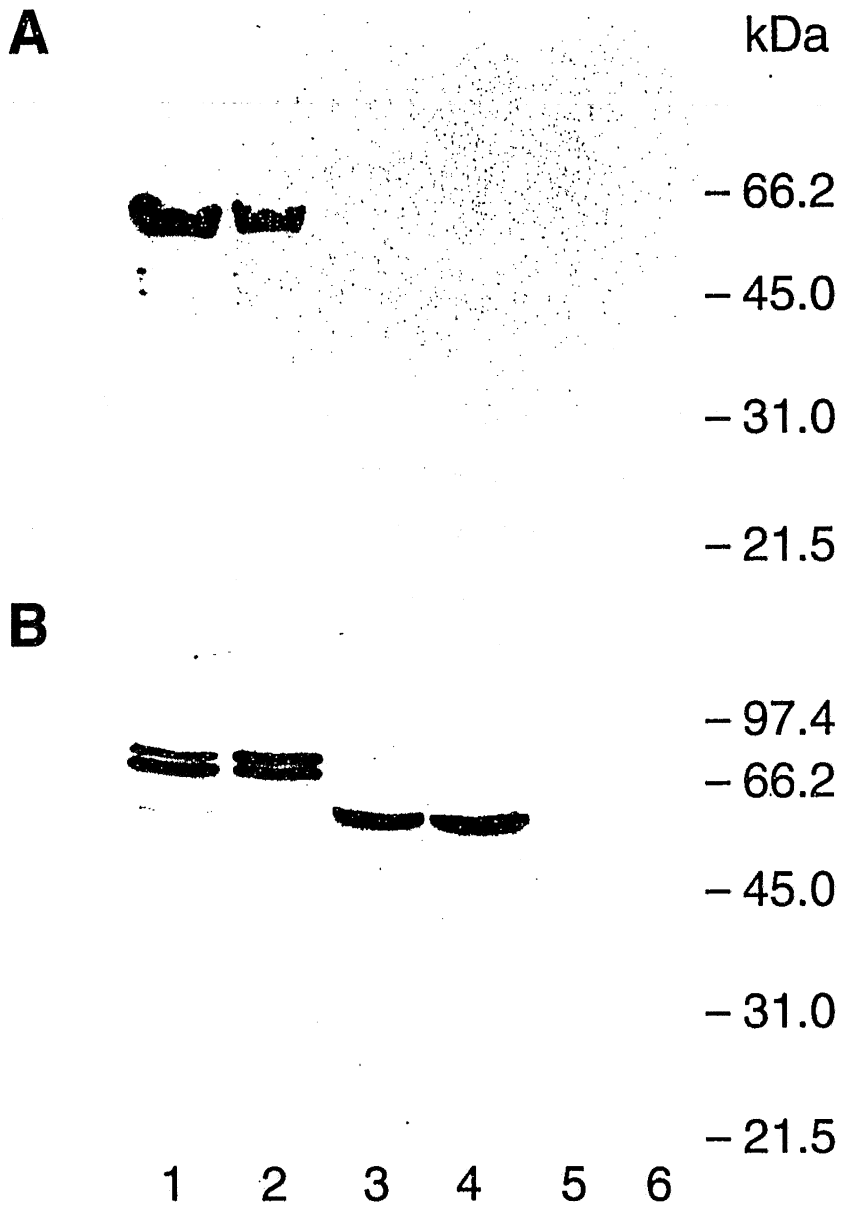

A
kDa
66.2
45.0
31.0
21.5
B
97.4
66.2
45.0
31.0
21.5
1 2 3 4 5 6

Figure 2.4 Immunoblot of intracellular proteins from *S. cerevisiae* strain BJ2168 before and after treatment with endoglycosidase H for 12 h. Lanes 1 and 2, extract from cells carrying plasmid pMAL3.1 (pre-pro α-factor~PDI~KDEL); lanes 3 and 4, extract from cells carrying plasmid pMAL5.1 (pre-pro α-factor~PDI~HDEL); lanes 5 and 6, extract from cells carrying plasmid pMAL6.1 (pre α-factor~PDI~KDEL); lanes 7 and 8, extract from cells carrying plasmid pMAL7.1 (pre α-factor~PDI~HDEL).

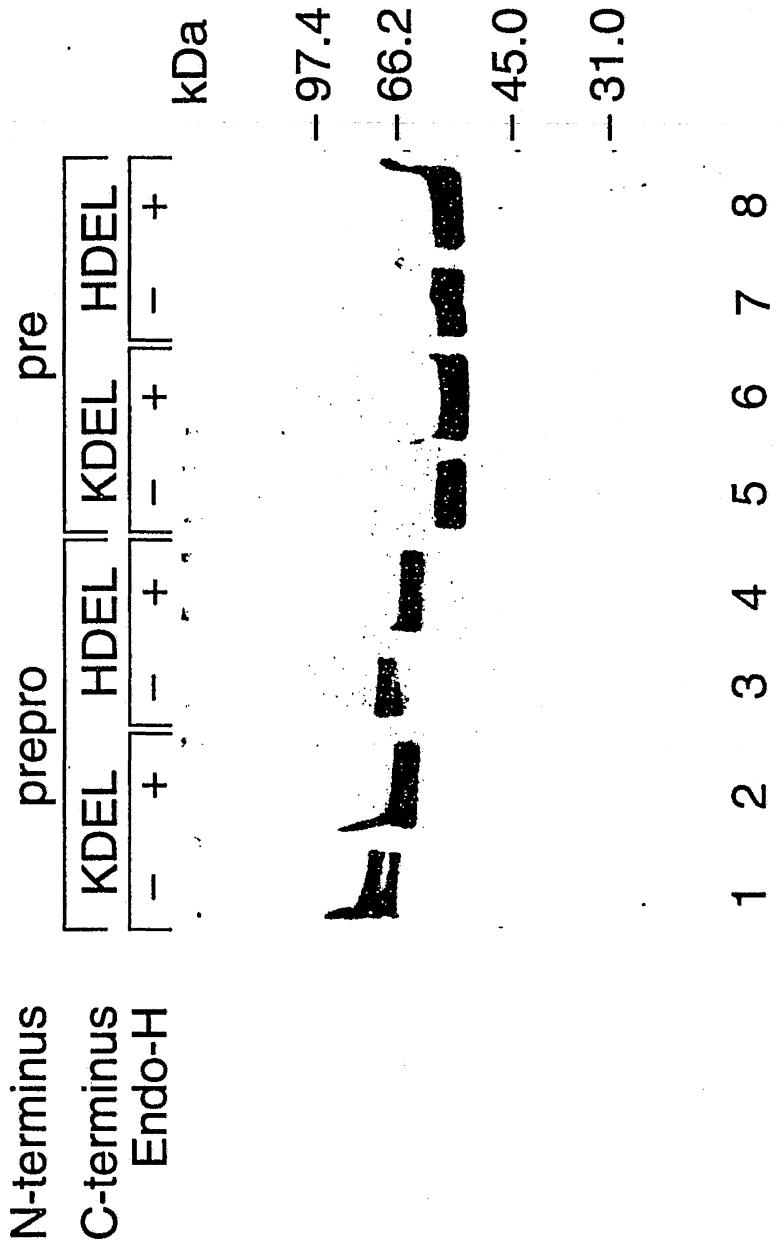

N-terminus
C-terminus
Endo-H
prepro
pre
KDEL
HDEL
KDEL
HDEL
−
+
−
+
−
+
−
+
kDa
97.4
66.2
45.0
31.0
1
2
3
4
5
6
7
8

Table 2.1

Summary of Purification of Rat Protein Disulfide Isomerase from *S. cerevisiae* [a]

Purification step	Protein (mg)	Total activity (U)	Specific activity (U/mg) [b]	Fold purification	Yield (%)
concentrated crude extract	177	0.87	0.0049	—	100
gel filtration chromatography	12.8	0.60	0.047	9.6	69
anion exchange chromatography	2.13	0.70	0.33	67	80

[a] From a 1.0 L culture.

[b] One unit of PDI will catalyze the reduction of 1 µmol of insulin per min at pH 7.2 in the presence of 0.5 mM GSH. PDI purified from bovine liver has a specific activity of 0.41 U/mg.

Chapter III

Importance of Protein Disulfide Isomerase to Spore Germination and Cell Division

ABSTRACT

Protein disulfide isomerase (PDI) is a protein of the endoplasmic reticulum (ER) that is essential for the unscrambling of nonnative disulfide bonds. Here, we have determined the importance of PDI to cell division during both spore germination and vegetative cell division. To vary the concentration of PDI in the ER, plasmids were constructed that direct the expression of rat PDI fused at the N-terminus to either the α-factor pre–pro segment or the α-factor pre sequence, and fused at the C-terminus to either the mammalian (KDEL) or the yeast (HDEL) ER retention signal. Classical yeast genetic (tetrad) analyses, and plasmid loss and plasmid shuffling experiments were used to evaluate the ability of these constructs to complement haploid *Saccharomyces cerevisiae* cells in which the endogenous *PDI* gene had been deleted. We find that basal levels of PDI in the ER are sufficient for vegetative growth. In contrast, high levels of PDI in the ER are required for efficient spore germination. Thus, the formation of native disulfide bonds in cellular proteins is apparently more important during spore germination than during cell division.

INTRODUCTION

A protein disulfide isomerase (PDI) activity was predicted to exist before the enzyme itself was isolated (Goldberger et al., 1963; Venetianer & Straub, 1963). The observed contrast between the slow formation of native disulfide bonds *in vitro* and their apparently rapid formation during protein biosynthesis indicated that the process was catalyzed by an enzyme. Subsequently, PDI was found to be an abundant protein of the endoplasmic reticulum (ER), the cellular compartment in which disulfide bonds are most most often formed in eukaryotic cells.

In vitro, PDI catalyzes the oxidation of dithiols to form disulfide bonds, and the reduction and isomerization of existing disulfide bonds (Hu & Tsou, 1991; Freedman et al., 1994). The sequence of the cDNA that codes for PDI has been determined for several species (Edman et al., 1985; Morris & Varandani, 1988; Scherens et al., 1991). The mammalian protein contains two active sites with the sequence WCGHCK, which act independently of each other (Vuori et al., 1992a). The C-terminus of mammalian PDI ends with the tetrapeptide KDEL, which has been implicated as the signal for retention of a protein in the ER of mammalian cells (Munro & Pelham, 1987). The sequence of the cDNA that codes for *Saccharomyces cerevisiae* PDI has also been determined (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991). The amino acid sequence contains an N-terminal signal peptide, two putative active sites, five putative N-glycosylation sites, and a C-terminus ending with HDEL, the *S. cerevisiae* equivalent of KDEL (Pelham et al., 1988; Scherens et al., 1991). Although the 522 amino acid residues of the encoded protein share only approximately 30% identity to mammalian PDI's, the active-site sequences are conserved completely.

PDI has other established roles in cell biology. It has been identified as the β subunit of prolyl hydroxylase (Koivu & Myllyla, 1987) and as a component of the microsomal

triglyceride transfer protein (MTP) complex (Wetterau et al., 1990). The precise role of PDI in these protein complexes is not known. In addition, PDI has been identified as the thyroid hormone binding protein (Cheng et al., 1987; Yamauchi et al., 1987), the cellular function of which is currently unknown.

The *PDI* gene is essential for the viability of *S. cerevisiae* (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991). Recently, we described an *S. cerevisiae* expression system for PDI that allows for the study of protein structure – function relationships *in vitro* and *in vivo* (Laboissière et al., 1995a). We used this system to demonstrate that the essential function of PDI in *S. cerevisiae* is to unscramble non–native disulfide bonds in the ER (Laboissière et al., 1995b). Here, we analyze the ability of four rat *PDI* constructs to complement a null mutation of the *S. cerevisiae PDI* gene. These constructs vary in their abilities to target PDI to the ER, and to retain it there. Our results show that the passage of low levels of PDI through the ER is sufficient for vegetative cell division, whereas the retention of high levels of PDI in the ER is necessary for efficient spore germination. We conclude that the formation of native disulfide bonds in cellular proteins is apparently more important during germination than during vegetative growth.

RESULTS

PDI is an essential gene in *S. cerevisiae*. To determine the role of PDI in *S. cerevisiae* growth, plasmids that direct the expression of rat PDI were transformed into *S. cerevisiae* cells in which the endogenous PDI1 gene had been deleted (*pdi1Δ*). Suppression of the mutant phenotype by the production of rat PDI was assessed in 3 ways. First, we determined the ability of plasmids encoding rat PDI to rescue haploid spores that had inherited the *pdi1Δ*::*HIS3* allele. Second, we monitored the ability of *pdi1Δ*/haploid cells to lose an

essential episomal copy of the yeast *PDI1* gene on pCT37 by the alternate retention of plasmids that direct the expression of rat PDI variants (non-selective plasmid loss). Third, we monitored the rate of growth of *pdi1Δ*/pCT37 cells containing plasmids that direct the expression of rat PDI under conditions that forced the loss of pCT37 (plasmid shuffling).

Plasmids pMAL3.1 and pMAL5.1 encode the pre–pro region of the α-factor leader segment. In pMAL5.1, the ER retention sequence of mammalian PDI in pMAL3.1, KDEL, is replaced with that of yeast, HDEL. Plasmids pMAL6.1 and pMAL7.1 encode only the pro region of the α-factor pre–pro segment. In pMAL7.1, the ER retention sequence from PDI encoded by pMAL6.1 is replaced with HDEL.

Plasmid dependent spore germination.

Plasmids encoding *S. cerevisiae* or rat PDI were transformed into a heterozygous *PDI/pdi1Δ::HIS3* diploid strain. After dissection of tetrad asci and germination 30°C on solid medium containing 2.0% (w/v) dextrose, the haploid progeny of these transformants were analyzed for spore viability, segregation of the *pdi1Δ::HIS3* disruption allele (as indicated by histidine protrophy, HIS^+), segregation of the plasmid encoding rat PDI (as indicated by tryptophan protrophy, TRP^+), and spore mating type.

As shown in Table 3.1, only the plasmids encoding *S. cerevisiase* PDI (pMAL9) or rat PDI fused to the α-factor pre–pro segment and the yeast retention signal (pMAL5.1), and grown at 30 °C on medium containing 2% (w/v) dextrose were able to rescue the *PDI* deficiency. Tetrads complemented with *S. cerevisiae* PDI grew within 3 days. As shown in Fig. 3.1, in tetrads complemented with this construct, 2 spores per tetrad that were his^- (and thus PDI^+ at the chromosomal locus) grew within 3 days. An additional 1 or 2 spores became visible only after 4 to 7 days, and gave rise to slowly growing colonies that were trp^+ (pMAL5.1) and his^+ (*pdi1Δ::HIS3*). The same experiments were done where after dissection of tetrad asci spores were germinated at 15 or 37°C on solid medium containing

2.0% (w/v) dextrose or at 30°C on solid medium containing 0.5 or 1.0% (w/v) dextrose. No complementation was observed under these conditions (results not shown). No difference in the production of rat PDI by the diploid parents was observed by immunoblot analysis (data not shown).

Low levels of PDI in the ER are sufficient for cell division.

We monitored *pdi1Δ*/pCT37 cells (which are haploid cells that express *S. cerevisiae* PDI from plasmid pCT37 rather than from a chromosome) containing plasmids that direct the expression of rat PDI for the loss of pCT37. Haploid *pdi1Δ* cells complemented by *S. cerevisiae* PDI were obtained by transforming *PDI/pdi1Δ::HIS3* with pCT37, sporulating the transformants, dissecting the segregants, and isolating his^+/ura^+ colonies. *pdi1Δ*/pCT37 haploid cells were transformed with plasmids pMAL3.1, pMAL5.1, pMAL6.1, pMAL7.1, or pMAL9. Transformants were selected on solid medium deficient in tryptophan but containing uracil. Single isolates were grown in liquid medium lacking only tryptophan to allow cells to lose pCT37 while forcing them to retain their *TRP1* plasmid. After several rounds of growth, cells were grown on tryptophan dropout plates to isolate single colonies. These colonies were grown on tryptophan dropout plates, and then replica plated onto tryptophan and uracil dropout plates to check for the presence of the *TRP1* and *URA3* plasmids. Table 3.2 shows that cells containing pMAL5.1 (rat PDI fused to the α-factor pre–pro segment and HDEL) often lost pCT37. In contrast, this loss was infrequent for cells containing pMAL3.1 (KDEL), and never occurred in cells containing plasmids pMAL6.1 or pMAL7.1 (α-factor pre sequence).

In a related approach, we monitored the growth of *pdi1Δ*/pCT37 cells containing plasmids that direct the expression of rat PDI under conditions that forced the loss of pCT37. Transformants were cultured and grown on plates containing 5-FOA, which selects for uracil auxotrophs (Sikorski & Boeke, 1991) and thus forces *pdi1Δ*/pCT37 cells to lose pCT37 in

order to grow. Under these conditions only cells that produce rat PDI form colonies. Cells containing plasmids that encode any variant of rat PDI were able to form colonies, though at different times after plating on 5-FOA medium. Cells containing plasmids pMAL3.1 or pMAL5.1 (α-factor pre–pro segment) formed colonies within 2 – 3 days. Cells containing pMAL7.1 (pre α-factor sequence and HDEL) formed colonies in 5 – 6 days. Cells containing plasmid pMAL6.1 (α-factor pre sequence and KDEL) formed colonies after 8 days. Colonies on the 5-FOA plates were prototrophic for histidine and tryptophan (his^+trp^+) but auxotrophic for uracil (ura^-), which indicated the presence of the *pdi1Δ::HIS3* allele, a plasmid encoding rat PDI, and the loss of plasmid pCT37.

Combined, the results from the plasmid loss and plasmid shuffling experiments indicate that rat PDI fused to the α-factor pre–pro segment and HDEL retention motif complemented *pdi1Δ* S. *cerevisiae* cells. Rat PDI fused to the α-factor pre–pro segment and KDEL retention motif was also able to complement, but with lower efficiency. Finally, plasmids encoding rat PDI fused to the α-factor pre sequence were poor in their complementation abilities. The analysis of the subcellular location of rat PDI from these constructs before plasmid shuffling demonstrated that the α-factor pre sequence was relatively ineffective in directing PDI to the ER (Fig. 3.2).

Growth characteristics conferred by mammalian PDI expression.

To quantify the ability of our different constructs of rat PDI to complement *pdi1Δ S. cerevisiae*, we grew the ura^-trp^+ cells that resulted from plasmid shuffling in YEP(1%)D and YEP(8%)D media and measured their doubling times. These results are shown in Table 3.3. All cells complemented with rat PDI grew more slowly than did those complemented with yeast PDI. Cells containing pre–pro α-factor~PDI~KDEL or pre–pro α-factor~PDI~HDEL had doubling times that were indistinguishable from each other and significantly lower than those of cells containing pre α-factor~PDI~KDEL or pre α-factor~PDI~HDEL. These results

are in gratifying agreement with the results obtained by the plasmid shuffling. Although growth in the presence of a high concentration of glucose represses expression from the *ADH2–GAPDH* promoter that controls the production of rat PDI, no significant difference in cell growth at 1 and 8% (w/v) glucose was detectable.

High levels of PDI in the ER are necessary for spore germination.

To determine if germination depends on PDI, we mated haploid *pdi1Δ* cells complemented with rat PDI with strain YPH252 to form diploids. These diploids were cultured and sporulated, and the tetrads were dissected. The results obtained were similar to those seen with direct dissection of a disrupted cell line that had been tranformed with the same plasmids (data not shown). Spores that did not form colonies were examined by microscopy. Approximately half of the nonviable spores did not germinate at all. The remainder proceeded through two or three cell divisions, and then died. We also dissected the tetrads at different times after the induction of sporulation, and found no change in viablity. Thus, viability does not depend on the time spent sporulating, as had been observed in the complementation of a ubiquitin deficiency (Finley et al., 1987).

DISCUSSION

Protein disulfide isomerase was isolated in 1964 on the basis of its ability to catalyze the isomerization of disulfide bonds (Givol et al., 1964). Since then, it has been shown to be the β subunit of prolyl hydroxylase (Koivu et al., 1987), the β subunit of the MTP complex (Wetterau et al., 1990), and a thyroid hormone binding protein (Cheng et al., 1987; Yamauchi et al., 1987). This multiplicity of roles provokes the question of what cellular process or processes are impaired by a lack of PDI. At least one of these processes must be

essential to *S. cerevisiae* cells because disruption of the *PDI* gene is lethal to these cells (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991).

The role of PDI in the triglyceride transfer protein complex is not known. At a minimum, PDI appears to be necessary to maintain the structural integrity of the transfer protein within the lumen of the ER. PDI may also play a more direct role in the lipid transfer protein, perhaps by contributing residues to the active site of MTP or catalyzing the oxidative folding of MTP (Wetterau et al., 1991b). Alternatively, PDI may provide high fidelity ER retention to the MTP complex by virtue of its C-terminal KDEL motif. Nonetheless, the absence of an MTP system in yeast as well as the viability of humans that lack MTP suggest that an MTP system, including its PDI component, is not essential for the life of eukaryotic organisms (Wetterau et al., 1992).

As with the MTP system, PDI appears to be required to keep prolyl 4-hydroxylase in a catalytically active, non-aggregated conformation within the lumen of the ER. Tetramers formed between the α subunit and mutated PDI subunits show that the enzymatic activity of the PDI subunit is not necessary for tetramer assembly or prolyl 4-hydroxylase activity (Vuori et al., 1992c). In addition, prolyl hydroxylase that lacks the PDI subunit have been found in species of green algae (Kaska et al., 1988; Kaska et al., 1990). Thus, the role of PDI in prolyl hydroxylase activity is not essential for cell viability.

S. cerevisiae is an optimal system to examine the role of PDI *in vivo* because *S. cerevisiae* cells need PDI to live (Scherens et al., 1991). We have developed a system that allows for both *in vivo* and *in vitro* studies of PDI (Laboissière et al., 1995a). Here, we have used this system to determine the importance of ER import and retention of PDI in spore germination and cell division.

Rat PDI fused to the α-factor pre–pro segment and either the mammalian retention sequence KDEL or the yeast retention sequence HDEL enabled *PDI*-deficient cells to divide (Table 3.2). On the other hand, if PDI was fused to the α-factor pre sequence [that is, a

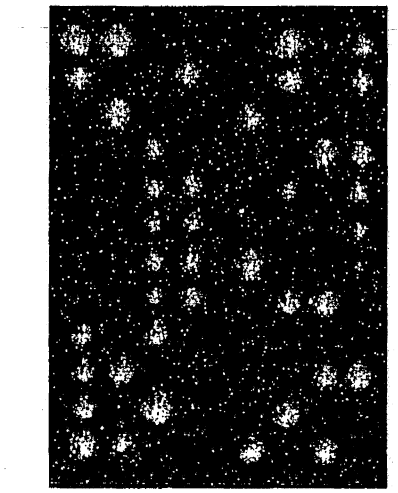
truncated form of the α-factor pre–pro segment that does not direct the protein to the external medium], doubling times were longer indicating that rescue was less efficient (Table 3.3). Immunoblots demonstrated that PDI is not properly targetted to the ER by the α-factor pre sequence (Fig. 3.2), but is retained in the cytosol (result not shown).

Rat PDI fused to the α-factor pre–pro segment and the yeast retention sequence HDEL enabled *PDI*-deficient spores to germinate (Table 3.1), albeit slowly (Fig. 3.1). No other PDI construct was able to support germination. Further, half of the spores not rescued by PDI failed to germinate while the remainder proceeded through only a few cell divisions before ceasing growth. This phenotype, which has been found in similar experiments with other yeast genes (Naumovski & Friedberg, 1983; Mann et al., 1987; Rose & Fink, 1987; Haggren & Kolodrubetz, 1988), suggests that nascent cells have a dire need for PDI. In a similar experiment, the germination of *pdi1Δ* spores containing a truncated PDI was observed to be inefficient (LaMantia et al., 1991).

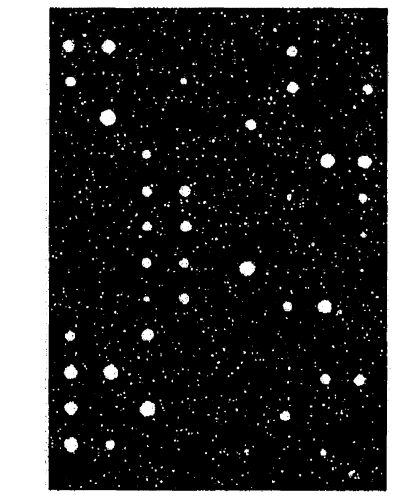
Our results indicate that passage of low levels of PDI through the ER is sufficient for vegetative cell division. In contrast, spore germination requires the efficient retention (via the HDEL motif) of large amounts (from the α-factor pre–pro segment) of PDI in the ER. Tetrad analysis does not look at as many cells as the other methods. However, the stricking difference in results between complementation by rat PDI and by yeast PDI during tetrad analysis argues against jnumbers looked at being the sole thing responsible for this result. In addition the backcrosses rule out the possibility that during plasmid shuffling we selected for up–mutants of Eug1p or any other protein that would be able to rescue a PDI deficiency independent of the presence of rat PDI. The essential role of PDI is to catalyze the unscrambling of nonnative disulfide bonds in ER proteins(Laboissière et al., 1995b). The contribution from a mother cell is limiting: in a budding cell the ER gets passed on to the cell, probably moving with the nucleus through the bud neck into the daughter. There's the possibility that during sporulation the ER gets dissolved and PDI would have no role in

germination. Alternatively, the shuffling of protein disulfide bonds could be more important to germinating spores than to dividing cells.

Figure 3.1 Germination of haploid *pdi1Δ S. cerevisiae* spores complemented with pMAL5.1 (rat PDI fused to the α-factor pre–pro segment and HDEL). Panel A, 3 days after dissection of tetrads. Panel B, 7 days after dissection of tetrads.



B



A

Figure 3.2 Protein transport to the ER is inefficient when directed by the pre α-factor sequence. Immunoblot of membrane-bound protein from *S. cerevisiae* strain *pdi1Δ*/pCT37 transformed with plasmids that direct the production of rat PDI. Each lane contains the same amount of total protein. Lane 1, pMAL6.1 (pre α-factor~PDI~KDEL); lane 2, pMAL7.1 (pre α-factor~PDI~HDEL); lane 3, pMAL3.1 (pre–pro α-factor~PDI~KDEL); lane 4, pMAL5.1 (pre–pro α-factor~PDI~HDEL); lane 5, pMAL9 (*S. cerevisiae* PDI).

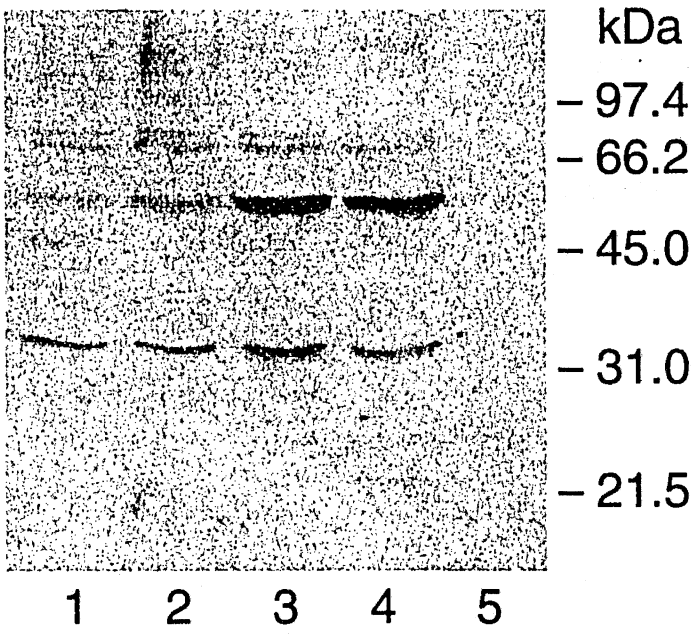

kDa
97.4
66.2
45.0
31.0
21.5
1
2
3
4
5

Table 3.1. Tetrad analysis of *PDI/Δpdi1::HIS* cells transformed with plasmids encoding rat PDI

		Segregation of spores (viable:nonviable)					Viable spores	
Plasmid	Tetrads	4:0	3:1	2:2	1:3	0:4	His^+/Trp^+	His^+/Trp^-
pMAL3.1	71	0	0	45	20	6	0	0
pMAL5.1	70	2	4	50	9	5	9 [a]	0
pMAL6.1	77	0	0	51	23	3	0	0
pMAL7.1	85	0	0	43	38	4	0	0
pMAL9	24	15	6	2	1	0	35	0

[a] One of the 3:1 tetrads had 2 His^+ and 1 His^- spore.

Table 3.2. Loss of plasmid encoding *S. cerevisiae* PDI from cells producing rat PDI [a]

Plasmid	Total colonies	trp^+/ura^- colonies	Plasmid loss (%)
pMAL3.1	90	3	3.3
pMAL5.1	306	146	48
pMAL6.1	249	0	0
pMAL7.1	229	0	0
pMAL9	208	14	6.7

[a] From 4 or 5 independent cultures.

Table 3.3

Doubling times of haploid *pdi1Δ S. cerevisiae* cells complemented with plasmids encoding rat PDI

	Medium	
Plasmid	1% Dextrose	8% Dextrose
pMAL3.1	1.7 ± 0.3	1.8 ± 0.2
pMAL5.1	1.8 ± 0.2	1.7 ± 0.3
pMAL6.1	3.4 ± 0.6	nd
pMAL7.1	2.8 ± 0.3	nd
pMAL9	1.0	1.0

nd, not determined

Chapter IV

The Essential Function of Protein Disulfide Isomerase is to Unscramble Nonnative Disulfide Bonds

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ABSTRACT

Protein disulfide isomerase (PDI) is an abundant protein of the endoplasmic reticulum that catalyzes dithiol oxidation, and disulfide bond reduction and isomerization using the active site: CGHC. Haploid *pdi1Δ S. cerevisiae* are inviable, but can be complemented with either a wild-type rat *PDI* gene or a mutant gene coding for CGHS PDI (shufflease). In contrast, *pdi1Δ* yeast cannot be complemented with a gene coding for SGHC PDI. *In vitro*, shufflease is an efficient catalyst for the isomerization of existing disulfide bonds but not for dithiol oxidation or disulfide bond reduction. SGHC PDI catalyzes none of these processes. These results indicate that *in vivo* protein folding pathways contain intermediates with nonnative disulfide bonds, and that the essential role of PDI is to unscramble these intermediates.

INTRODUCTION

Protein disulfide isomerase (PDI; EC 5.3.4.1) constitutes approximately 2% of the protein in the endoplasmic reticulum (ER). PDI has been shown to catalyze the *in vitro* oxidation of protein sulfhydryl groups, and reduction and isomerization of protein disulfide bonds (1, 2; Fig. 4.1). The products of catalysis by PDI depend on the dithiol/disulfide reduction potential of the substrate and the solution. The enzyme itself can exist in either a reduced or oxidized state, or as a mixed disulfide with a substrate. Although PDI is the most efficient known catalyst of oxidative protein folding (Freedman, 1989; Noiva & Lennarz, 1992), it also participates in cellular processes that do not exploit its enzymatic activity (Koivu & Myllyla, 1987; Wetterau et al., 1990).

Mature PDI from rat contains two active sites with the sequence WCGHCK (Edman et al., 1985). The *C*-terminus of rat PDI ends with the sequence KDEL, the signal for retention of proteins in the mammalian ER (Munro & Pelham, 1987). PDI also has at least one site that can bind to peptides (Morjana & Gilbert, 1991; Noiva et al., 1991b). The amino acid sequence of *Saccharomyces cerevisiae* PDI is approximately 30% identical to that of rat PDI, and the regions containing the active sites are conserved completely (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991). Mature PDI from *S. cerevisiae* contains five putative *N*-glycosylation sites and a *C*-terminus ending with HDEL, the *S. cerevisiae* equivalent of KDEL (Pelham et al., 1988; Scherens et al., 1991).

The role of PDI *in vivo* is unclear. In *S. cerevisiae*, *pdi1Δ* mutants are inviable (Farquhar et al., 1991; LaMantia et al., 1991; Scherens et al., 1991; Tachikawa et al., 1991). In *E. coli*, the *PDI* analog *dsbC* is necessary for the formation of native disulfide bonds in many periplasmic proteins (Missiakas et al., 1994). Studies based on the complementation of *pdi1Δ S. cerevisiae* have provided some clues as to the role of PDI. Tachibana and Stevens showed that the overexpression of *EUG1*, which codes for an ER protein with active-site sequences

WCLHSQ and WCIHSK, allows *pdi1Δ* cells to grow (Tachibana & Stevens, 1992). Also, LaMantia and Lennarz found that *pdi1Δ* cells can be rescued by a mutant PDI that cannot catalyze dithiol oxidation (LaMantia & Lennarz, 1993).

To determine why the *PDI* gene is essential for the growth of *S. cerevisiae*, we have mutated a cDNA that codes for PDI, and have tested the ability of the resulting mutant proteins to support the growth of *pdi1Δ S. cerevisiae*. These results, coupled with *in vitro* analyses of catalysis, demonstrate that the essential role of PDI is not related to the net formation of protein disulfide bonds. Rather, the role of PDI is to act as a "shufflease"—a catalyst of the isomerization of existing disulfide bonds (Fig. 4.1, bottom).

RESULTS

CGHS PDI but not SGHC PDI Complements pdi1Δ S. cerevisiae

Plasmids that direct the expression of mutant forms of rat PDI were made by oligonucleotide-mediated site-directed mutagenesis. Four constructs were tested: mutations in either the first cysteines of both active sites or the second cysteines of both active sites of proteins [designated SGHC PDI and CGHS PDI (or shufflease), respectively] containing the mammalian *C*-terminal ER retention signal KDEL or the yeast *C*-terminal retention signal HDEL. These plasmids were transformed into *pdi1Δ S. cerevisiae* to test by plasmid shuffling for suppression of the lethal phenotype. Cells that produce CGHS PDI form colonies, a result similar to that reported previously with a CLHS/CIHS PDI (LaMantia & Lennarz, 1993). In contrast, cells that produce SGHC PDI were inviable once cured of the *URA3* plasmid (Fig. 4.2). The identity of the *C*-terminus of PDI (KDEL or HDEL) had no effect on complementation by this assay (Fig. 4.2).

Immunoblots of membrane fractions extracted before plasmid shuffling showed that rat PDI was present in all transformants (Fig. 4.3*A*). Immunoblots of membrane fractions from cells that complemented the *pdi1Δ* deficiency showed that rat PDI was also present in these cells (Fig. 4.3*B*). Immunoblots probed with antibodies to *S. cerevisiae* PDI show that this protein is absent from the rat PDI complemented cells (data not shown).

The abilities of wild-type and mutant PDI's to support the growth of *pdi1Δ S. cerevisiae* is shown in Table 4.1. Cells complemented with PDI containing a *C*-terminal KDEL or HDEL sequence had indistinguishable doubling times (data not shown). The data described hereafter were obtained from the KDEL constructs.

Wild-Type and CGHS PDI, but not SGHC PDI, are Efficient Catalysts of Disulfide Bond Isomerization

SGHC PDI and CGHS PDI had physical properties similar to those of wild-type PDI and were purified by identical procedures. Wild-type PDI had been shown to be an efficient catalyst of dithiol oxidation (LaMantia & Lennarz, 1993), and disulfide bond reduction and isomerization (Vuori et al., 1992a). In addition, CLHS/CIHS PDI had been shown not to have dithiol oxidation activity (LaMantia & Lennarz, 1993). Finally, SGHC PDI had been shown not to have disulfide reduction or isomerization activity (Vuori et al., 1992a).

Here, we have expanded the assessment of PDI mutants to include all three enzymatic assays on wild-type PDI and the two relevant mutant enzymes. Our results are listed in Table 4.1, and the results of PDI assays are summarized in Table 4.2. Briefly, we found that wild-type rat PDI had dithiol oxidation activity (measured by an increase in activity of reduced RNase A) comparable to that of PDI isolated from bovine liver. SGHC and CGHS PDI had negligible dithiol oxidation activity. Wild-type rat PDI had disulfide reduction activity (measured by the cleavage of porcine insulin) comparable to that of PDI isolated from bovine liver. SGHC PDI and CGHS PDI had negligible disulfide reduction activity. Wild-type and

CHGS rat PDI had isomerization activity (measured by an increase in activity of scrambled RNase A) comparable to PDI from bovine liver. SGHC PDI had negligible isomerization activity.

DISCUSSION

Early work on PDI suggested that its cysteine residues were essential for its enzymatic activity. Carboxymethylation or carbamoylmethylation caused irreversible inactivation (Fuchs et al., 1967; Hawkins & Freedman, 1991). In addition, PDI was shown to be inhibited by arsenite or Cd^{2+}, behavior diagnostic of enzymes with active site dithiol groups (Ramakrishna Kurup et al., 1966; Hillson & Freedman, 1980). Thus, a cysteine residue was suspected to be responsible for the enzymatic activity of PDI.

In 1991, *pdi1Δ S. cerevisiae* cells was shown to be inviable (Scherens et al., 1991). Still, the question remained: What cellular process is impaired by the absence of PDI? PDI catalyzes dithiol oxidation, and disulfide bond reduction and isomerization (Freedman et al., 1994). On the other hand, PDI can bind to peptides (Morjana & Gilbert, 1991; Noiva et al., 1991b), and is part of cellular complexes in which the role of its enzymatic activity has not been explored (Koivu & Myllyla, 1987; Wetterau et al., 1990). These data have lead many to suggest that the essential role of PDI is unrelated to its enzymatic activities (LaMantia & Lennarz, 1993; Noiva et al., 1993; Puig & Gilbert, 1994a; Puig & Gilbert, 1994b; Wang & Tsou, 1994; Quan et al., 1995).

To illuminate the cellular process that is impaired by the absence of PDI, we have mutated each cysteine residue in its active site and studied the resultant proteins *in vitro* and *in vivo*. Since each PDI monomer has two CGHC active sites, our two mutant proteins are actually double mutants in which either the first or second cysteine residue in each active site is

replaced by a serine. We find that CGHS PDI and wild-type PDI are able to complement a *pdi1Δ* strain of *S. cerevisiae* (Table 4.1). In contrast, SGHC PDI is unable to compensate for this deficiency.

Replacing two sulfur atoms with oxygen atoms is unlikely to have a significant impact on the ability of PDI to bind to peptides, or to otherwise act in nonenzymic roles. The mutations do, however, have a significant effect on catalysis by PDI. The results of *in vitro* PDI assays show that CGHS PDI catalyzes the shuffling of disulfide bonds with efficiencies comparable to that of the wild-type enzyme (Table 4.1). But unlike the wild-type enzyme, CGHS PDI does not catalyze the oxidation of dithiols or the reduction of disulfide bonds. Apparently, the ability of PDI to form an intramolecular disulfide bond is necessary for it to catalyze efficiently the oxidation or reduction of a substrate. SGHC PDI catalyzes none of these three processes. Thus, the essential function of PDI is enzymic, but does not relate to the net formation of disulfide protein bonds. Rather, the role of PDI *in vivo* is to act as a shufflease (Fig. 4.1, bottom).

The properties of two homologs of PDI support our conclusion. The *S. cerevisiae EUG1* gene complements *pdi1Δ S. cerevisiae*. Each active site of Eug1p (WCLHSQ and WCIHSK) contains only a single cysteine residue (Tachibana & Stevens, 1992). Wild-type Eug1p is therefore analogous to the shufflease mutant of PDI. Thioredoxin (Trx), catalyzes disulfide bond reduction in the cytosol of eukaryotes and prokaryotes in the active site: WCGPCK (Holmgren, 1985). Although the three-dimensional structure of PDI is unknown, that of *E. coli* Trx has been determined by both X-ray diffraction analysis and NMR spectroscopy (Holmgren et al., 1975; Katti et al., 1990). In the Trx structure, the most pronounced deviation from an almost spherical surface is a protrusion formed by residues 29 – 37, which includes the active site. The sulfhydryl group of Cys32, which has a low pK_a and hence high nucleophilicity, is exposed to the solvent while that of Cys35 is recessed. The results of chemical modification studies and pK_a determinations on PDI are parallel to those on Trx

(Freedman et al., 1988), suggesting that the reactivity of the active sites is similar. In addition, PDI is a substrate for thioredoxin reductase, which suggests that the three-dimensional structures of the active sites are similar. Recently, we demonstrated that CGPS Trx but not SGPC Trx can complement *pdi1Δ S. cerevisiae* (Hawkins et al., 1991). Thus, the essential functional group in the CXXC motif is the sulfhydryl group of the *N*-terminal cysteine residue.

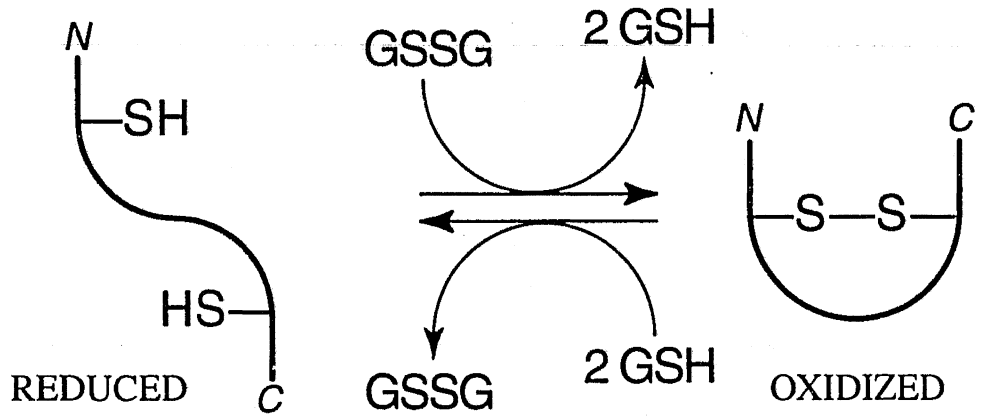
If a CXXS sequence can replace the CXXC motif, why does PDI have a CXXC motif? A CXXC motif could be less susceptible to inactivation by adventitious oxidation to a hindered mixed disulfide or a sulfenic acid (S–OH) because it can escape by forming an intramolecular disulfide bond. In addition, having CXXC and CXXS motifs from endogenous PDI and Eug1p, respectively, could provide cells with a selective advantage.

Catalysis of dithiol oxidation or disulfide bond reduction depends on the redox environment (Gilbert, 1990; Gilbert, 1995). In contrast, during catalysis of disulfide bond isomerization, the substrate does not undergo a net change in oxidation state (Fig. 4.1). The simplest mechanism for catalysis of an isomerization reaction begins with the attack of a thiolate ion on a protein disulfide, forming a mixed disulfide (Darby & Creighton, 1995). Then, the protein thiolate produced can attack another protein disulfide bond. Finally, the resulting thiolate can attack the mixed disulfide to release the catalyst, unaltered. Such an isomerization reaction would be driven by the search for the most stable conformation of the substrate protein.

Much evidence suggests that dithiol oxidation is random during the early stages of protein folding (Creighton, 1988; Kim & Baldwin, 1990). Classic studies on the oxidative folding of reduced bovine protease trypsin inhibitor suggest that non–native intermediates accumulate during the folding process (Creighton, 1977). In contrast, recent work using modern techniques argue that the well-populated intermediates contain only native disulfide bonds (Weissman & Kim, 1991). Still, to reach the final conformation, these intermediates

must rearrange by forming species with nonnative disulfide bonds. PDI has been shown to catalyze this process by rescuing such kinetically trapped intermediates (Weissman & Kim, 1993). Thus, PDI activity may be required either in a normal protein folding pathway or for rescuing proteins that have become misfolded or aggregated. Our results link the disulfide bond isomerization activity of PDI with cell viability. Thus, as proposed by Anfinsen (Givol et al., 1964) more than thirty years ago, the essential function of PDI is to isomerize nonnative disulfide bonds—to be a shufflease.

Figure 4.1 Reactions catalyzed by PDI *in vitro*. PDI catalyzes the oxidation of dithiols and reduction of disulfide bonds (top), and the isomerization of disulfide bonds (bottom). In this work, the substrate for dithiol oxidation was reduced RNase A, that for disulfide reduction was insulin, and that for disulfide bond isomerization was scrambled RNase A.


N
SH
HS
C
REDUCED
GSSG
2 GSH
GSSG
2 GSH
N
C
S—S
OXIDIZED

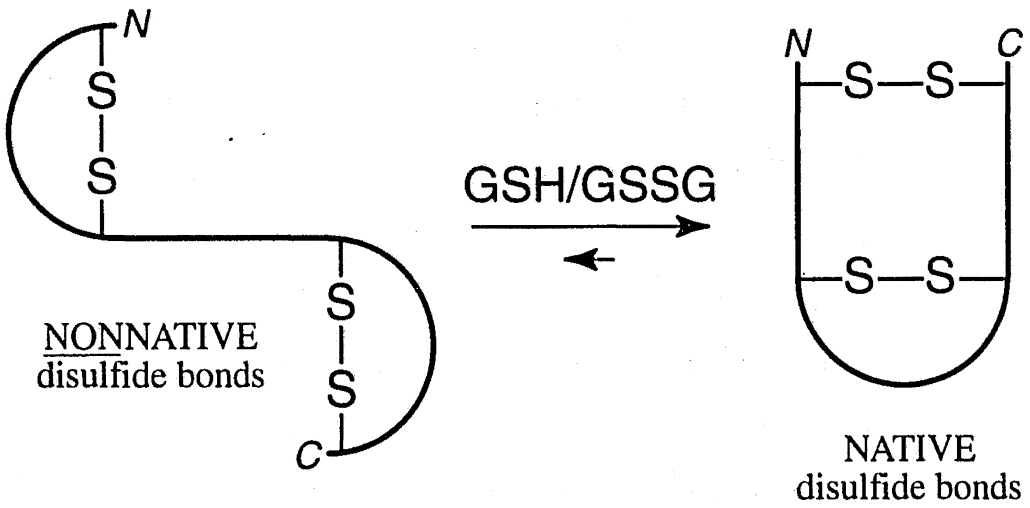

N
S
S
S
S
C
NONNATIVE
disulfide bonds
GSH/GSSG
N
C
S—S
S—S
NATIVE
disulfide bonds

Figure 4.2 Complementation of *pdi1Δ S. cerevisiae*. Haploid cells carrying a *URA3* plasmid that directs the production of *S. cerevisiae* PDI were transformed with a *TRP1* plasmid that directs the production of a test PDI. Transformants (5 for each plasmid) were cultured, and then grown for 4 days on solid tryptophan dropout medium (left) or solid medium containing 5-FOA (right), which selects for cells that have lost the *URA3* plasmid. In each panel: Row 1, SGHC PDI; row 2, CGHS PDI; row 3, SGHC PDI (HDEL); row 4, CGHS PDI (HDEL); row 5, *S. cerevisiae* PDI.

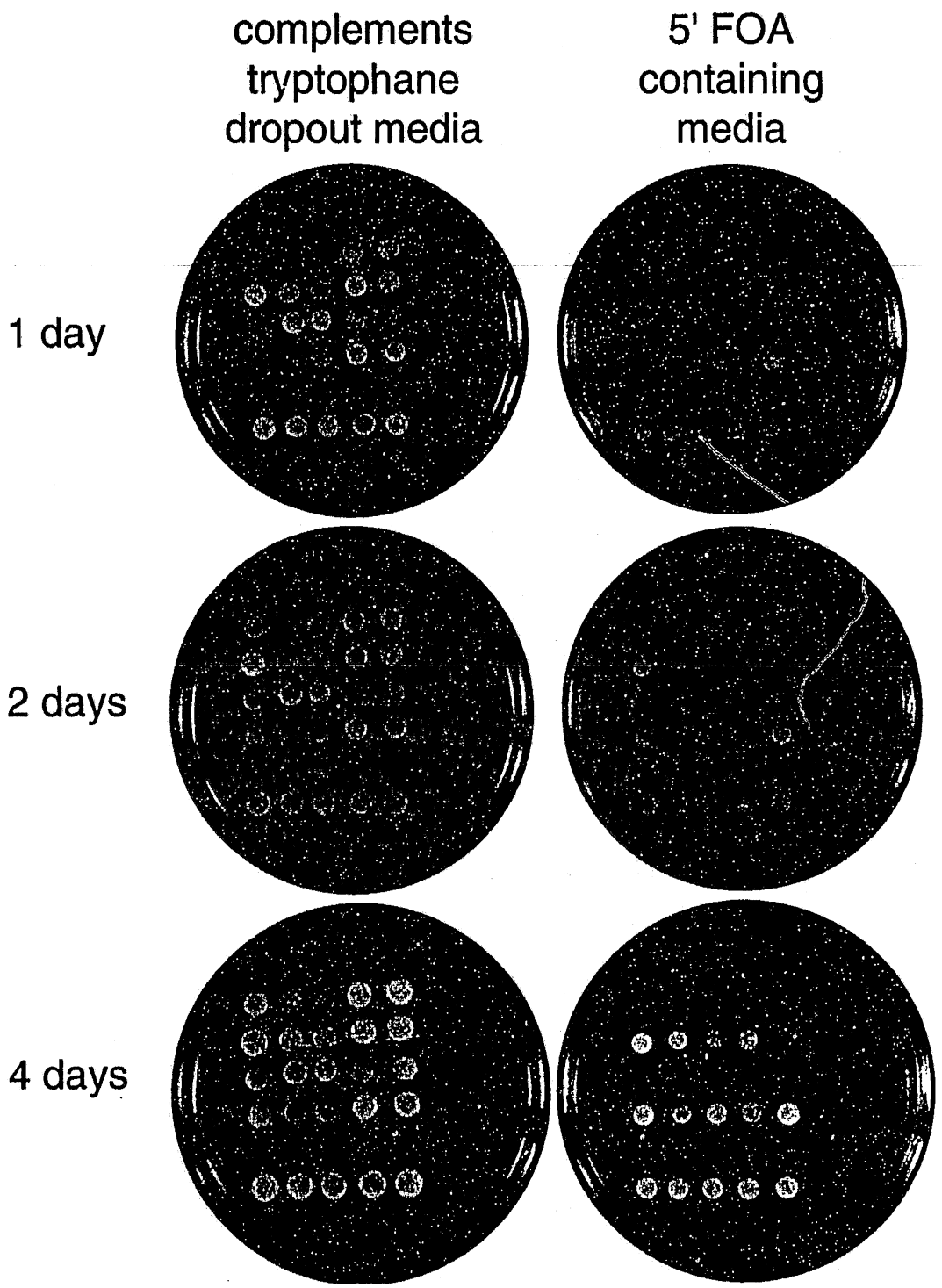
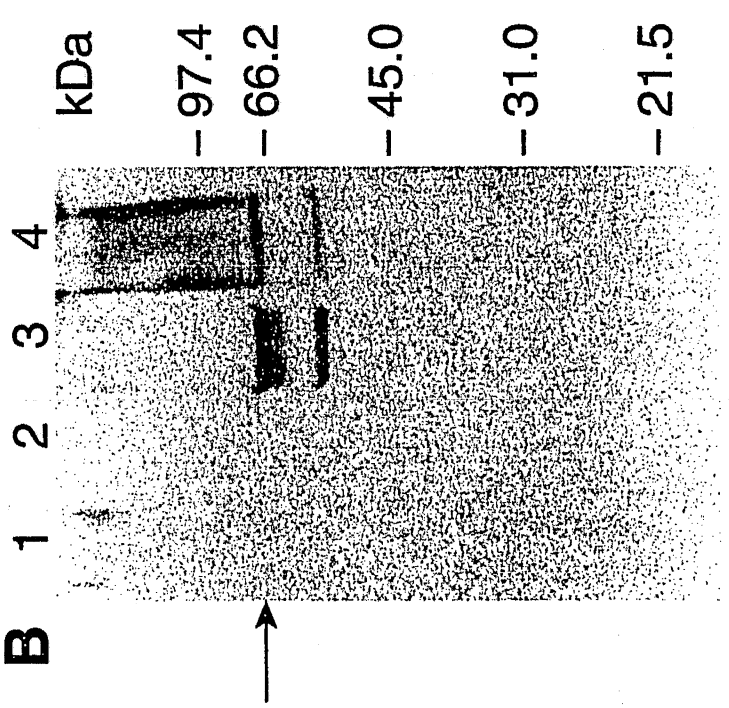

complements tryptophane dropout media
5' FOA containing media
1 day
2 days
4 days

Figure 4.3 Membrane-bound protein in complemented *pdi1Δ S. cerevisiae*. Immunoblots were probed with antibodies to bovine PDI. A. Before plasmid shuffling. Lane 1, SGHC PDI; lane 2, CGHS PDI; lane 3, SGHC PDI (HDEL); lane 4, CGHS PDI (HDEL); lane 5, *S. cerevisiae* PDI. B. After plasmid shuffling. Lane 1, *S. cerevisiae* PDI; lane 2, empty; lane 3, CGHS PDI; lane 4, CGHS PDI (HDEL).


B
1
2
3
4
kDa
97.4
66.2
45.0
31.0
21.5

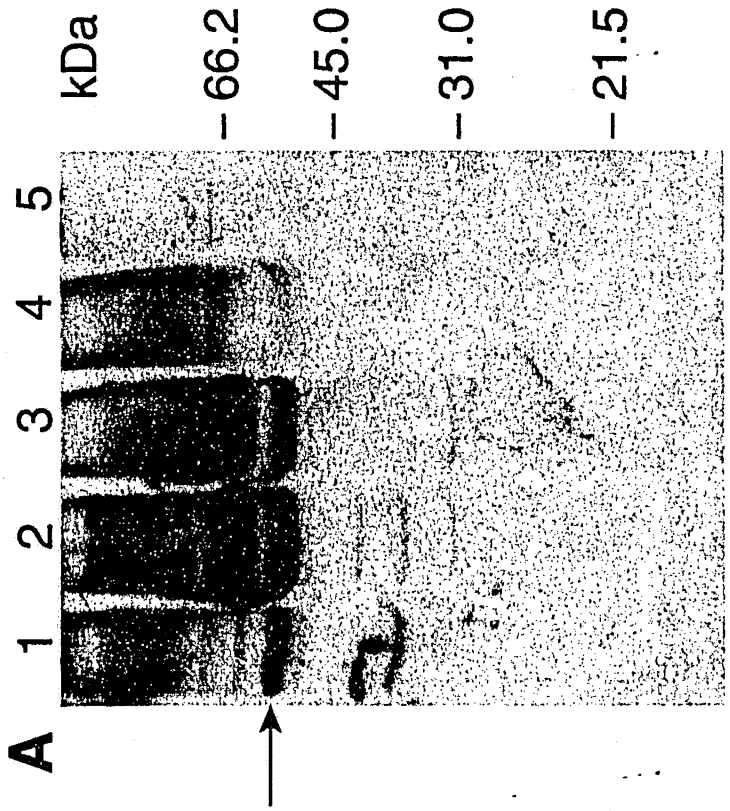

A
1
2
3
4
5
kDa
66.2
45.0
31.0
21.5

Table 4.1

Properties of wild-type and mutant PDI's

PDI	Doubling time of complemented *pdi1Δ* *S. cerevisiae* [a]	Dithiol oxidation activity (units/mg) [b]	Disulfide reduction activity (units/mg) [c]	Disulfide isomerization activity (units/mg)[d]
wild-type (bovine)	nd	8.5 ± 0.8	0.41 ± 0.04	0.65 ± 0.01
wild-type (rat)	1.8 ± 0.2	7.0 ± 0.7	0.33 ± 0.03	0.55 ± 0.05
CGHS (rat)	2.3 ± 0.6	0.18 ± 0.02	0.02 ± 0.01	0.51 ± 0.16
SGHC (rat)	nc	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02

[a] Relative to cells complemented with *S. cerevisiae* PDI.

[b] One unit will catalyze the reactivation of 1 nmol of reduced RNase A per min at pH 7.6 in the presence of GSH (1.0 mM) and GSSG (0.20 mM).

[c] One unit will catalyze the reduction of the disulfide bonds in 1 μmol of insulin per min at pH 7.2 in the presence of GSH (0.50 mM).

[d] One unit will catalyze the reactivation of 1 nmol of scrambled RNase A per min at pH 7.6 in the presence of GSH (1.0 mM) and GSSG (0.20 mM).

nd, not determined; nc, no complementation

Table 4.2
Summary of reactions catalyzed by wild-type and mutant PDI's

PDI	Dithiol oxidation	Disulfide reduction	Disulfide isomerization
Wild-type	yes (LaMantia & Lennarz, 1993)	yes (Vuori et al., 1992a)	yes (Vuori et al., 1992a)
CGHS	no (this work)[a]	no (this work)	yes (this work)
SGHC	no (this work)	no (Vuori et al., 1992a)	no (Vuori et al., 1992a)

[a] The same result was obtained when the two active sites in PDI were changed to CLHS and CIHS, as in Eug1p (LaMantia & Lennarz, 1993).

Chapter V

Materials and Experimental Methods

GENERAL

Materials

Escherichia coli strain CJ236 (*dut ung thi relA*; pCJ105[Cm^r]) and helper phage M13K07 were from Bio-Rad (Richmond, CA). *Escherichia coli* strain JM109 was from Promega (Madison, WI). Protease-deficient *S. cerevisiae* strain BJ2168 (a *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) was from Yeast Genetic Stock Center (Berkeley, CA). Yeast strains YPH 250 (a *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) and YPH 252 (α *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) were from the Yeast Genetic Stock Center (Berkeley, CA). Diploid yeast strain YPH274 α/a *pdi1Δ::HIS3* (which is homozygous for *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) and plasmid pCT37 (which is a *URA3* plasmid that encodes yeast PDI under the control of a GAL promoter) (Tachibana & Stevens, 1992) were generous gifts from Dr. Tom Stevens. Plasmid pFL44 *Bam*HI-*Bam*HI which encodes *S. cerevisiae* PDI was a generous gift from Dr. Bart Scherens. Haploid *S. cerevisiae* strains 125 (α *hom3*) and 126 (a *hom3 ilv*) and plasmid pRS424 were generous gifts from Dr. Lucy Robinson. 5′-Fluoroorotic acid (5-FOA, 1 mg/ml) addition to defined media was as described (Sikorski & Boeke, 1991).Sporulation of diploid yeast was induced by growth on potassium acetate plates (which contained 1% w/v potassium acetate, 0.1% w/v yeast extract, 0.05% w/v dextrose, and 2% w/v agar), and the resulting tetrads were dissected by micromanipulation without selection for plasmids, as described (Shermann, 1991).

Plasmid prpdi, which contains the cDNA that codes for rat PDI (Edman et al., 1985), was the generous gift of Dr. P. Tekamp-Olson. Plasmid pMP36 (Phillips et al., 1990), which contains an ADH2–GAPDH hybrid promoter, DNA that codes for the α-factor leader region (pre-pro segment), and the GAPDH terminator, was the generous gift of Dr. M. A. Phillips. Plasmid YEpWL.RNase A was from D. J. Quirk (delCardayré et al., 1995).

Enzymes for the manipulation of DNA were from Promega (Madison, WI) or New England Biolabs (Beverly, MA). Ampicillin (sodium salt) was from International Biotechnologies (New Haven, CT).

Bacto yeast extract, Bacto peptone, and Bacto yeast nitrogen base without amino acids (YNB) were from Difco (Detroit, MI). Yeast minimal medium (SD) contained (in 1.0 L) Bacto YNB (6.7 g), dextrose (2% w/v), and a supplemental nutrient mix (Ausubel et al., 1989). Yeast rich medium (YEPD) contained (in 1.0 L) Bacto yeast extract (10 g), Bacto peptone (20 g) , and dextrose (2% w/v). Variations of SD and YEPD media were also used. For example, S(8%)D – trp medium contained four times the concentration of dextrose and lacked tryptophan in the supplemental nutrient mix. Filtered medium was prepared by removing high molecular mass material from YEP(1%)D medium by tangential flow over a 5 kDa-cutoff membrane using a Minitan apparatus (Amicon; Beverly, MA). All media were prepared in distilled, deionized water and autoclaved before use.

DNA sequencing was performed with a Sequenase 2.0 kit from United States Biochemical (Cleveland, OH). DNA fragments were purified from agarose gels with a GeneClean II kit from BIO-101 (La Jolla, CA). Site-directed mutagenesis was performed by the method of Kunkel (Kunkel et al., 1987) on single-stranded DNA isolated from *E. coli* strain CJ236.

Bovine PDI was from PanVera (Madison, WI). Reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH, and scrambled ribonuclease A (sRNase A) were from Sigma Chemical (St. Louis, MO). 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was from Calbiochem (La Jolla, CA). Poly(C) was from Midland Certified Reagents (Midland, TX), and was precipitated from aqueous ethanol (70% v/v) before use. All other chemicals were of reagent grade or better, and were used without further purification.

pH was measured with a Beckman pH meter fitted with a Corning electrode, calibrated at room temperature with standard buffers from Fisher (Chicago, IL). Ultraviolet and visible

absorbance measurements were made on a Cary 3 spectrophotometer equipped with a Cary temperature controller. DNA oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer by using the phosphoramidite method (Sinha et al., 1984) and reagents from Applied Biosystems (Foster City, CA) except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Synthetic oligonucleotides were purified with Oligonucleotide Purification Cartridges from Applied Biosystems.

S. cerevisiae cells were transformation as described (Ito et al., 1983). The cell density of cultures was determined by measuring the absorbance at 600 nm after diluting the culture so that $A_{600} = 0.1 - 0.5$ O.D. Other manipulations of DNA, *E. coli*, and *S. cerevisiae* were performed as described (Ausubel et al., 1989; Shermann, 1991).

Protein concentration was determined with the Protein Assay Kit from Bio-Rad (Richmond, CA). The concentration of purified PDI was determined by using an absorption coefficient of $\varepsilon^{1.0\%}_{1cm} = 47{,}300\ M^{-1}\ cm^{-1}$. Free sulfhydryl groups wre detected by titration with 5,5'-dithiobis(2-nitrobenzoic acid) (Creighton, 1989).

Polyacrylamide gel electrophoresis (PAGE) was performed in the presence of sodium dodecyl sulfate (SDS; 0.1% w/v) according to Ausubel *et al.* (1989). Gels were fixed and stained by washing with aqueous methanol (40% v/v), containing acetic acid (10% v/v) and Coomassie brilliant blue (0.1% w/v). The molecular weight standards were from Bio-Rad (Richmond, CA): phosphorylase B (97.4 kDa unstained; 106 kDa prestained), serum albumin (66.2; 80.0), ovalbumin (45.0; 49.5), carbonic anhydrase (31.0; 32.5), trypsin inhibitor (21.5; 27.5), and lysozyme (14.4; 18.5).

Methods

Assays for enzymatic activity. The ability of PDI to catalyze the reduction of insulin by GSH was assayed as described (Lu et al., 1992). The ability of PDI to catalyze the isomerization of the disulfide bonds of scrambled RNase A to those of native RNase A was

assayed as described (Ibbetson & Freedman, 1976), with minor modifications. Assays were performed at 30 °C in 50 mM Tris-HCl buffer, pH 7.6, containing PDI (0.7 – 1.4 μM), GSH (1 mM), and GSSG (0.2 mM). Reactions were initiated by the addition of scrambled RNase A (1.0 μL of a 0.50 mg/mL solution in 10 mM acetic acid). At 10-minute intervals, aliquots (10 μL) were removed and added to 500 μL of 100 mM Mes-HCl buffer, pH 6.0, containing poly(C) (10 mM). Ribonuclease activity was monitored by following the change in absorbance at 238 nm. Catalysis of dithiol oxidation was assayed by monitoring the regain in activity of reduced RNase A as follows. Reduced RNase A was prepared by incubating RNase A (5 mg; Sigma Chemical) in 1.0 mL of 0.10 M Tris-AcOH, pH 8.0, containing EDTA (2 mM), guanidinium chloride (6 M), and DTT (0.14 M) for 3 h. The protein was purified by gel filtration chromatography on Biogel P4 resin, and then dialyzed exhaustively against 0.10 M acetic acid. Assays were performed at 30 °C in 50 mM Tris-HCl buffer, pH 7.6, containing PDI (0.7 – 1.4 μM), GSH (1 mM), and GSSG (0.2 mM). Reactions were initiated by the addition of reduced RNase A (1.0 μL of a 0.50 mg/mL solution in 10 mM acetic acid). At 10-min intervals, aliquots (10 μL) were removed and added to 500 μL of 100 mM Mes-HCl buffer, pH 6.0, containing poly(C) (10 mM). Ribonuclease activity was monitored by following the change in absorbance at 238 nm.

Values of specific activity were precise to ±10%.

S. cerevisiae *cell extracts.* Intracellular protein was isolated from *S. cerevisiae* cells as described (Bostian et al., 1983).

Polyclonal antibodies against bovine PDI. Bovine PDI was obtained from PanVera (Madison, WI). A chicken was injected with bovine PDI (200 μg), and 7 days later boosted with additional bovine PDI (100 μg). Eggs were collected 21 days after the initial injection, and IgY isolated as described (Polson et al., 1980).

Immunoblots. Yeast cultures were grown to late log phase in liquid medium. Cells were removed by centrifugation, and the supernatant was concentrated with a 10 kDa-cutoff Centriprep concentrator. Denaturing protein gel electrophoresis was performed as described (Ausubel et al., 1989). Western blotting was performed using the Renaissance kit (DuPont NEN; Boston, MA) as directed by the manufacturer. Antibodies against *S. cerevisiae* PDI were the generous gift of Dr. Tom Stevens. Antibodies to bovine PDI were used at a 1:500 dilution for immunoblots. Peroxidase-conjugated rabbit affinity purified antibody to chicken IgG was from Cappel (Durham, NC), and was used as directed by the manufacturer.

Protein Sequence Analysis. Protein sequencing was performed by Dr. G. Grant (Department of Molecular Biology, Washington University, St. Louis, MO).

EXPERIMENTAL FOR CHAPTER II

Plasmid for production of pre-pro α-factor~PDI~KDEL. Plasmid pMAL3.1, which directs the production in *S. cerevisiae* of rat PDI with an α-factor pre-pro segment at its N-terminus and a KDEL sequence at its C-terminus, was constructed as follows. Plasmid prpdi was digested with *Nar*I, treated with T4 DNA polymerase to generate blunt ends, and then digested with *Sal*I. The resulting fragment was inserted into plasmid pMP36 that had been digested with *Bal*I (which creates a blunt end) and *Sal*I, to yield plasmid pMAL1. An adaptor made from oligonucleotides RR44 and RR45 (Table 1) was inserted into plasmid pMAL1 that had been digested with *Kpn*I and *Asp*I, to yield plasmid pMAL2. The *Bam*HI and *Sal*I fragment of pMAL2 was inserted into the appropriate *Bam*HI and *Sal*I sites of plasmid YEpWL.RNase A to yield plasmid pMAL3. The *Nco*I site in the *LEU2*-d gene of plasmid

pMAL3 was destroyed by site-directed mutagenesis using oligonucleotide ML1, to yield plasmid pMAL3.1 (pre-pro α-factor~PDI~KDEL).

Plasmid for production of pre-pro α-factor~PDI~HDEL. A plasmid, pMAL5.1, that directs the production in *S. cerevisiae* of rat PDI with an α-factor pre-pro segment at its N-terminus and an HDEL sequence at its C-terminus was constructed as follows. An adaptor made from oligonucleotides ML9 and ML10 was inserted into plasmid M13mp18 that had been digested with *Eco*RI and *Hind*III, to yield plasmid pMAL10. The *Nhe*I/*Sal*I fragment of pMAL3.1 was inserted into pMAL10 that had been digested with *Nhe*I and *Sal*I, to yield plasmid pMAL13. The cDNA in pMAL3.1 that codes for the KDEL sequence at the C-terminus of PDI was changed to code for an HDEL sequence by site-directed mutagenesis using oligonucleotide ML19. The region in the pMAL13-derived vector containing the desired mutation was amplified by using the PCR. The amplified region was digested with *Nhe*I and *Sal*I, and ligated to the *Nhe*I/*Sal*I fragment of pMAL3.1, to yield plasmid pMAL5.1 (pre-pro α-factor~PDI~HDEL).

Plasmid for production of pre α-factor~PDI~KDEL and pre α-factor~PDI~HDEL. The overlap extension method of Pease and coworkers (Ho et al., 1989) was used to excise from pMAL3.1 the DNA that codes for the pro region of the pre-pro α-factor segment. Plasmid pMAL3.1 was digested with *Bam*HI and *Nhe*I. The resulting 2577 bp fragment was used as a template in two PCR's—first with oligonucleotides ML25 and ML26, and then with oligonucleotides ML27 and ML28. The resulting fragments were purified and again subjected to PCR conditions using oligonucleotides ML25 and ML28. This PCR product was digested with *Sac*I and *Nco*I and ligated to plasmid pGEM5f(-) (Promega) that had been digested with *Sac*I and *Nco*I, to yield plasmid pMAL14. The *Sac*I/*Nco*I fragment of pMAL14 was inserted

into pMAL3.1 and pMAL5.1 that had been digested with *Sac*I and *Nco*I, to yield plasmids pMAL6.1 (pre α-factor~PDI~KDEL) and pMAL7.1 (pre α-factor~PDI~HDEL).

Production and purification of recombinant rat PDI. S. cerevisiae cells that had been transformed with pWL (delCardayré et al., 1995) served as a control for all experiments. *S. cerevisiae* BJ2168 cells were transformed with plasmids pMAL3.1 (which codes for pre-pro α-factor~PDI~KDEL) and transformants were selected by plating on SD – trp medium. Stock cultures were prepared in S(8%)D – trp medium, which contained a high concentration of dextrose to repress expression from the ADH2–GAPDH hybrid promoter. These cultures were allowed to grow for 1 day with shaking at 30 °C. The stock culture was diluted to A_{600} = 0.05 O.D. in filtered YEP(1%)D medium, and the resulting culture (1.0 L) was grown with vigorous shaking at 30 °C until A_{600} = 4.0 O.D. The cells were removed by centrifugation, and the culture medium was concentrated to 10 mL by tangential flow over a 5 kDa-cutoff membrane. The concentrate was loaded on a Hi Load 26/60 G-75 gel filtration column (Pharmacia; Uppsala, Sweden) that had been equilibrated with 20 mM imidazolium chloride buffer, pH 7.2. The loaded column was eluted with the same buffer at a flow rate of 0.75 mL/min. Peak fractions were analyzed by SDS-PAGE, and fractions containing PDI were pooled and concentrated to 10 mL by ultrafiltration.

The concentrated sample was loaded on a Mono-Q column (Pharmacia) that had been equilibrated with the same buffer. PDI was eluted with a linear gradient (30 mL + 30 mL) of NaCl (0.15 – 0.75 M) in 20 mM imidazolium chloride buffer, pH 7.2. Fractions containing PDI eluted at 0.25 – 0.28 M NaCl, and were analyzed individually by SDS-PAGE. Those fractions containing homogeneous PDI were pooled and concentrated with a 10 kDa-cutoff Centriprep concentrator (Amicon; Beverly, MA).

EXPERIMENTAL FOR CHAPTER III

Plasmid for the production of S. cerevisiae PDI. A plasmid, pMAL9, that directs the production of *S. cerevisiae* PDI under the control of its own promoter was constructed as follows. A fragment that contains the cDNA that codes for *S. cerevisiae* PDI was isolated from plasmid pFL44 *Bam*HI-*Bam*HI by digestion with *Apa*I and *Pst*I, and was inserted into plasmid pRS424 that had been digested with *Apa*I and *Pst*I.

Complementation of pdi1Δ S. cerevisiae with rat PDI. Complementation for viability of *S. cerevisiae* cells in which the endogenous *PDI* gene had been deleted was tested in three ways: tetrad analysis, plasmid loss, and plasmid shuffling.

Tetrad analysis. Strain YPH 274α/a:Δ*pdi* was transformed with plasmid pMAL3.1, pMAL5.1, pMAL6.1, pMAL7.1, or pMAL9. Transformants were selected on tryptophan dropout plates. Sporulation was induced on potassium acetate plates. Tetrads were dissected onto solid medium containing dextrose (2.0% w/v) and incubated at 30°C. Spores obtained from tetrad dissection were analyzed by growth on solid tryptophan, uracil, or histidine dropout medium. Mating types of strains were identified by mating capability with strains 125 and 126.

Non-selective loss of plasmids encoding yeast PDI. Strain YPH 274α/a:Δ*pdi*::*HIS3* was transformed with plasmid pCT37, which contains the *S. cerevisiae PDI* gene under the control of the *GAL* promoter. Transformants were selected on solid uracil dropout medium. Sporulation was induced and tetrads dissected onto solid YEP medium containing galactose (2% w/v) and raffinose (1% w/v). *pdi1Δ* haploids complemented by yeast PDI (*pdi1Δ*/pCT37) were identified by growth on histidine and uracil dropout plates. *pdi1Δ*/pCT37 haploids were transformed with plasmids pMAL3.1, pMAL5.1, pMAL6.1, or pMAL7.1. Transformants were selected by growth on uracil/tryptophan dropout plates

containing galactose (2% w/v) and raffinose (1% w/v). Transformants were transferred to tryptophan dropout medium containing dextrose (1% w/v). The resulting culture was allowed to grow for 24 h, and was then diluted so that A_{600} = 0.1 O.D. The dilution/growth cycle was repeated, and aliquots of the resulting culture were grown on solid tryptophan dropout medium containing 1% (w/v) dextrose. The resulting colonies were replica plated onto tryptophan or uracil dropout plates.

Selective loss of plasmids encoding yeast PDI by plasmid shuffling. pdi1Δ/pCT37 haploids were transformed with plasmids pMAL3.1, pMAL5.1, pMAL6.1, pMAL7.1, or pMAL9 and grown on tryptophan dropout plates. Colonies were replica plated onto plates containing 5-FOA. Once growth was observed (which varied from 2 to 5 days), colonies were replica plated onto tryptophan and uracil dropout plates and onto plates containing 5-FOA, to repeat the selection cycle. trp^+/ura^- colonies were isolated from the second round of selection.

Mating of complementants. trp^+ ura^- cells were grown on solid YEPD medium and mated with cells from strain YPH252. After 24 h, the resulting cells were streaked for single colonies onto tryptophan dropout plates. Single colonies were picked, cultured, and mated with strains 125 or 126 for 24 h, and then replica plated onto minimal plates to select for diploids. Colonies that did not mate were considered diploids. Cells from these non-mating colonies were sporulated on potassium acetate plates. Tetrads were dissected and analyzed as described above.

Doubling times. Haploid *pdi1Δ* cells complemented with rat or yeast PDI were inoculated into YEP(1%)D medium (25 mL) at an initial A = 0.025 at 600 nm (= 7 x 10^6 cells/mL). Samples were removed at 2-h intervals, diluted to read A = 0.1 – 0.5, and then grown until the cells reached the stationary phase (1.2 x 10^8 cells/mL). Log A was plotted versus time,

and the slope of the linear portion of the curve was determined by linear least squares analysis. At least 5 different clones from each construct were analyzed. Doubling time was calculated by dividing log 2 by the slope of the curve. Mean and standard deviation for the doubling times of each construct were calculated. Growth rates were normalized to the growth rate of wild-type cells by dividing the doubling time of haploid *pdi1Δ* cells complemented with a rat PDI construct by the doubling time of haploid *pdi1Δ* cells complemented with pMAL9.

Cell extracts. Cultures (50 mL) of *S. cerevisiae* were grown at 30 °C until $A = 0.5 - 0.8$ at 600 nm. The cells were collected by centrifugation, washed with distilled water (20 mL), and resuspended in 100 mM Tris-H_2SO_4 buffer, pH 9.4, containing DTT (20 mM) such that $A = 20$. This suspension was incubated for 10 min at 30 °C. Cells were then washed in 1.2 M sorbitol and resuspended in spheroplasting buffer, which was 10 mM potassium phosphate buffer, pH 7.2, containing sorbitol (1.2 M) and DTT (2 mM), such that $A = 50$. Cells were converted into spheroplasts by adding Novozym 234 (Novo BioLabs; Bagsvaerd, Denmark) to a final concentration of 3 mg/mL, and incubating the resulting solution at 30 °C for 30 min. Spheroplasts were washed twice with 2 volumes of spheroplasting buffer. Spheroplast lysis and protein isolation were then performed as described (Bostian et al., 1983).

EXPERIMENTAL FOR CHAPTER IV

Plasmid for Production of CGHS PDI and SGHC PDI. To mutate both active sites in rat PDI, we used a subcloning strategy that took advantage of the *Xma*III site that lies between the regions in pMAL3.1 that code for the two active sites. The *NcoI*/*Xma*III fragment of pMAL3.1 was inserted into an M13mp18-derived plasmid, pMAL10, that had been digested

with *Nco*I and *Xma*III, to yield plasmid pMAL11. The codons for cysteine residues in the first active site of the *PDI* cDNA in pMAL11 were changed to those for a serine residue by using oligonucleotide ML11 or ML12. The regions in the resulting plasmids that code for PDI were amplified by using the PCR. The amplified fragments were digested with *Nco*I and *Xma*III, and ligated to the *Nco*I/*Xma*III fragment of pMAL3.1, generating plasmids pMAL112 (which codes for C35S PDI) and pMAL122 (C38S PDI).

The *Nhe*I/*Xma*III fragment of pMAL3.1 was inserted into pMAL10 that had been digested with *Nhe*I and *Xma*III, to yield plasmid pMAL12. The codons for cysteine residues in the second active site of the *PDI* cDNA in pMAL12 were changed to those for a serine residue using oligonucleotide ML13 or ML14. The regions in the resulting plasmids that code for PDI were amplified by using the PCR. The amplified fragments were digested with *Nhe*I and *Xma*III, and ligated to the *Nhe*I/*Xma*III fragment of pMAL112 and pMAL122 generating plasmids pMAL312 (C35S/C379S PDI) and pMAL322 (C38S/C382S PDI).

To discern whether the *C*-terminal HDEL sequence of *S. cerevisiae* PDI is important for its function *in vivo*, we replaced the *C*-terminal KDEL sequence of C35S/C379S and C38S/C382S PDI with HDEL. The *Nco*I/*Nhe*I fragments of pMAL312 and pMAL322 were inserted into pMAL5.1 (Laboissière et al., 1995a) that had been digested with *Nco*I and *Xma*III, to yield plasmids pMAL512 (C35S/C379S/K486H) and pMAL522 (C38S/C382S/K486H).

Complementation of pdi1Δ S. cerevisiae. To determine the role of PDI *in vivo*, we attempted to complement *pdi1Δ S. cerevisiae* by plasmid shuffling using plasmids that code for active-site mutants of rat PDI. Briefly, strain YPH 274α/a:Δ*pdi*::*HIS3* was transformed with plasmid pCT37, which contains the yeast *PDI* gene under the control of the *GAL1* promoter (Tachibana & Stevens, 1992). Transformants were selected on solid uracil dropout medium. Sporulation was induced by growth on solid medium containing potassium acetate,

and tetrads dissected onto solid YEP medium containing galactose (2% w/v) and raffinose (1% w/v). Haploid cells complemented by *S. cerevisiae* PDI (*pdi1Δ*/pCT37) were identified by growth on solid medium lacking histidine or uracil. *pdi1Δ*/pCT37 haploids were transformed with plasmids pMAL9, pMAL312, pMAL322, pMAL512 or pMAL522 and plated onto solid tryptophan/uracil dropout medium. Transformants were cultured, and then grown on solid medium containing 5-FOA, which selects for cells that have lost the *URA3* plasmid (Sikorski & Boeke, 1991). Once growth was observed (which varied from 2 to 5 days), colonies were replica plated onto solid medium without tryptophan or uracil, and onto solid medium containing 5-FOA to repeat the cycle. trp^+/ura^- colonies were isolated from the second round. Cultures of the resulting his^+ trp^+ ura^- cells (5 for each construct) were grown in YEP(1%)D medium, and doubling times were determined as described (Hawkins et al., 1991).

Yeast Cell Extracts. Cultures (50 mL) of *S. cerevisiae* were grown at 30 °C until O.D. = 0.5 – 0.8 at 600 nm. The cells were collected by centrifugation, washed with distilled water (20 mL), and resuspended such that O.D. = 20 at 600 nm in 100 mM Tris-H_2SO_4 buffer, pH 9.4, containing DTT (20 mM). This suspension was incubated for 10 min at 30 °C. Cells were then washed in 1.2 M sorbitol and resuspended such that O.D. = 50 at 600 nm in spheroplasting buffer, which was 10 mM potassium phosphate buffer, pH 7.2, containing sorbitol (1.2 M) and DTT (2 mM). Cells were converted into spheroplasts by adding Novozym 234 (Novo BioLabs; Bagsvaerd, Denmark) to a final concentration of 3 mg/mL, and incubating the resulting solution at 30 °C for 30 min. Spheroplasts were washed twice with 2 volumes of spheroplasting buffer. Spheroplast lysis and protein isolation were then performed as described (Bostian et al., 1983).

Table 5.1

DNA Oligonucleotides used in This Work

Name	Sequence
ML1	GCTTGGTACGGTGCCAAGACGAGG
ML9	AATTCGGATCCCCATGGGTCGACATATATATACGGCCGAT ATATATAGCATGCGCTAGCTCTAGAA
ML10	AGCTTTCTAGAGCTAGCGCATGCTATATATATCGGCCGTATATAT ATGTCGACCCATGGGGATCCG
ML11	GAATTCGGATCGCCCATGG(G/T)CCGGACACTGCAAAGC
ML12	GAATTCGGATCGCCATGGTGTGGGCAT(G/T)CGAAAGCACTGG
ML13	ATGCTCCCTGG(G/T)CCGGACACTGCAAGCAGCTAGCGCTAGAA AGCTT
ML14	TCCTGGTGTGGGCAT(G/T)CGAA GCAGCTAGCGCTAGAAAGCTT
ML19	CAGAAAGCCGTGCACGATGAACTGTAG
ML25	AGTGGCCACACCATGGGGCATAGAACTCCA
ML26	CATCCTCCGCATTAGCTGACGCTCTGGAGGAGGA
ML27	CCTCCTCCAGAGCGTCAGCTAATGCGGAGGATG
ML28	TTCGTCTTCAGAGCTCATTGTTTGTT TGAA
RR44	CTTTGGATAAAAGAGACGCTCT GGAGGAGGAGGACA
RR45	TTGTCCTCCTCCTCCAGAGCGTCTCTTTTATCCAAAGGTAC

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