

**Molecular Mechanism of Oxidative Protein Folding
by Soybean Protein Thiol Disulfide Oxidoreductases / ERO1 Pathway**

Motonori MATSUSAKI

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GENERAL INTRODUCTION

Seed proteins of major crops such as maize, wheat, rice, and soybean supply energy and amino acids to humans and domestic animals. Among these, soybean seed storage proteins, with their high capacity for accumulation, have significant potential applications. Soybean is protein-rich crop; >35% of dry matter weight is protein, the quality of which is equal to that of animal protein sources because of a superior amino acid balance. Most of the soybean produced in the world is utilized as oilseed, and soybean powder defatted with organic solvent is used to feed domestic animals. This means soybean is a potentially significant protein source for human. In addition, soybean seed could potentially be used in a variety of applications in the future, including the production of oral vaccines, monoclonal antibodies, and therapeutic proteins instead of seed storage proteins, since the cost of production in soybean is very low comparing to that in animal culture cells, and soybean cultivation farms already exist in developed countries as well as developing/underdeveloped countries, where sufficient medical care is often unaffordable.

Around 70% of the total proteins in soybean are seed storage proteins. There are two major seed storage proteins, glycinin and β -conglycinin, which consist of >70% of total storage proteins. They are synthesized in the endoplasmic reticulum (ER) of the cotyledon cell. In eukaryotes, the proper folding of most secretory and membrane proteins synthesized in the ER is accompanied by intramolecular disulfide bond formation. Folding coupled with disulfide bond formation is called oxidative folding. Most soybean storage proteins also undergo folding accompanied by intramolecular disulfide bond formation in the ER. The folded storage proteins are transported via the Golgi apparatus and accumulate in protein bodies (Kermode and Bewley, 1999; Jolliffe *et al.*, 2005). In contrast to normally folded proteins, misfolded and unfolded proteins are retained in the ER and degraded by an ER-associated degradation or vacuolar system (Smith *et al.*, 2011; Pu and Bassham, 2013).

Therefore, quick and efficient oxidative folding of nascent seed storage proteins is required for their accumulation in protein bodies.

During oxidative folding, protein disulfide isomerase (PDI; EC 5.3.4.1) and other ER protein thiol disulfide oxidoreductases (ER oxidoreductases) are thought to catalyze the formation and isomerization of disulfide bonds in nascent proteins (Hatahet and Ruddock, 2009; Feige and Hendershot, 2011; Lu and Holmgren, 2014). The disulfide bond in the active site of ER oxidoreductases is reduced as a result of catalyzing disulfide bond formation in an unfolded protein. The reduced active site of PDI was discovered to be oxidized again by ER oxidoreductin-1 (Ero1p) in yeast (Frand and Kaiser, 1998; Pollard *et al.*, 1998). Ero1p orthologs are present universally in eukaryotes. This sequential reaction is termed the PDI/Ero1 pathway. Over 20 human ER oxidoreductases have been discovered, and the enzymatic properties and three-dimensional structures of some of these have been studied in detail (Hatahet and Ruddock, 2009; Galligan and Petersen, 2012). In plant, 10 classes of ER oxidoreductases have been identified by phylogenetic analysis (Houston *et al.*, 2005). In soybean, a PDI ortholog, GmPDIL-1, and other ER oxidoreductases, GmPDIL-2, GmPDIL-3, GmPDIM, GmPDIS-1, and GmPDIS-2, have been identified and shown to have oxidative refolding activity by the Urade group at Kyoto University (Wadahama *et al.*, 2007, 2008; Kamauchi *et al.*, 2008; Iwasaki *et al.*, 2009).

In this study, I have identified soybean Ero1 and have successfully expressed recombinant soybean Ero1. Using recombinant soybean Ero1, I have successfully reconstituted multiple *in vitro* ER oxidoreductase/Ero1 pathways. In addition, my research has uncovered cooperative oxidative folding by Ero1 and multiple ER oxidoreductases, and I have studied the underlying molecular mechanisms using recombinant mutant proteins. CHAPTER 1 describes the identification of soybean Ero1 orthologs (GmERO1a and GmERO1b), the cloning of cDNA-encoded soybean Ero1 orthologs, and determination of their subcellular localization and expression in soybean tissues. CHAPTER 2 describes the

establishment of a recombinant soybean Ero1 ortholog (GmERO1a) and the activation of multiple soybean ER oxidoreductases by recombinant GmERO1a. CHAPTER 3 details findings regarding the synergistic mechanism by which GmPDIM and GmPDIL-2 cooperatively fold unfolded proteins using oxidizing equivalents provided by GmERO1a *in vitro*, and the electron relay between GmPDIM and GmPDIL-2.

CHAPTER 1

Identification of Soybean Ero1

Introduction

Yeast and flies have a single copy of the *Ero1* gene, which is essential for survival (Pollard *et al.*, 1998; Frand and Kaiser, 1999; Tien *et al.*, 2008). Mammals have two genes encoding Ero1 α (Cabibbo *et al.*, 2000) and Ero1 β (Pagani *et al.*, 2000) that function as major disulfide donors to nascent proteins in the ER, but are not critical for survival (Zito *et al.*, 2010).

Only rice Ero1 (OsERO1) has been identified as a plant ortholog of Ero1p (Onda *et al.*, 2009). OsERO1 is necessary for disulfide bond formation in rice endosperm. The formation of native disulfide bonds in the major seed storage protein proglutelin was demonstrated to depend upon OsERO1 by RNAi knockdown experiments. However, no plant protein thiol disulfide oxidoreductases that are oxidized by a plant Ero1 ortholog have been identified to date.

In this chapter, it is described that soybean Ero1 orthologs (GmERO1a and GmERO1b) were identified. The cDNA encoded soybean Ero1 orthologs were cloned, and their subcellular localization and expression in soybean tissues were determined.

Materials and Methods

Plants

Soybean (*Glycine max* L. Merrill cv. Jack) seeds were planted in 5-L pots and grown in a controlled environmental chamber at 25°C under 16-h day/8-h night cycles. All samples taken were immediately frozen and stored in liquid nitrogen until use.

Expression and Purification of the Luminal Domain of Soybean Calnexin (GmCNX)

Cloning of the cDNA for *GmCNX* was performed by the rapid amplification of cDNA ends (5'RACE) method. Soybean trifoliolate center leaves were frozen in liquid nitrogen and then ground into a fine powder using a micropestle SK-100 (Tokken, Inc.). Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen Inc.) according to the manufacturer's protocol. Messenger RNA was isolated from total RNA with the PolyATtract® mRNA Isolation System (Promega Corporation). The 5' RACE was performed using the SMART™ RACE cDNA Amplification kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol using primer 5'-GGTGATGATGATACTAAACAGGGCGCCAC-3', which was designed based on the sequence of a calnexin homolog (Genbank U20502). The amplified DNA fragments were subcloned into the pT7Blue vector (TaKaRa Bio Inc.), and the inserts were sequenced using the fluorescence dideoxy chain termination method and an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems). Expression plasmids encoding GmCNX luminal domain Ser32–Leu474, excluding the putative signal peptide, (Δ TMC) was constructed as follows. DNA fragments were amplified from *GmCNX* cDNAs by PCR using forward primer 5'-GCCCCTGGGATCCGGTGACGCCGACGACGCGATC-3' and reverse primer 5'-CTCTACCGCGGCCGCTTAGAGATTTGGCTGTTTCTCAGC-3'. Amplified DNA fragments were subcloned into the pGEX-6P-2 vector (GE Healthcare) between the *Bam*H1 and *Not*I restriction sites and expressed in *Escherichia coli* (*E. coli*) Origami

(Novagen) as a GST fusion protein. Induction of recombinant Δ TMC-GST was performed with the addition of 0.4 mM isopropyl thiogalactoside at 30°C for 4 h. The fusion protein extracted from *E. coli* was subjected to affinity chromatography on glutathione Sepharose resin in the presence of 1 mM CaCl_2 ; tags were removed by cleavage with PreScission protease at 4°C for 19 h (GE Healthcare), leaving N-terminal Δ TMC. Recombinant Δ TMC was further purified to apparent homogeneity by gel filtration chromatography on a TSK gel G3000SW column (Tosoh; EMD Biosciences, Inc.) and sequenced. Guinea pig antiserum specific for GmCNX was prepared using purified recombinant Δ TMC, which was conjugated to keyhole limpet hemocyanin by Operon Biotechnologies, K.K.

cDNA Cloning of *GmERO1a* and *GmERO1b*

The cDNAs transcribed from *GmERO1a* and *GmERO1b* mRNAs were amplified from total RNA extracted from 100 mg immature soybean cotyledon by RT-PCR using forward primer 5'-CCTCAGTGTTCTTCAGCGATCTTCTTCTGGGTCTG-3' and reverse primer 5'-CCAAACCACGACAGTGGTTTTTCCTATGTCTAGTC-3', and forward primer 5'-ATGGTGAAAGCGGAGATTGAGAAAAAGGGTTGCAGC-3' and reverse primer 5'-CCACGACGGTGGTTTTTCCTATGTCTAGTCTCTCTAG-3', respectively. The amplified DNA fragments were subcloned into the pUC19 vector. The inserts in the plasmid vectors were sequenced.

Expression and Purification of His-tagged GmERO1a

An expression plasmid encoding His-tagged GmERO1a K³⁵-T⁴⁶⁵ was constructed as follows. The DNA fragment was amplified from *GmERO1a* cDNA by PCR using primers 5'-GACGACGACAAGATGGCCATGGCCATGTCTTCCAAAACCTCTCC-3' and 5'-GAGGAGAAGCCCGGTAAAGTTTTAGATACATAGACCAAA-3'. The amplified DNA fragment was subcloned into the pET46 Ek/LIC vector (Novagen). The recombinant protein

has the His-tag linked to the amino terminus. *E. coli* Rosseta 2 (DE3) cells (Takara Bio, Inc.) were transformed with the expression vector described above. Expression of recombinant His-tagged GmERO1a was induced in LB containing 0.4 mM isopropyl thiogalactoside, 500 µg/mL ampicillin, 15 µg/mL kanamycin, and 12.5 µg/mL tetracycline at 15°C for 120 h. The cells were collected by centrifugation and disrupted by sonication in phosphate-buffered saline containing 20 mM imidazole, 8 M urea, and 5 mM dithiothreitol (DTT). The pellet fraction was washed with urea twice. The pellet was solubilized in 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl, 20 mM imidazole, 6 M guanidine hydrochloride, and 5 mM DTT, and applied to His-Bind resin (EMD Biosciences, Inc.). Recombinant His-tagged GmERO1a was eluted with 20 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl, 500 mM imidazole, 6 M guanidine hydrochloride, and 5 mM DTT. The solution was dialyzed against distilled water. The insoluble recombinant GmERO1a was collected by centrifugation and solubilized in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 9.0), 0.2 M DTT, 2% SDS.

Western Blot Analysis

Soybean roots, leaves, and stems were collected from 10-day-old seedlings. Seeds were collected from plants, and cotyledons were isolated. Tissues were frozen in liquid nitrogen and then ground into a fine powder with a micropestle SK-100. Proteins were extracted by boiling for 5 min in SDS-PAGE buffer (Laemmli, 1970) containing a 1% cocktail of protease inhibitors (Sigma-Aldrich, Inc.). To cleave N-glycans, proteins were extracted from the cotyledons in 0.1% SDS/50 mM phosphate buffer (pH 5.5). Protein concentration in the sample was measured with a RC DC protein assay kit (Bio-Rad Laboratories). Proteins (0.4 mg) were treated with 10 mU endoglycosidase F or endoglycosidase H (Sigma-Aldrich Inc.) at 37°C for 16 h. To separate the supernatant and membrane fractions, the frozen cotyledons from three 100-mg seeds in liquid nitrogen were

crushed with a micropestle SK-100 and sonicated with a Sonifier II (Branson Ultrasonic Corporation) in 200 mM Tris-HCl, pH 7.8, five times for 30 sec on ice. The homogenate was placed into a cell strainer (BD Biosciences) and centrifuged at $824 \times g$ for 40 min at 4°C. The filtrated suspension was divided two fractions, diluted with 200 mM Tris-HCl, pH 7.8, with or without 1% Triton X-100, and centrifuged at $100,000 \times g$ for 1 h at 4°C. Proteins were subjected to SDS-PAGE (Laemmli, 1970) and blotted onto a polyvinylidene difluoride membrane. Blots were immunostained first with antiserum and then with horseradish peroxidase-conjugated IgG antiserum (Promega Corporation) as the secondary antibody. Anti-GmERO1a guinea pig serum was prepared using recombinant His-tagged GmERO1a by Operon Biotechnologies, K.K. Anti-GmPDIL-1 serum was prepared previously (Kamauchi *et al.*, 2008). Blots were developed with the Western Lightning Chemiluminescence Reagent (Perkin Elmer Life Sciences).

Confocal Microscopy

Cotyledons from developing soybean seeds (195 mg) were cut into $3 \times 3 \times 1$ -mm cubes. The pieces of tissue were fixed with 4% formaldehyde for 2 h at room temperature. The fixed cotyledon pieces were dehydrated with a series of ethanol dilutions, embedded in Histoiresin (Leica Microsystems), and sliced into sections. The sections were stained with anti-GmPDIL-1 rabbit serum (Wadahama *et al.*, 2007) and then biotin-anti-rabbit IgG goat serum (Cortex Biochem), followed by incubation with Cy5-streptavidin (GE Healthcare). For detection of GmERO1, specimens were stained with guinea pig antiserum against recombinant GmERO1a, followed by staining with Cy3-conjugated anti-guinea pig IgG goat serum (Chemicon International). The specimens were examined on a FV1000-D laser scanning confocal imaging system (Olympus).

Measurement of mRNA

Total RNA was isolated from young leaves treated with or without 5 µg/mL tunicamycin at 25°C for 5 h using an RNeasy Plant Mini kit (Qiagen Inc.). Quantification of mRNA was performed by real-time PCR with a Thermal Cycler Dice™ Real Time System with SYBR®Premix *Ex Taq*™ (TaKaRa Bio Inc.). Forward primers 5'-CATGCTCTCTTCTGAGTATTTGTCA-3' and 5'-TCATCCAAGAAATGGGACCT-3' and reverse primers 5'-CAGTGGTTTTGGTATGTCTAGTCTCTC-3' and 5'-CGACGGTGGTTTTTCCTATGT-3' were used for detection of GmERO1a mRNA and GmERO1b, respectively.

Results and Discussion

To find soybean proteins homologous to human *ERO1 α* (NP_055399), the BLAST program was used to search for sequence similarity against the soybean genome databases. *Glycine max* have *GmERO1a* (AB622257) and *GmERO1b* (AB622258) showed 38% identity with *HsERO1 α* . The *GmERO1a* cDNA and *GmERO1b* cDNA encoding the soybean Ero1 orthologs were cloned. *GmERO1a* and *GmERO1b* encoded proteins composed of 465 amino acids containing 19 cysteine residues (Figure 1-1 and Figure 1-2). GmERO1 proteins were expressed as a single 53-kDa band in soybean root, stem, leaf, and cotyledon (Figure 1-3). The band shifted to 46-kDa under nonreducing SDS-PAGE, suggesting that GmERO1 proteins have intramolecular disulfide bonds. I confirmed that GmERO1 proteins, which have a putative transmembrane region (Trp16 - Ser34) near the N-termini and two putative N-glycosylated asparagine residues, are type I membrane-bound glycoproteins targeted to the ER. When the extract from the cotyledon was treated with endoglycosidase H or F, the size of GmERO1 proteins decreased from 53 kDa to 50 kDa (Figure 1-4A), suggesting that one or more high-mannose type N-glycan(s) were attached to GmERO1 proteins. To confirm whether GmERO1 is membrane binding protein, cell fractionation was performed, and GmERO1 was compared to well-known membrane protein, soybean calnexin (GmCNX). Anti-GmCNX serum was prepared against C-terminal truncated GmCNX (Figure 1-5). GmERO1 proteins were recovered in the precipitate of the cell homogenate, but not the supernatant, after ultra-centrifugation at 100,000 $\times g$, and pretreatment with Triton X-100 caused solubilization of GmERO1 proteins in the supernatant along with soybean calnexin (Figure 1-4B). In confocal microscopic images, GmERO1 was observed upon immunostaining to colocalize with GmPDIS-1, which is localized in the ER (Wadahama *et al.*, 2007) (Figure 1-4C).

The expression levels of GmERO1 proteins and their mRNAs were high when seed

storage proteins β -conglycinin and glycinin were synthesized (70–100 mg and 150–200 mg cotyledon weight, respectively) (Figure 1-6A and B). *GmERO1a* and *GmERO1b* mRNAs were upregulated following ER stress induced by tunicamycin (Figure 1-7). The ER stress response elements (Oh *et al.*, 2003; Iwata and Koizumi, 2005; Iwata *et al.*, 2008; Hayashi *et al.*, 2013; Sun *et al.*, 2013) were not found within the 2500-bp upstream and downstream sequences of the transcriptional region of either *GmERO1a* or *GmERO1b*. These Results suggest that GmERO1 is involved in protein production in the ER.

GmERO1a	1	MVKSEIEKKG	<u>CSTRQWLWL</u> V	<u>MALVAVFVAM</u>	<u>AMSSKTSPKA</u>	LFGAIDRACP	50
GmERO1b	1	MVKAEIEKKG	<u>CSTRRWLWL</u> V	<u>MALVAVFVAM</u>	<u>VMSSRTSPKA</u>	LFGAIDRACP	50
HsEro1a			1	MGRGWGFLF	GLLGAVWLLS	SGHGEEQPPE	TA----- 31
		***	.*****	****.*****	*****	:***:*****	*****
GmERO1a		CARGTPKYS	G	MVEDCCCDYE	TVDRNLNEEV	L	HPSLQELVKT PFFRYFKVKL 100
GmERO1b		CARGTPKYS	G	MVEDCCCDYE	TVDRNLNEEV	L	HPSLQELVKT PFFRYFKVKL 100
HsEro1a		AQRCF	CQVSG	YLDDCTCDVE	TIDRFNNYRL	FPRLQKLLES	DYFRYYKVN 81
		*****	*****	*****	*****	*****	*****
GmERO1a		WCD	CPFWPDD	GM	CRLRDCSV	CE	CPENEFPE SFKKPDRRLS MTDLV----C 146
GmERO1b		WCD	CPFWPDD	GM	CRLRDCSV	CE	CPSEFEPE SFKKPDHRLS MTDLV----C 146
HsEro1a		KRP	CFPWNDI	SQ	CGRRDCAV	KP	CQSDEVDP GIKSASYKYS EEANNLIEEC 131
		*****	*****	*****	.****	*****:***	*****----
GmERO1a		QEGKPQAAVD	RTLDS---KA	FRGWTEIDNP	WTNDDETDND	---	EMTYVNL 190
GmERO1b		QEGKPQAAVD	RTLDS---KA	FRGWTEIDNP	WTNDDETDND	---	EMTYVNL 190
HsEro1a		EQAERLGAVD	ESLSEETQKA	VLQWTKHDDS	SDNF	C	EADDI QSPEAEYVDL 181
		*****	*****	**	*****	*****	*****
GmERO1a		QLNPERYTG	Y	TGPSARRIWD	AVYSEN	C	PKY PSQE----- 224
GmERO1b		QLNPERYTG	Y	TGPSARRIWD	AVYSEN	C	PKY PSQE----- 224
HsEro1a		LLNPERYTG	Y	KGPDAWKIWN	VIYEEN	C	FKP QTIKRPLNPL ASGQGTSEEN 231
		*****	*****	*****	*****	****	*****
GmERO1a		-----	L	C	QEEKILYKLI	SGLHSSISIH	IASDYLLDEA TN--LWGQNL 264
GmERO1b		-----	L	C	HEEKILYKLI	SGLHSSISIH	IASDYLLDEA TN--LWGQNL 264
HsEro1a		TFYSWLEGL	C	VEKRAFYRLI	SGLHASINVH	LSARYLLQET	WLEKKWGHNI 281
		**	*****	*****	*****	:**	** *****
GmERO1a		TLMYDRVL--	---	RYPDRV	RNLYFTFLFV	LRAVTKASDY	LEQ--AEYDT 306
GmERO1b		TLMYDRVL--	---	RYPDRV	RNLYFTFLFV	LRAVTKASDY	LEQ--AEYDT 306
HsEro1a		TEFQQRFDGI	L	TEGEGPRRL	KNLYFLYLIE	LRALSKVLPF	FERPDFQLFT 331
		*****	*****	*****	*****	*****	*** *****
GmERO1a		GNPNEDLTTQ	SLIKQLLYNP	KLQAAC	PIPF	DEANLWKG--	QSGPELKQKI 354
GmERO1b		GNPNEDLTTQ	SLIKQLLYNP	KLQAAC	PIPF	DEANLWKG--	QSGPELKQKI 354
HsEro1a		GNKIQDEENK	MLLLEILHEI	KS---	FPLHF	DENSFFAGDK	KEAHKLKEDF 378
		*****	*****	*****	*****	*****	*****
GmERO1a		QQQFR	ISAL	MD	CVGCEKCR	LWGKLQVLGL	GTALKILFSV D-----GQEN 399
GmERO1b		QQQFR	ISAM	MD	CVGCEKCR	LWGKLQVLGL	GTALKILFSV D-----GQEN 399
HsEro1a		RLHFRNISRI	MD	CVGCFKCR	LWGKLQTQGL	GTALKILFSE	KLIANMPESG 428
		*****:	*****	*****	*****	*****	* *****
GmERO1a		SSHTLQLQRN	EVIALTNLLN	RLSESVKFVH	EVGPTAERIM	EGGHFSAHTR	449
GmERO1b		SSHTLQLQRN	EVIALTNLLN	RLSESVKFIQ	EMGPTAERIM	EGGHFSAHTR	449
HsEro1a		PSYEFHLTRQ	EIVSLFNAFG	RISTSVKELE	NFRNLLQNIH	468	
		*****	*****	*****:	.	*:*****	*****
GmERO1a		TLISSWKKIW	SYVSKT	465			
GmERO1b		TLISSWKKIW	SYVSKT	465			
		*****	*****				

Figure 1-1. Amino acid sequences of GmERO1a, GmERO1b, and HsEro1a.

GmERO1a and *GmERO1b* mRNAs are products transcribed from genes *Glyma15g23150* and *Glyma09g10830*. The numbers refer to the amino acid number. The putative transmembrane region (underlined) and N-glycosylation consensus asparagine residues (shaded in gray) are indicated. Asterisks indicate positions that have a single, fully conserved residue between GmERO1a and GmERO1b. Colons indicate conservation between groups of strongly similar properties. Periods indicate conservation between groups of weakly similar properties

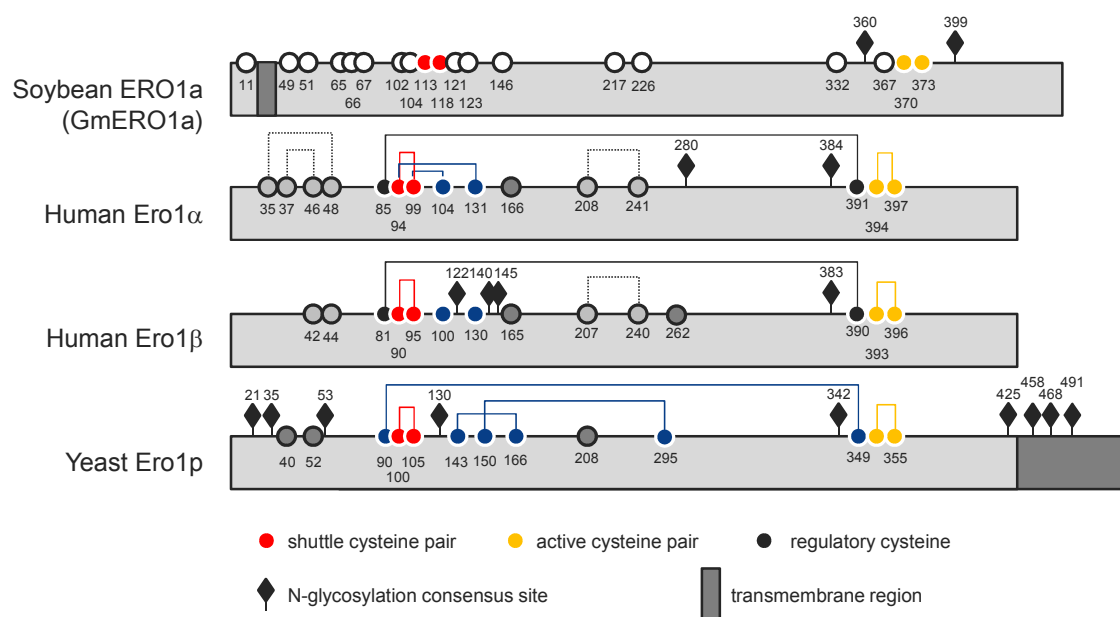


Figure 1-2. Schematic representation of Ero1 orthologs.

Numbered circles represent the positions of the cysteine residues. Lines indicate disulfide bonds.

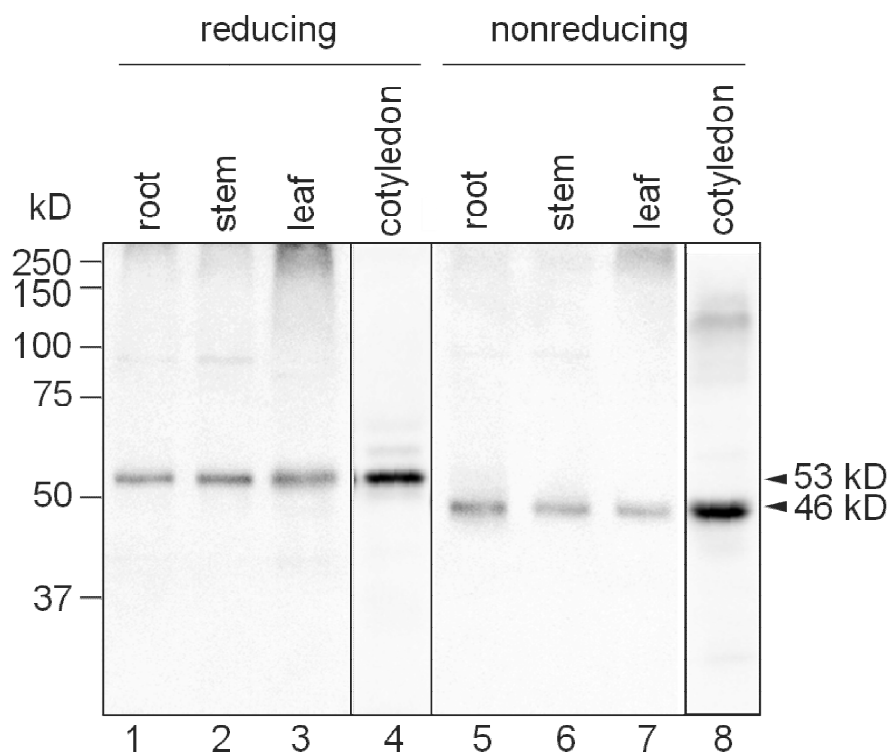


Figure 1-3. GmERO1 is ubiquitously expressed in tissues.

The proteins extracted from root (4.5 μ g protein) (lanes 1 and 5), stem (10 μ g protein) (lanes 2 and 6), leaf (30 μ g protein) (lanes 3 and 7), and immature cotyledon (100 mg) (30 μ g protein) (lanes 4 and 8) were separated under reducing (lanes 1–4) and nonreducing SDS-PAGE (lanes 5–8) and subjected to western blot analysis with an antiserum prepared against recombinant GmERO1a.

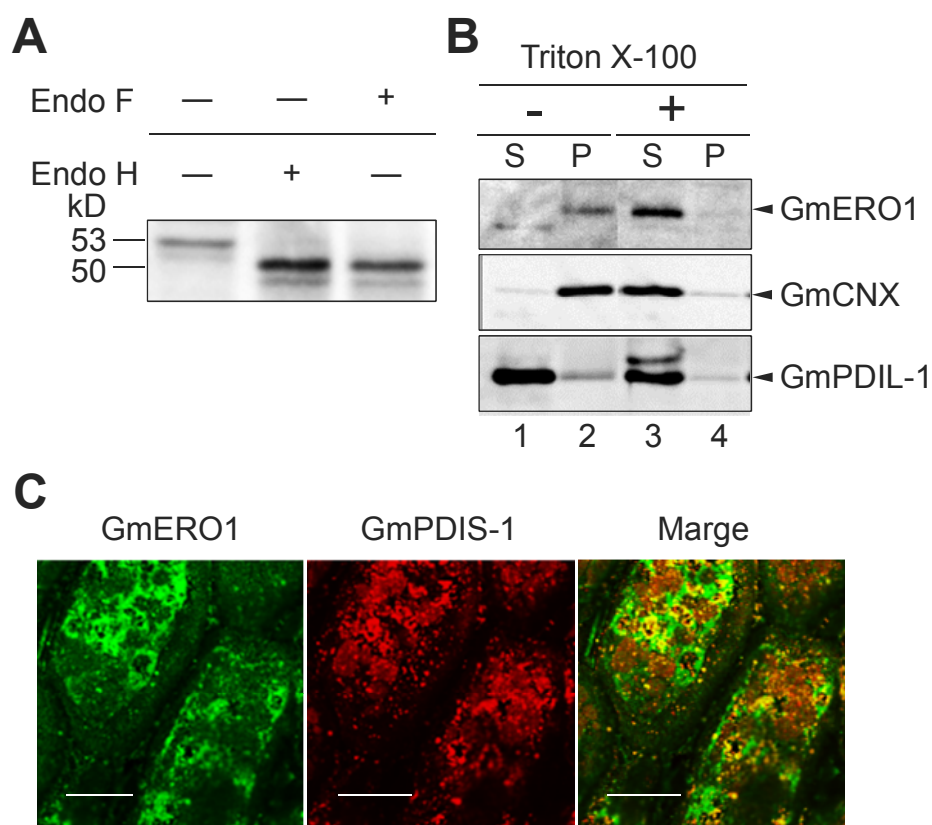


Figure 1-4. Subcellular localization of GmERO1.

(A) GmERO1 is N-glycosylated. GmERO1 in the immature cotyledon (30 mg) was detected by western-blot analysis with the anti-GmERO1 serum after treatment with (+) or without (–) endoglycosidase H (Endo H) or endoglycosidase F (Endo F). The 53-kD band of GmERO1 was shifted to 50 kD after Endo H and Endo F treatment.

(B) GmERO1 is a membrane-bound protein. An immature cotyledon (100 mg) was homogenized by sonication. The homogenate was centrifuged at 100,000 ×g for 2 h at 4°C in the absence (–; lanes 1 and 2) or presence (+) of 1% Triton X-100 (lanes 3 and 4). GmERO1 protein in the supernatant (S; lanes 1 and 3) and pellet (P; lanes 2 and 4) was detected by western-blot analysis with anti-GmERO1 serum, anti-GmCNX serum, or anti-GmPDIL-1 serum.

(C) GmERO1 localizes in the ER. The immature cotyledon (195 mg) was fixed and embedded in resin. The sections were cut with a microtome and immunostained with anti-GmERO1 guinea pig serum and anti-GmPDIL-1 rabbit serum, and observed under a confocal microscope. Bars = 20 µm.

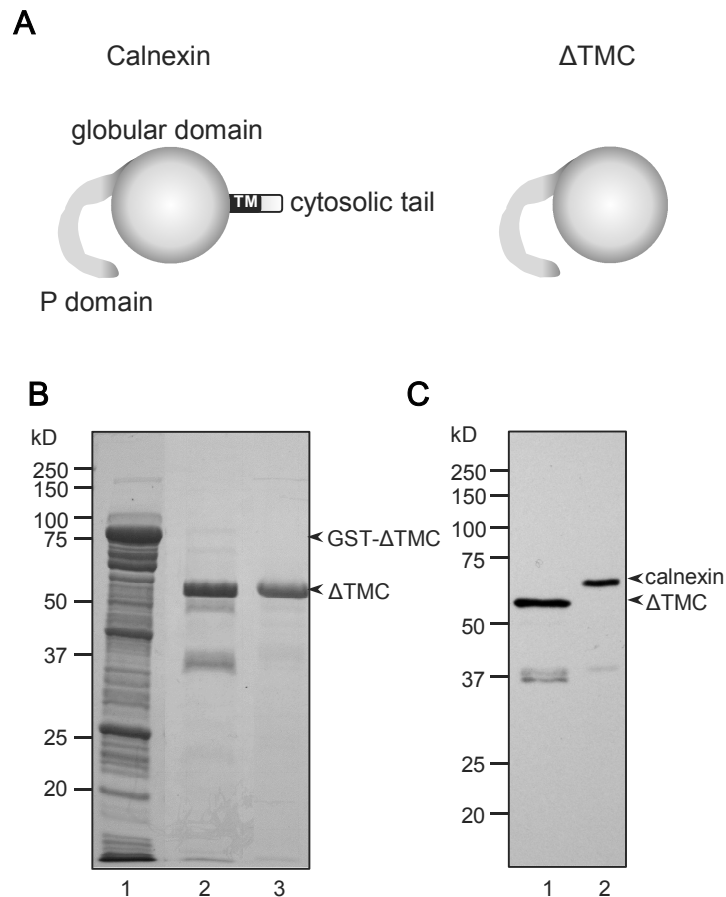


Figure 1-5. Expression and purification of recombinant C-terminal truncated soybean calnexin.

(A) Schematic representation of soybean calnexin and recombinant C-terminal truncated GmCNX (ΔTMC) (Ser32-Leu474). TM, transmembrane region. Sequence data of *GmCNX* can be found in the DDBJ/EMBL/GenBank databases under accession numbers AB196933.

(B) Expression of recombinant ΔTMC in *E. coli* and purification. Recombinant GST-ΔTMC in *E. coli* (lane 1) were adsorbed onto a glutathione resin column and the ΔTMC fragment was eluted by cleavage with PreScission protease from the resin (lane 2), followed by gel filtration chromatography (lane 3). Proteins in each eluate were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue.

(C) Immunodetection of recombinant ΔTMC and calnexin in cotyledons. Recombinant ΔTMC (100 ng) (lane 1) and protein extracted from the cotyledon (30 μg) (lane 2) were analyzed by western blot using anti-GmCNX serum.

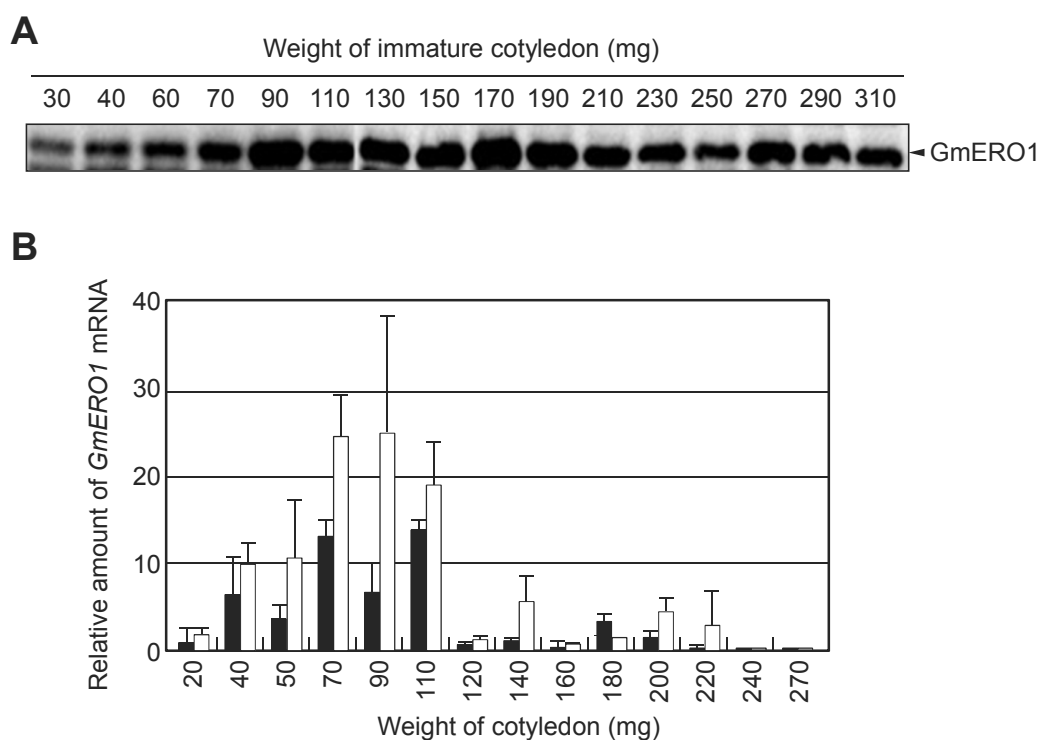


Figure 1-6. Expression of GmERO1 in cotyledons during development.

(A) The proteins (25 μ g) extracted from cotyledons were analyzed by western blot with anti-GmERO1a serum.

(B) Relative amounts of *GmERO1a* (black bars) and *GmERO1b* (white bars) mRNA in cotyledons during development were quantified by real-time PCR. Data are represented as mean $\pm$ SE of $n = 3$.

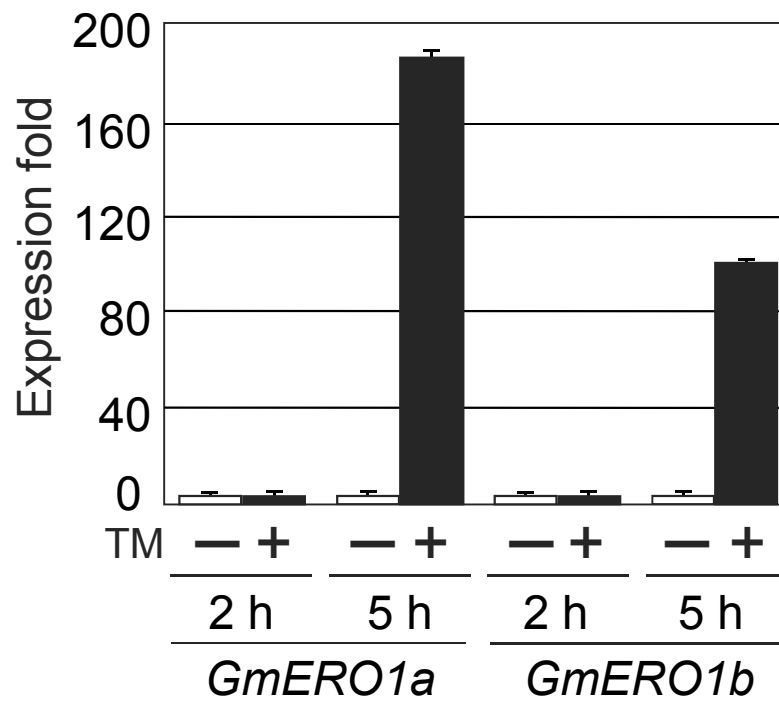


Figure 1-7. Expression levels of *GmERO1a* and *GmERO1b* mRNA are up-regulated under ER stress.

Young soybean leaves were treated with (+, black bars) or without (-, white bars) 5 μ g/mL TM for 2 or 5 h. After treatment, *GmERO1a* and *GmERO1b* mRNA were quantified by real-time PCR. Data are represented as mean $\pm$ SE of $n = 3$. TM, tunicamycin.

CHAPTER 2

Enzymatic Properties of GmERO1a

Introduction

After phylogenetic analysis of the *Arabidopsis* genome, 10 classes of ER oxidoreductases (classes I–X) were identified (Houston *et al.*, 2005). Among them, class I ER oxidoreductase, a plant PDI ortholog, has been studied in a wide variety of plants. Class I ER oxidoreductases have two catalytically active domains **a** and **a'**, containing active sites composed of Cys-Gly-His-Cys and two catalytically inactive domains **b** and **b'**. An *Arabidopsis* ortholog of class I ER oxidoreductases is required for proper seed development and regulates the timing of programmed cell death by chaperoning and inhibiting Cys proteases (Andème Ondzighi *et al.*, 2008). OaPDI, a PDI from *Oldenlandia affinis*, a coffee family (*Rubiaceae*) plant, is involved in the folding of knotted circular proteins (Gruber *et al.*, 2007). The rice ortholog (PDIL1-1) was suggested to be involved in the maturation of the major seed storage protein glutelin (Takemoto *et al.*, 2002). Furthermore, rice PDIL1-1 plays a role in regulatory activities for various proteins that are essential for the synthesis of grain components as determined by analysis of a T-DNA insertion mutant (Satoh-Cruz *et al.*, 2010).

The oxidative refolding ability of class I ER oxidoreductases was confirmed in recombinant soybean (GmPDIL-1) and wheat proteins produced by an *E. coli* expression system established from cDNAs (Kamauchi *et al.*, 2008; Kimura *et al.*, 2015).

Class II and III ER oxidoreductases have an **a–b–b'–a'** domain structure. Class II ER oxidoreductases have an acidic amino acid-rich sequence in the N-terminal region ahead of the **a** domain. Recombinant soybean (GmPDIL-2) and wheat class II ER oxidoreductases have oxidative refolding activities similar to that of class I (Kamauchi *et al.*, 2008; Kimura *et al.*, 2015). Class III ER oxidoreductases contain the nonclassical redox-center

Cys-X-X-Ser/Cys motifs, as opposed to the more traditional CGHC sequence, in the **a** and **a'** domains. Recombinant soybean (GmPDIL-3) and wheat proteins lack oxidative refolding activity *in vitro* (Iwasaki *et al.*, 2009; Kimura *et al.*, 2015). Class IV ER oxidoreductases are unique to plants and have an **a–a'–ERp29** domain structure, which is homologous to the C-terminal domain of mammalian ERp29 (Demmer *et al.*, 1997).

Recombinant soybean class IV ER oxidoreductases (GmPDIS-1 and GmPDIS-2) and wheat class IV ER oxidoreductase possess an oxidative refolding activity that is weaker than that of classes I and II (Wadahama *et al.*, 2007; Kimura *et al.*, 2015). Class V ER oxidoreductases are plant orthologs of mammalian P5 and have an **a–a'–b** domain structure. A rice class V ER oxidoreductase, consisting of OsPDIL2 and OsPDIL3, plays an important role in the accumulation of the seed storage protein Cys-rich 10-kD prolamin (crP10; Onda *et al.*, 2011). Recombinant soybean class V ER oxidoreductase (GmPDIM) and wheat class V ER oxidoreductase possess an oxidative refolding activity similar to that of class IV (Wadahama *et al.*, 2008; Kimura *et al.*, 2015).

Domain **a** of yeast PDI is the most favored substrate of yeast Ero1p (Vitu *et al.*, 2010), whereas **a'** of human PDI is specifically oxidized by human Ero1 α (Chambers *et al.*, 2010) and Ero1 β (Wang *et al.*, 2011). Electrons from Cys residues of the active sites of PDI are transferred to oxygen by Ero1 (Tu and Weissman, 2004; Sevier and Kaiser, 2008). The reaction mechanisms of yeast Ero1p and human Ero1s have been intensively investigated; their regulation by PDI has been extensively studied as well (Tavender and Bulleid, 2010; Araki and Inaba, 2012; Benham *et al.*, 2013; Ramming *et al.*, 2015). After the flavoprotein human Ero1 α transfer disulfide bond to active site of PDI, the flavin adenine dinucleotide (FAD) cofactor in Ero1 α is reduced to FADH₂. FADH₂ returns to oxidized form by transferring two electrons to O₂ (Ramming and Appenzeller-Herzog, 2012). Thus, two electrons from PDI are transferred to shuttle cysteine pair of Ero1 α (Cys⁹⁴/Cys⁹⁹), and via active cysteine pair (Cys³⁹⁴/Cys³⁹⁷) finally to FAD (Figure 1-1). This process is regulated by

the reversible formation of two inhibitory disulfide bonds (Cys⁹⁴–Cys¹³¹ and Cys⁹⁹–Cys¹⁰⁴) between regulatory cysteines (Cys¹⁰⁴/Cys¹³¹) and shuttle cysteines catalyzed by PDI (Appenzeller-Herzog *et al.*, 2008; Baker *et al.*, 2008; Hansen *et al.*, 2012). In plant, OsERO1 has been reported to be required for disulfide bond formation in rice endosperm (Onda *et al.*, 2009). However, which ER oxidoreductase is oxidized by a plant Ero1 ortholog is unclear.

In this chapter, it is described the establishment of a recombinant soybean Ero1 ortholog (GmERO1a) and the activation of multiple soybean ER oxidoreductases by recombinant GmERO1a.

Materials and Methods

Preparation of Recombinant GmERO1a

An expression plasmid encoding GST-fused GmERO1a E⁷⁰-G⁴²² was constructed as follows. The DNA fragment was amplified from *GmERO1a* cDNA by PCR using primers 5'-GCGATGGATCCTATGAAACTGTGGATCGTCTTAATG-3' and 5'-GCGATGTCGACTATCCTCCTTCCATGATTCTTTCAGC-3'. The amplified DNA fragment was subcloned into the pGEX6p-2 vector (GE Healthcare). The recombinant protein has glutathione-S-transferase (GST) linked to the amino terminus. *E.coli* Rosseta-gami cells (Takara Bio, Inc.) were transformed with the GST-fusion vector described above. Expression of recombinant GST-GmERO1a was induced in LB containing 0.5 mM isopropyl thiogalactoside, 20 µM FAD, 100 µg/mL ampicillin, 15 µg/mL kanamycin, and 12.5 µg/mL tetracycline at 15°C for 120 h. The cells were collected by centrifugation and disrupted by sonication in phosphate-buffered saline. Recombinant GST-GmERO1a was adsorbed to a glutathione Sepharose 4B column and digested by PreScission protease (GE Healthcare). The eluted recombinant GmERO1a was purified by gel filtration chromatography on a TSK gel G3000SW column (Tosoh Co., Ltd.) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 10% glycerol as described previously (Wadahama *et al.*, 2007). The concentration of purified recombinant GmERO1a was determined by amino acid analysis with norleucine as an internal standard.

Far-UV Circular Dichroism Analysis

Circular Dichroism (CD) spectra of recombinant proteins in 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 10% glycerol were obtained using a J-720 spectropolarimeter (JASCO Corp.) in a 1-mm path-length cell with a scan speed of 20 nm/min at 14°C.

Expression of ER oxidoreductases

Wild-type GST-GmPDIL-2 and GST-GmPDIM were expressed in *E. coli* BL21 (DE3) cells (Merck KGaA) in the presence of 0.5 mM IPTG at 15°C for 72 h. Recombinant proteins were separated on a glutathione Sepharose 4B column equilibrated with phosphate-buffered saline, cleaved from GST with PreScission protease at 4°C for 16 h, and eluted from the column with cleavage buffer according to the manufacturer's protocol. The eluted recombinant proteins were purified by gel filtration chromatography on a TSK gel G3000SW column equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 10% glycerol. The concentrations of the purified recombinant proteins were determined by absorbance at 280 nm using the molar extinction coefficient calculated according to the modified method of Gill and von Hippel (Pace *et al.*, 1995). Recombinant proteins, including wild-type GmPDIL-1, GmPDIS-1, and GmPDIS-2, were prepared and purified as described previously (Wadahama *et al.*, 2007, 2008; Kamauchi *et al.*, 2008). LB containing 50 µg/mL ampicillin was incubated at 37°C until IPTG was added to the medium at a final concentration of 0.5 mM at an A_{600} of 0.5. After an additional 4 h of shaking at 30°C, cells were collected. The cell lysate supernatant was applied to His-Bind resin (EMD Biosciences, Inc.) the glutathione Sepharose 4B column. Fractions eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 1 M imidazole after PreScission protease digestion were further purified by gel filtration chromatography on a TSK gel G3000SW column. Each construct was quantified using the molar extinction coefficient at 280 nm.

ER Oxidoreductase Oxidation Assays

Oxygen consumption was measured using a Clark-type oxygen electrode system (OXYT-1) (Hansatech Instruments Ltd.). All experiments were performed at 25°C in 100 mM [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 7.5), 2 mM CaCl₂, and 150 mM NaCl. Catalytic oxygen consumption was initiated by the addition of GmERO1a in a

reaction mixture containing recombinant ER oxidoreductases or its variants, and 10 mM GSH.

The activity was measured using a coupled assay following the decrease in absorbance at 340 nm due to the consumption of NADPH by glutathione reductase (Sigma-Aldrich, Inc.) with the reaction being started by the addition of GmERO1a to initiate the reaction (Nguyen *et al.*, 2011; Sato *et al.*, 2013). A molar extinction coefficient of 6200 M⁻¹cm⁻¹ for NADPH was used for calculations. All experiments were performed in 100 mM HEPES (pH 7.5) containing 150 mM NaCl and 2 mM CaCl₂.

RNase A Refolding Assays

Thiol oxidative refolding activity was assayed as previously described by measuring RNase activity following regeneration of the active form of the enzyme from its reduced and denatured form in the presence of the recombinant proteins (Creighton, 1977; Lyles and Gilbert, 1991). Reduced and denatured RNase A was prepared as described previously. Each reaction mixture contained 100 mM HEPES buffer (pH 7.5), 150 mM NaCl, 2 mM CaCl₂, 0.5 mM glutathione disulfide, 2 mM glutathione, reduced RNase A, and recombinant oxidoreductases and their variants, and were incubated at 25°C. The formation of active RNase A was measured spectrophotometrically by monitoring the hydrolysis of the RNase A substrate cCMP at 284 nm. The isomerase activities of oxidoreductases were calculated as described previously (Kulp *et al.*, 2006). Briefly, isomerase activity was determined from the linear increase in the amount of enzymatically active RNase A with time after a lag. Oxidation of free thiol residues to disulfides on reduced RNase A were also determined by measuring the amounts of free thiol groups in the reaction mixtures (Ellman, 1959).

Gel-based RNase A Refolding Experiments

RNase A oxidation analyses were performed by the addition of 1 μM GmERO1 in

100 mM HEPES buffer (pH7.5), 150 mM NaCl, 2 mM CaCl₂, and 3 μ M recombinant ER oxidoreductases and their variants containing 8 μ M denatured and reduced RNase A. At the indicated time points, free thiols were blocked by the addition of Laemmli's 4 $\times$ SDS-loading buffer (Laemmli, 1970) containing 8 mM 4-acetamido-4-maleimidylstilbene-2,2 disulfonic acid and separated on a 15% polyacrylamide gel by SDS-PAGE (Laemmli, 1970) without reducing reagent. Proteins were detected by Coomassie Brilliant Blue staining after electrophoresis.

Results and Discussion

Recombinant GmERO1a Oxidizes Various ER Oxidoreductases

The soluble and folded 48-kDa recombinant GmERO1a, which is missing 60 N-terminal residues including the putative transmembrane region and the C-terminal 23 residues (Figure 2-1A), was expressed in *E. coli*. Its mobility as visualized by SDS-PAGE shifted to 38 kDa under nonreducing conditions, suggesting that the recombinant protein has intramolecular disulfide bonds (Figure 2-1B). The recombinant protein had CD spectra typical of a well-folded protein (Figure 2-1C).

The UV-visible absorption spectrum of the recombinant GmERO1a showed a typical unresolved flavin envelope with a maximum absorbance at 454 nm and a shoulder at 485 nm (Figure 2-2A). The spectrum, identical to that of authentic flavin adenine dinucleotide (FAD), was obtained after denaturation with guanidine hydrochloride, indicating that FAD is noncovalently bound to recombinant GmERO1a. The molar ratio of GmERO1a to bound FAD was calculated to be 1:1 with a molecular extinction coefficient of $12.9 \text{ mM}^{-1}\text{cm}^{-1}$. The FAD moiety bound to GmERO1a was reduced by DTT (Figure 2-2B), suggesting that FAD bound to GmERO1a acts as a cofactor to transfer electrons from a substrate.

To determine the ability of recombinant GmERO1a to oxidize five soybean ER oxidoreductases (Figure 2-3A), oxidation of ER oxidoreductases by GmERO1a was monitored by oxygen consumption (Figure 2-3B). The reaction was performed in the presence of GSH, as a substrate for oxidation by ER oxidoreductases oxidized by GmERO1a. In contrast to human Ero1 α and Ero1 β , which predominantly oxidize PDI, GmERO1a oxidized GmPDIL-1, GmPDIS-1, GmPDIS-2, and GmPDIM to comparable levels (Figure 2-3C). GmPDIL-2 was negligibly oxidized by GmERO1a. These results suggest that the plant Ero1 ortholog has a broad substrate specificity. In contrast, human Ero1 α and Ero1 β preferentially oxidize PDI. Since another pathway that oxidizes other ER oxidoreductases likely exists in

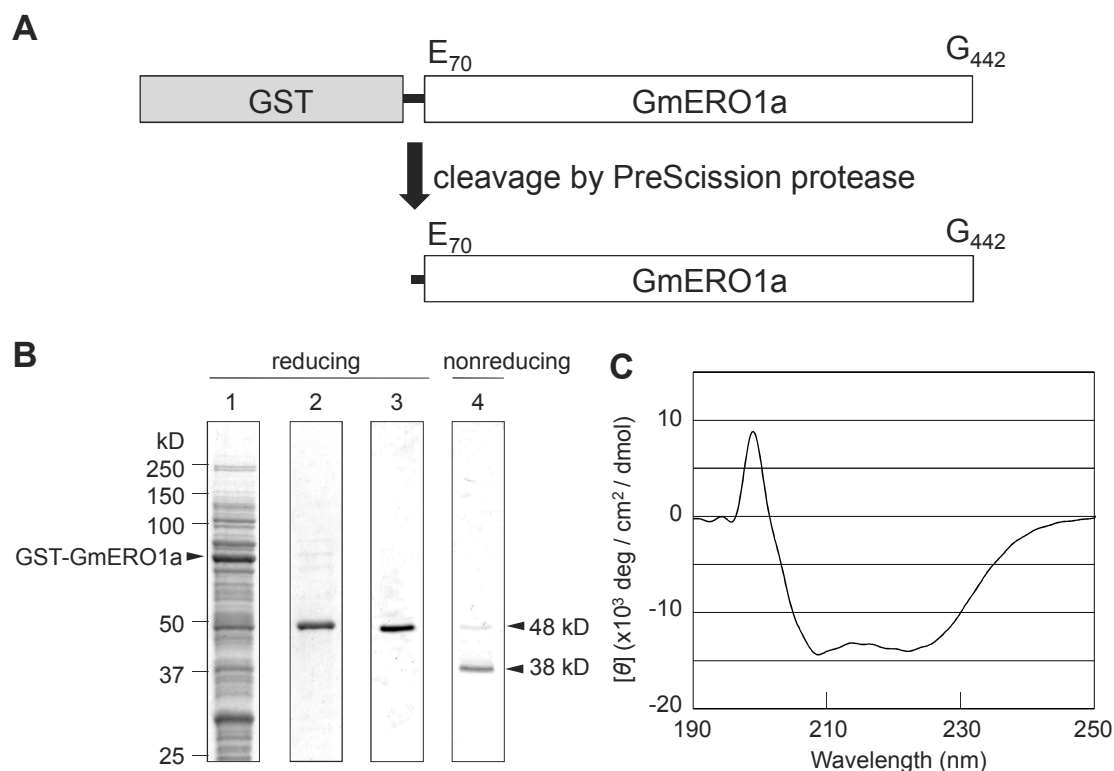


Figure 2-1. Expression of recombinant GmERO1a.

(A) Cleavage of recombinant GST-fused GmERO1a by PreScission protease. The gray box indicates GST, and the black line denotes the PreScission cleavage sequence. The N-terminal GST was cleaved from the fusion protein after protease treatment.

(B) Expression and purification of recombinant GmERO1a. Recombinant GST-fused GmERO1a expressed in *E. coli* (lane 1) was purified by a glutathione Sepharose 4B affinity column chromatography. GmERO1a was cleaved by PreScission protease and eluted from the column (lane 2), followed by gel filtration chromatography (lanes 3 and 4). Proteins in each eluate were separated by reducing SDS-PAGE (lanes 1–3) and nonreducing SDS-PAGE (lane 4), and stained with Coomassie Brilliant Blue.

(C) Far-UV circular dichroism spectrum of recombinant GmERO1a in 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 10% glycerol was obtained using a J-720 spectropolarimeter in a 1-mm path-length cell with a scan speed of 20 nm/min at 14°C.

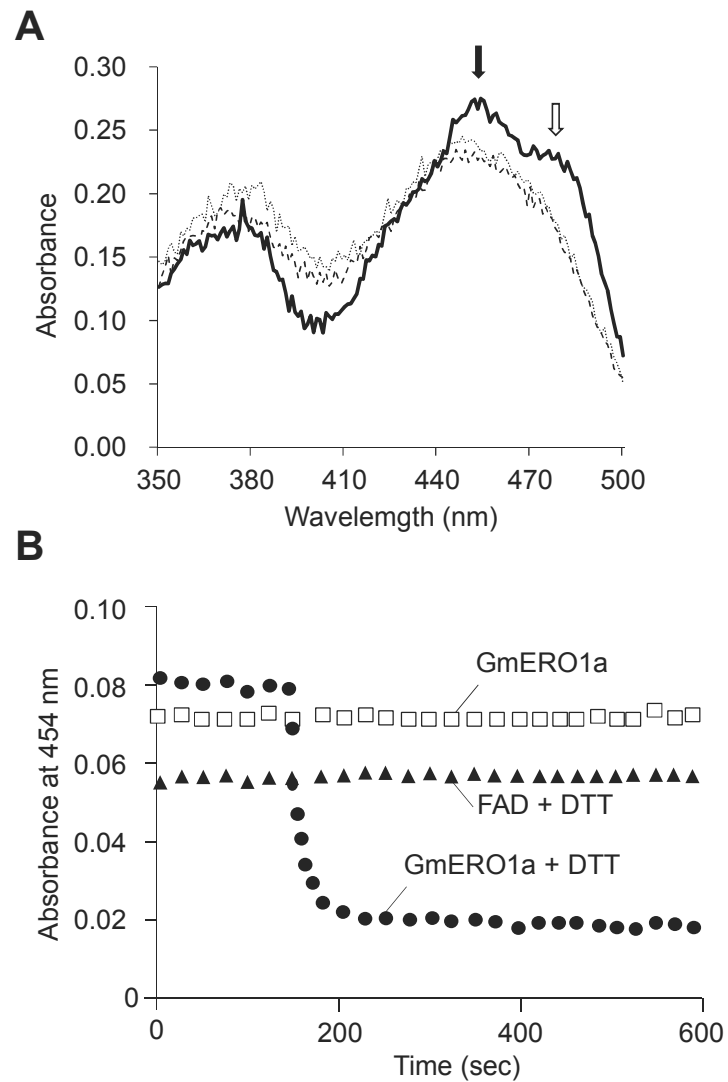


Figure 2-2. FAD bound to GmERO1a acts as a cofactor to transfer electrons from a substrate.

(A) Absorbance spectrum of recombinant GmERO1a was measured in the absence (black line) or presence (gray line) of 6 M guanidine hydrochloride. Free FAD spectrum (dashed line) was also measured. Black and white arrows show the maximum A_{454} and the shoulder at 485 nm of GmERO1a, respectively.

(B) GmERO1a-bound FAD is reduced by DTT. Absorbance changes at 454 nm of 10 μ M GmERO1a (circles) and 10 μ M free FAD (triangles) were monitored in the presence of 2 mM DTT. DTT was added at zero time. GmERO1a in the absence of DTT was also measured (rectangles).

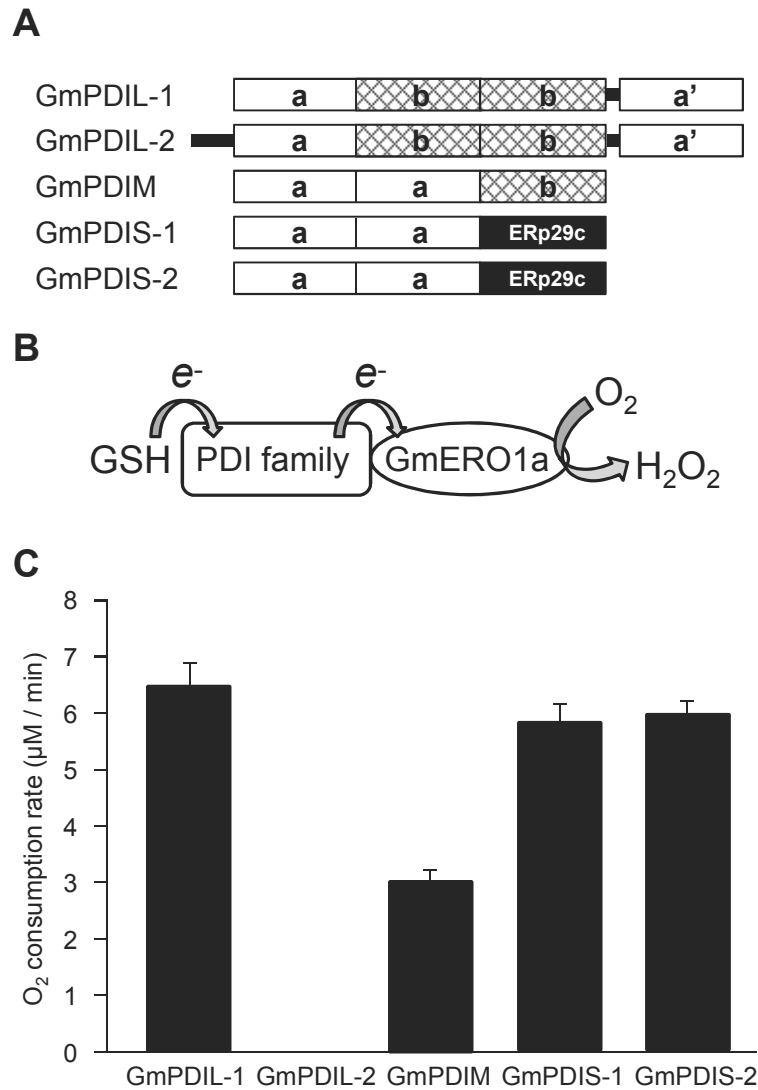


Figure 2-3. GmERO1a shows broad specificity for ER oxidoreductases.

(A) Schematic representation of soybean ER oxidoreductases.

(B) Schematic representation of the continuous oxidation of ER oxidoreductases by GmERO1a in the presence of GSH and the reduction of O₂. PDI family, ER oxidoreductases.

(C) Oxygen consumption rate of GmERO1a (1 μM) in the presence of 3 μM each ER oxidoreductase and 10 mM GSH at 25°C. Data are represented as mean ± SE of *n* = 3.

human, the lack in oxidation of other ER oxidoreductases may be compensated by other oxidation pathway. Peroxiredoxin-4 primarily oxidizes two ER oxidoreductases, ERp46 and P5, using H₂O₂ generated during the oxidation of PDI by human Ero1 α (Sato *et al.*, 2013). Conversely, in the plant ER, a peroxiredoxin-4 ortholog has not been found. In rice, a lack of OsERO1 causes the aggregation of glutelin because of deficient disulfide bond formation (Onda *et al.*, 2009), suggesting that disulfide bond formation depends on the Ero1 system for protein folding in the plant ER. Therefore, plant Ero1 may induce efficient oxidative folding in the ER by supplying disulfide bonds to multiple ER oxidoreductases.

Next, oxidative refolding by these ER oxidoreductases was determined in the presence of GmERO1a. ER oxidoreductases other than GmPDIL-2 were able to refold reduced and denatured RNase A (Figure 2-4A). Lag times prior to the initiation of refolding (Figure 2-4B–E, white bars) and refolding rates (black bars) differed between the ER oxidoreductases. The highest refolding rates were observed in the presence of GmPDIL-1. The rate of refolding by GmPDIL-1 increased with increasing concentrations of GmERO1a. The lag time of refolding by GmPDIL-1 did not change at different concentrations of GmERO1a. The rates of refolding by GmPDIM, GmPDIS-1, and GmPDIS-2 were slower than that by GmPDIL-1, and underwent very little change at different concentrations of GmERO1a. In contrast to the refolding rates, the lag times decreased with increasing concentrations of GmERO1a. These results suggest that dithiol oxidation is rate limiting in the reaction by GmPDIL-1, whereas disulfide isomerization was rate limiting in the reactions by GmPDIM, GmPDIS-1, and GmPDIS-2.

This observation was confirmed by analysis of intermediates during refolding. RNase A molecules during refolding were separated by nonreducing SDS-PAGE after modification of the free thiol groups with 4-acetamido-4'-maleimidylstilbene- 2,2'-disulfonic acid (AMS) (Figure 2-5A). GmPDIL-1 converted most of the reduced and denatured RNase A molecules (D_{red}) into an intermediate with non-native disulfide bonds (D_{ox}) within the first 5 min. During

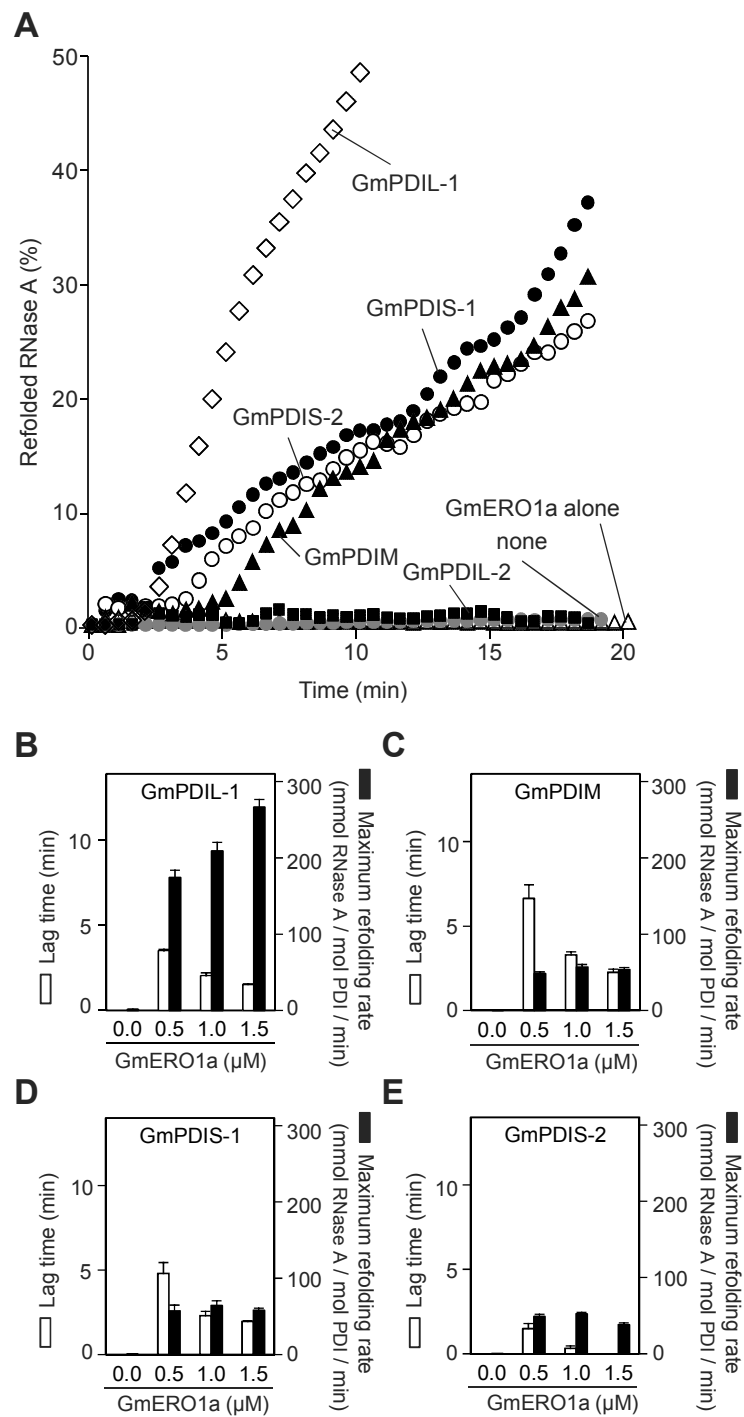


Figure 2-4. Reconstitution of oxidative protein folding with ER oxidoreductases and GmERO1a *in vitro*.

(A) GmERO1a (1 μ M) without (GmERO1a alone), or with 3 μ M of each ER oxidoreductase, was incubated with reduced and denatured RNase A (8 μ M), and the recovered RNase A activity was assayed. “None” shows refolding of RNase A alone.

(B–E) Lag time (white bars) and maximum refolding rate (black bars) of reduced and denatured RNase A (8 μ M) by 3 μ M each ER oxidoreductase in the presence of GmERO1a. Data are represented as mean $\pm$ SE of $n = 3$.

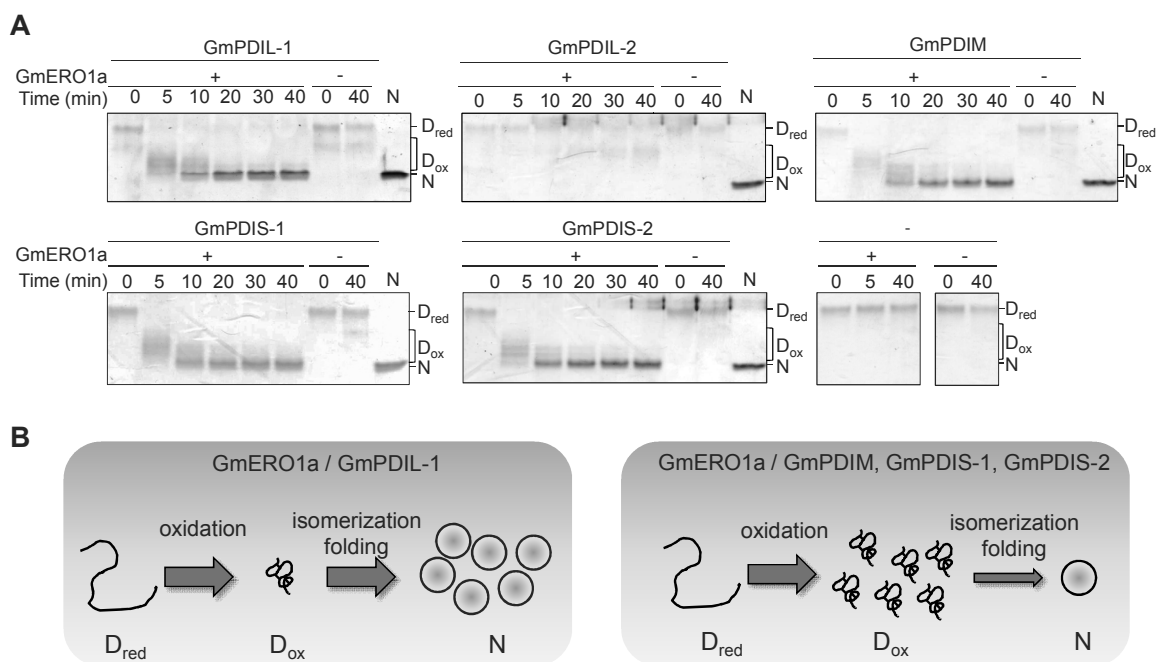


Figure 2-5. Oxidative refolding of reduced and denatured RNase A by GmPDIL-1 and GmPDIM, GmPDIS-1 or GmPDIS-2

(A) Refolding of reduced and denatured RNase A in the absence (–) or presence (+) of GmERO1a and each ER oxidoreductase, quenched with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, and was analyzed by nonreducing SDS-PAGE. D_{red} , reduced and denatured RNase A; D_{ox} , denatured RNase A with nonnative disulfides; N, native RNase A.

(B) Model of refolding of RNase A by ER oxidoreductases in the presence of GmERO1a. Left, GmPDIL-1 transfers nonnative disulfide bonds to D_{red} . D_{ox} is promptly folded into the native form accompanied by the isomerization of disulfide bonds. Right, GmPDIM, GmPDIS-1, or GmPDIS-2 transfers nonnative disulfide bonds to D_{red} . D_{ox} accumulates due to rate-limiting isomerization activities of GmPDIM, GmPDIS-1, and GmPDIS-2.

the next 15 min, "D_{ox}" was converted into a native form with four disulfide bonds (N) (Figure 2-5B, left). GmPDIM, GmPDIS-1, or GmPDIS-2 converted most of the "D_{red}" into "D_{ox}" within the first 5 min, and conversion of "D_{ox}" into "N" was slower than by GmPDIL-1 (Figure 2-5A and B, right). Neither "D_{ox}" nor "N" was generated by GmPDIL-2 (Figure 2-5A).

Oxidation Rates of ER Oxidoreductases by GmERO1 Depend on Various Factors

I predicted that difference of oxidation rates between ER oxidoreductases by GmERO1a depends on the affinity of ER oxidoreductases to GmERO1a and/or their abilities to activate GmERO1. In human Ero1 α , the thiol-disulfide statuses of all inhibitory disulfide bonds (Cys⁹⁴–Cys¹³¹ and Cys⁹⁹–Cys¹⁰⁴) are governed by PDI or other ER oxidoreductases (Shepherd *et al.*, 2014; Ramming *et al.*, 2015). In the case of soybean GmERO1a, Cys¹²¹ and Cys¹⁴⁶ were predicted to be regulatory cysteine residues (Sevier *et al.*, 2007) also exists in GmERO1a by cysteine mutation experiments (Data not shown). Therefore, the oxidation activity of GmERO1a, of which both Cys¹²¹ and Cys¹⁴⁶ residues were replaced with alanine (C121/146A), was assayed. GmERO1a(C121/146A) showed the oxidation activity higher than that of wild-type GmERO1a for GmPDIL-1, GmPDIM, GmPDIS-1 and GmPDIS-2 (Figure 2-6), indicating that the Cys¹²¹ and Cys¹⁴⁶ residues are certainly regulatory cysteine residues. On the other hands, GmPDIL-2 was hardly oxidized by GmERO1a(C121/146A). Although C121/146A lacks the regulatory cysteines and is always activated, the oxidation rates between ER oxidoreductases by C121/146A differed, meaning that factors other than the ability of each ER oxidoreductase to activate GmERO1a affect on the oxidation rates. One possibility is difference in affinity of GmERO1a to each ER oxidoreductase. The association of GmERO1a and ER oxidoreductases was examined by far-western blot analysis (Figure 2-7). GmERO1a tightly bound to GmPDIL-1, GmPDIS-1, and GmPDIS-2, which are good substrates of GmERO1a, while very weakly bound to GmPDIL-2 and GmPDIM. These

patterns of the association between GmERO1a and ER oxidoreductases were consistent with the oxidation rates.

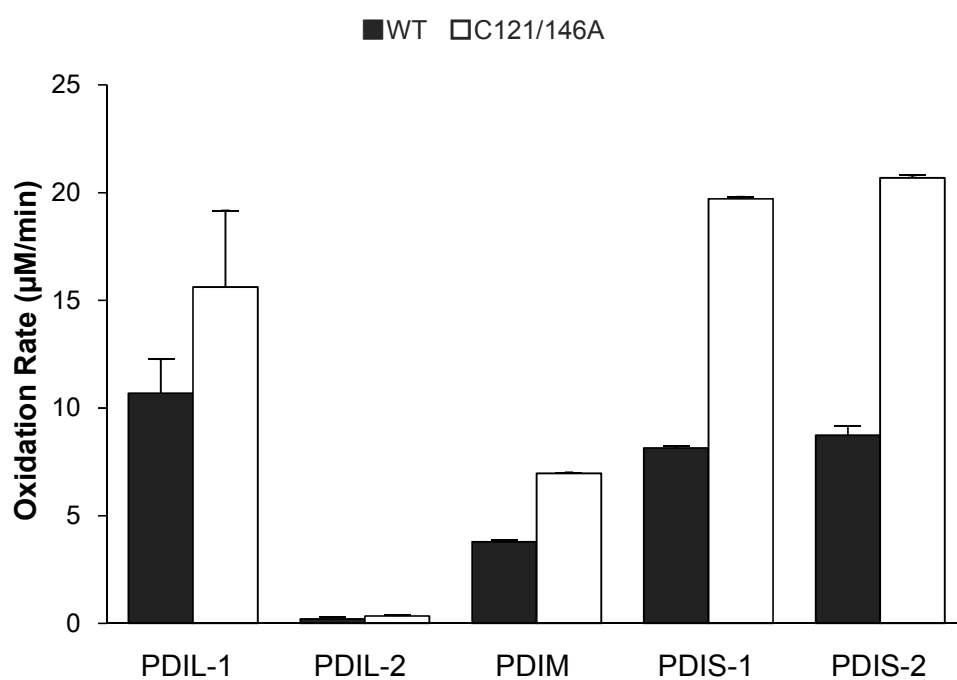


Figure 2-6. Oxidation rates of ER oxidoreductases by mutated GmERO1a.

Decrease in NADPH in the presence of 1 μ M wild-type GmERO1a (■) or its mutant (□), and 3 μ M indicated ER oxidoreductase (3 mM GSH, 120 μ M NADPH, and 1 U/mL GR). Data are represented as mean $\pm$ SE of $n = 3$.

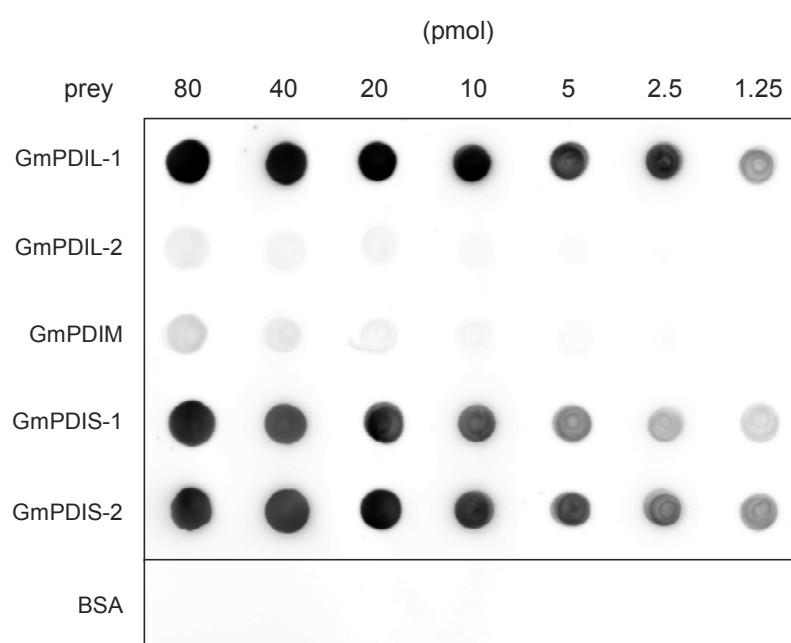


Figure 2-7. *In vitro* binding of GmERO1a to ER oxidoreductases.

The indicated amounts of GmPDIL-1, GmPDIL-2, GmPDIM, GmPDIS-1, and GmPDIS-2 (prey) were spotted onto a nitrocellulose membrane and incubated with 0.2 μ M GmERO1a for 3 h at 4 °C. Bound GmERO1a was immunostained. BSA, bovine serum albumin.

CHAPTER 3

Molecular Mechanisms of Cooperative Oxidative Folding by GmERO1a, GmPDIM, and GmPDIL-2

Introduction

Human Ero1 α and Ero1 β preferentially oxidize PDI. Therefore, another pathway that oxidizes other ER oxidoreductases likely exists in human. Peroxiredoxin-4 primarily oxidizes two ER oxidoreductases, ERp46 and P5, using H₂O₂ generated during the oxidation of PDI by human Ero1 α (Sato *et al.*, 2013). Conversely, in the plant ER, a peroxiredoxin-4 ortholog has not been found. In rice, a lack of OsERO1 causes the aggregation of glutelin because of deficient disulfide bond formation (Onda *et al.*, 2009), suggesting that disulfide bond formation depends on the Ero1 system for protein folding in the plant ER. In Chapter 2, I described that GmERO1a oxidizes, not only the conserved typical ER oxidoreductase GmPDIL-1, but also GmPDIM, GmPDIS-1, and GmPDIS-2. GmPDIL-1, GmPDIL-2, GmPDIM, GmPDIS-1, and GmPDIS-2 were found to associate transiently with a seed storage precursor protein, proglycinin and β -conglycinin, in the ER of the cotyledon by coimmunoprecipitation experiments, suggesting that multiple ER oxidoreductases are involved in the folding of the nascent proglycinin and β -conglycinin (Wadahama *et al.*, 2007, 2008; Kamauchi *et al.*, 2008). Therefore, I tried to find a cooperative system of multiple ER oxidoreductases and GmERO1a.

In this chapter, the findings about the synergistic mechanism by which GmPDIM and GmPDIL-2 cooperatively fold unfolded proteins using oxidizing equivalents provided by GmERO1a *in vitro* and the electron relay between GmPDIM and GmPDIL-2, are described.

Materials and Methods

Immunoprecipitation Experiments

Cotyledons (each 100 mg) were frozen under liquid nitrogen and homogenized with a Dounce homogenizer at 4°C in 20 mM HEPES buffer (pH 7.2) containing 150 mM NaCl, 1% digitonin, and 1% cocktail of protease inhibitors (Sigma-Aldrich). The homogenate was placed on ice for 1 h and centrifuged for 30 min at 10,000 ×g at 4°C. Immunoprecipitation was performed at 4°C for 1 h with preimmune serum or anti-GmPDIL-1, anti-GmPDIL-2, anti-GmPDIM, anti-GmPDIS-1, or anti-GmPDIS-2 serum. The immunoprecipitate was collected with protein A-conjugated Sepharose beads (Sigma-Aldrich), washed with 20 mM HEPES buffer (pH 7.2) containing 150 mM NaCl, and subjected to western blot analysis using a specific antiserum against ER oxidoreductases as primary antibodies and peroxidase-conjugated ImmunoPure Recombinant Protein A (Pierce Biotechnology).

Preparation of Recombinant GmERO1a

An expression plasmid encoding GST-fused GmERO1a E⁷⁰-G⁴²² was constructed as follows. The DNA fragment was amplified from *GmERO1a* cDNA by PCR using primers 5'-GCGATGGATCCTATGAACTGTGGATCGTCTTAATG-3' and 5'-GCGATGTCGACTATCCTCCTTCCATGATTCTTTCAGC-3'. The amplified DNA fragment was subcloned into the pGEX6p-2 vector (GE Healthcare). The recombinant protein has glutathione-S-transferase (GST) linked to the amino terminus. *E.coli* Rosseta-gami cells (Takara Bio, Inc.) were transformed with the GST-fusion vector described above. Expression of recombinant GST-GmERO1a was induced in LB containing 0.5 mM isopropyl thiogalactoside, 20 µM FAD, 100 µg/mL ampicillin, 15 µg/mL kanamycin, and 12.5 µg/mL tetracycline at 15°C for 120 h. The cells were collected by centrifugation and disrupted by sonication in phosphate-buffered saline. Recombinant GST-GmERO1a was adsorbed to a

glutathione Sepharose 4B column and digested by PreScission protease (GE Healthcare). The eluted recombinant GmERO1a was purified by gel filtration chromatography on a TSK gel G3000SW column (Tosoh Co., Ltd.) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 10% glycerol as described previously (Wadahama *et al.*, 2007). The concentration of purified recombinant GmERO1a was determined by amino acid analysis with norleucine as an internal standard.

Construction of Expression Plasmids of Wild-type and Mutant GmPDIL-2 and Wild-type and Mutant GmPDIM

Expression plasmids encoding wild-type and mutant GST-GmPDIL-2 and GST-GmPDIM were constructed. The DNA fragments were amplified from cDNAs of wild-type GmPDIL-2 and GmPDIM (Kamauchi *et al.*, 2008; Wadahama *et al.*, 2008) by PCR using the oligonucleotide primers listed in Table 3-1 and Table 3-2. The DNA fragments were subcloned into the pGEX-6P-2 vector. The DNA fragments for mutant GmPDIL-2 and GmPDIM were amplified from wild-type GST-GmPDIL-2 and GmPDIM by PCR using the oligonucleotide primers listed in Table 3-1 and Table 3-2, and were self-ligated using a ligation kit (Takara Bio, Inc.).

Expression and Purification of Recombinant Proteins

Wild-type and mutant GST-GmPDIL-2 and GST-GmPDIM were expressed in *E. coli* BL21 (DE3) cells (Merck KGaA) in the presence of 0.5 mM IPTG at 15°C for 72 h. Recombinant proteins were separated on a glutathione Sepharose 4B column equilibrated with phosphate-buffered saline, cleaved from GST with PreScission protease at 4°C for 16 h, and eluted from the column with cleavage buffer according to the manufacturer's protocol. The eluted recombinant proteins were purified by gel filtration chromatography on a TSK gel G3000SW column equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM

Table 3-1. List of primers and template plasmids for PCR of GmPDIL-2 variant preparation.

GmPDIL-2			
Variant	Forward primer	Reverse primer	Template plasmids
WT(full)	CGTAGAATTCTCACCAGCAGCAGGACC	TCCTCATAAATTCATCCTTCACATC	GmPDIL-2(full)/pT7Blue
C101/104A	GGCTGGCCACGCTCAGGCCCTCGCC CCCGAATACGCCG	TGAGCGTGGCCAGCCCAGGGCGCGT AGAACTCGACCAT	GmPDIL-2(full)/pGEX6p-2
C440/443A	GGCTGGCCACGCTCAAGCACTGGAG CCAATATACGACAAGC	TGAGCATGGCCAGCCCAGGGAGCAT AAATCTCGAGGAG	GmPDIL-2(full)/pGEX6p-2
C104A	GTGCGGCCACGCTCAGGCCCTCGCC CCCGAATACGCCG	TGAGCGTGGCCGACCAGGGCGCGT AGAACTCGACCAT	GmPDIL-2(full)/pGEX6p-2
C443A	GTGCGGCCACGCTCAAGCACTGGAG CCAATATACGACAAGC	TGAGCATGGCCACACCAGGGAGCAT AAATCTCGAGGAG	GmPDIL-2(full)/pGEX6p-2
C104/443A	GTGCGGCCACGCTCAAGCACTGGAG CCAATATACGACAAGC	TGAGCATGGCCACACCAGGGAGCAT AAATCTCGAGGAG	GmPDIL-2(C104A)/pGEX6p-2
C101/104/ 440/443A	GGCTGGCCACGCTCAAGCACTGGAG CCAATATACGACAAGC	TGAGCATGGCCAGCCCAGGGAGCAT AAATCTCGAGGAG	GmPDIL-2(C101/104A)/pGEX6p-2
a	TGAGGGTCGACTCGAGC	GCTACTTTTCTTAATCCACGTCAT TATAGC	GmPDIL-2(full)/pGEX6p-2
a-b	TGAGGGTCGACTCGAGC	GCTACTTGATTGGATTTTCGAAGAC TG	GmPDIL-2(full)/pGEX6p-2
a-b-b'	TGAGGGTCGACTCGAGC	GCTACACATCACCATCATTACTTTC AG	GmPDIL-2(full)/pGEX6p-2
a'	ATCGGACCTGGTATATACAACCTTGA C	CAGGGGCCCCCTGGAACAGAA	GmPDIL-2(full)/pGEX6p-2
b'-a'	ATCAAGAAACAGTTGTTGCTGTTTG C	CAGGGGCCCCCTGGAACAGAA	GmPDIL-2(full)/pGEX6p-2
b-b'-a'	GAAGACAAGCTAAAACCTTTTTTACA AGTC	CAGGGGCCCCCTGGAACAGAA	GmPDIL-2(full)/pGEX6p-2
b-b'	GGAATTCAAAAGAAAAAGATCGGAC CTGGT	TGTTTGGGCGGCCGCTTAATCACCA TCATTACTTTCAGGAAC	GmPDIL-2(full)/pT7Blue

Table 3-2. List of primers and template plasmids for PCR of GmPDIM variant preparation.

GmPDIM			
Variant	Forward primer	Reverse primer	Template plasmids
WT(full)	GTCAGGATCCCTCTATGGAGCATCC	TCATAGCTCATCCTTGCTTG	GmPDIM(full)/pT7Blue
C64/67A	GCTCTGACTCCTATATGGGAGAAG	CTGAGCGTGTCCAGCCCATGGAGCA AAGAATTCAACAAGAACAATC	GmPDIM(full)/pGEX6p-2
C192/195A	CTCCTGAGTGGAAGAAAGCATCC	CTAATTTCTTAGCATGTCCAGCCCA AGGTGCGAAAAATCCACAATCCAG AGTTC	GmPDIM(full)/pGEX6p-2
C67A	GCTCTGACTCCTATATGGGAGAAG	CTGAGCGTGTCCACACCATGGAGCA AAGAATTCAACAAGAACAATC	GmPDIM(full)/pGEX6p-2
C195A	CTCCTGAGTGGAAGAAAGCATCC	CTAATTTCTTAGCATGTCCACACCA AGGTGCGAAAAATCCACAATCCAG AGTTC	GmPDIM(full)/pGEX6p-2
a	TGAGGGTCGACTCGAGC	TTTTCCACTTAACCGATCC	GmPDIM(full)/pGEX6p-2
a-a'	TGAGGGTCGACTCGAGC	GCTATTGAGCTGCTCTAATGCAA	GmPDIM(full)/pGEX6p-2
a'	TGAGGGTCGACTCGAGC	TTCCAGCTGCTCTAATGCAA	GmPDIM(a'b)/pGEX6p-2
a'-b	GCAACAGGAGGATCTAGTG	CAGGGGCCCCCTGGAACAGAA	GmPDIM(full)/pGEX6p-2
b	ACAAACGTTGCTCCTCCAGAAG	CAGGGGCCCCCTGGAACAGAA	GmPDIM(full)/pGEX6p-2

NaCl and 10% glycerol. The concentrations of the purified recombinant proteins were determined by absorbance at 280 nm using the molar extinction coefficient calculated according to the modified method of Gill and von Hippel (Pace *et al.*, 1995). Recombinant proteins, including wild-type GmPDIL-1, GmPDIS-1, and GmPDIS-2, were prepared and purified as described previously (Wadahama *et al.*, 2007, 2008; Kamauchi *et al.*, 2008). LB containing 50 µg/mL ampicillin was incubated at 37°C until IPTG was added to the medium at a final concentration of 0.5 mM at an A_{600} of 0.5. After an additional 4 h of shaking at 30°C, cells were collected. The cell lysate supernatant was applied to His-Bind resin (EMD Biosciences, Inc.) or the glutathione Sepharose 4B column. Fractions eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 1 M imidazole from His-Bind resin or fractions obtained from the glutathione Sepharose 4B column after PreScission protease digestion were further purified by gel filtration chromatography on a TSK gel G3000SW column. Each construct was quantified using the molar extinction coefficient at 280 nm.

Far-UV CD Analysis

CD spectra of recombinant proteins in 20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 10% glycerol were obtained using a J-720 spectropolarimeter (JASCO Corp.) in a 1-mm path-length cell with a scan speed of 20 nm/min at 14°C.

RNase A Refolding Assays

Thiol oxidative refolding activity was assayed as previously described by measuring RNase activity following regeneration of the active form of the enzyme from its reduced and denatured form in the presence of the recombinant proteins (Creighton, 1977; Lyles and Gilbert, 1991). Reduced and denatured RNase A was prepared as described previously. Each reaction mixture contained 100 mM HEPES buffer (pH 7.5), 150 mM NaCl, 2 mM CaCl_2 , 0.5 mM glutathione disulfide, 2 mM glutathione, reduced RNase A, and recombinant

oxidoreductases and their variants, and were incubated at 25°C. The formation of active RNase A was measured spectrophotometrically by monitoring the hydrolysis of the RNase A substrate cCMP at 284 nm. The isomerase activities of oxidoreductases were calculated as described previously (Kulp *et al.*, 2006). Briefly, isomerase activity was determined from the linear increase in the amount of enzymatically active RNase A with time after a lag. Oxidation of free thiol residues to disulfides on reduced RNase A were also determined by measuring the amounts of free thiol groups in the reaction mixtures (Ellman, 1959).

Dot Far-western Blot Analysis

Purified recombinant ER oxidoreductases and their variants as prey proteins were spotted onto a nitrocellulose membrane (GenScript) in a volume of 4 µL. The membrane was dried, rinsed twice, and blocked with 20 mM Tris-HCl (pH 8) containing 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk (blocking solution) at 4°C for 16 h. After blocking, the membrane was incubated in 0.2 µM bait protein in blocking solution without nonfat dry milk (TBST) for 3 h at 4°C. The membrane was incubated with anti-bait antiserum and further with horseradish peroxidase-conjugated IgG antiserum (Promega Corporation) as the secondary antibody diluted with blocking solution. Blots were washed four times for 20 min with TBST and developed using the Western Lightning Chemiluminescence Reagent (Perkin Elmer Life Sciences).

Dithiol Oxidation Assays

Dithiol oxidation activities of GmPDIL-2, GmPDIM, and their variants were assayed using a synthetic peptide, NH₂-NRCSQGSCWN-COOH (Operon Biotechnology Ltd.), as described previously (Ruddock *et al.*, 1996; Alanen *et al.*, 2006). Briefly, the recombinant proteins (0.56 µM) were incubated in McIlvaine buffer (0.2 M disodium phosphate/0.1 M citrate buffer, pH 6.0), 2 mM reduced glutathione, 0.5 mM oxidized glutathione and 5 µM

synthetic peptide at 25°C, and fluorescence was monitored on a FP-750 fluorescence spectrophotometer (JASCO) at 350 nm, with excitation at 280 nm. The half-time was calculated by the following procedure. The mid-point fluorescence was calculated as the mean of the initial and final fluorescence intensities. The half-time was calculated from the midpoint fluorescence. A measure of the rate of the reaction was determined from the inverse of the half-time of the reaction.

Disulfide Reduction Assays

Disulfide reduction activities of GmPDIL-2, GmPDIM, and their variants were assessed by measuring the glutathione-dependent reduction of insulin according to the method as described previously (Morjana and Gilbert, 1991). Briefly, recombinant proteins were incubated at 25°C in 1 mL of 0.2 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 3.7 mM glutathione, 120 μ M NADPH, 1 U of glutathione reductase (Sigma-Aldrich Inc.) and 30 μ M bovine insulin (Sigma-Aldrich), and absorbance was monitored at 340 nm.

ER Oxidoreductase Oxidation Assays

Oxygen consumption was measured using a Clark-type oxygen electrode system (OXYT-1) (Hansatech Instruments Ltd.). All experiments were performed at 25°C in 100 mM [4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (pH 7.5), 2 mM CaCl_2 , and 150 mM NaCl. Catalytic oxygen consumption was initiated by the addition of GmERO1a in a reaction mixture containing recombinant ER oxidoreductases or its variants, and 10 mM GSH.

The activity was measured using a coupled assay following the decrease in absorbance at 340 nm due to the consumption of NADPH by glutathione reductase (Sigma-Aldrich, Inc.) with the reaction being started by the addition of GmERO1a to initiate the reaction (Nguyen *et al.*, 2011; Sato *et al.*, 2013). A molar extinction coefficient of 6200 $\text{M}^{-1}\text{cm}^{-1}$ for NADPH was used for calculations. All experiments were performed in 100 mM

HEPES (pH 7.5) containing 150 mM NaCl and 2 mM CaCl₂.

Gel-based RNase A Refolding Experiments

RNase A oxidation analyses were performed by the addition of 1 μ M GmERO1 in 100 mM HEPES buffer (pH7.5), 150 mM NaCl, 2 mM CaCl₂, and 3 μ M recombinant ER oxidoreductases and their variants containing 8 μ M denatured and reduced RNase A. At the indicated time points, free thiols were blocked by the addition of Laemmli's 4 $\times$ SDS-loading buffer (Laemmli, 1970) containing 8 mM 4-acetamido-4-maleimidylstilbene-2,2 disulfonic acid and separated on a 15% polyacrylamide gel by SDS-PAGE (Laemmli, 1970) without reducing reagent. Proteins were detected by Coomassie Brilliant Blue staining after electrophoresis.

Assay of Mixed Disulfide Complexes

A 4- μ L aliquot of purified GmPDIL-2 or GmPDIL-2 variants, and GmPDIM or GmPDIM variants, were incubated at 25°C for 60 min and quenched with N-ethyl maleimide. Proteins were subjected to 7.5% SDS-PAGE under nonreducing conditions. Lanes cut from the first SDS-polyacrylamide gel were incubated in SDS-sample buffer with 5% 2-mercaptoethanol for 120 min at 37°C. The gel slices were then subjected to 12.5% SDS-PAGE under reducing conditions. The separated proteins were stained with a silver stain II kit (Wako Pure Chemical Industries Ltd.).

Measurement of Redox Equilibrium Constants of GmPDIM and GmPDIL-2

GmPDIM, GmPDIL-2, and their variants (1 μ M) were incubated with 0.1 mM GSSG and 0.015–28 mM GSH at 25°C for 1 h in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 150 mM NaCl. After incubation under N₂ at 25°C for 1 h, further thiol-disulfide exchange was prevented by the addition of 10% TCA. The aggregated proteins

were precipitated by centrifugation and washed with 100% acetone. The protein pellet was solubilized and incubated in 0.1 M sodium phosphate buffer, pH 7.0, containing 2% SDS and 3 mM methoxypolyethylene glycol-maleimide (mPEG5000-mal) (Fluka Sigma-Aldrich) at 25°C for 30 min. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Values for the reduced fraction, oxidized fraction, and intermediate fraction (in the case of wild-type GmPDIM and GmPDIL-2, which have two active sites) were quantified using ImageJ software (National Institutes of Health). The values for the completely oxidized or reduced states were regarded as 0 or 100, respectively, and all intermediate states were recalibrated. In the case of wild-type GmPDIM and GmPDIL-2, the intensity of the band in which one of the two active sites was oxidized was calibrated as one-half of the reduced form. The sum of the completely reduced form and one-half of the half-reduced form was plotted. The K_{eq} was calculated by fitting the recalibrated fraction of the apparent reduced form to the following equation: $R = ([GSH]^2/[GSSG])/ \{K_{eq} + ([GSH]^2/[GSSG])\}$, in which R is the relative ratio of the reduced forms. The equilibrium redox potential of proteins was calculated using the Nernst equation $[E'_0 = E'_{0(GSH/GSSG)} - (RT/nF) * \ln K_{eq}]$ using the glutathione standard potential $E'_{0(GSH/GSSG)}$ of -0.240 V at pH 7.0 and 25°C.

Results and Discussion

GmPDIL-2 and GmPDIM Cooperatively Refold Denatured RNase A

It was previously found that GmPDIS-1, GmPDIM, GmPDIL-1, and GmPDIL-2 associate with nascent proglycinin in the ER, suggesting that these ER oxidoreductases cooperatively fold glycinin *in vivo* (Wadahama *et al.*, 2007, 2008; Kamauchi *et al.*, 2008). Associations between these ER oxidoreductases in the ER were detected by coimmunoprecipitation. GmPDIM and GmPDIS-1, GmPDIM and GmPDIS-2, GmPDIS-1 and GmPDIS-2, and GmPDIM and GmPDIL-2 were coimmunoprecipitated (Figure 3-1). Therefore, I examined the refolding of RNase A when these ER oxidoreductases were present simultaneously. No combination of GmPDIM, GmPDIS-1, and GmPDIS-2 showed an additive effect on the refolding rate (Figure 3-2). In contrast, the refolding rate of denatured RNase A when GmPDIM and GmPDIL-2 were present simultaneously was almost five times greater than that for GmPDIM alone, although GmPDIL-2 alone had no refolding activity (Figure 3-3A). The refolding rate drastically increased until a 1:1 molar ratio of GmPDIL-2 to GmPDIM was obtained (Figure 3-3B), suggesting that formation of the complex is important for the cooperation, and that these ER oxidoreductases play different roles, respectively. The difference in oxidative refolding activity between GmPDIL-2 and GmPDIM is thought to arise from differences in the catalytic activities of **a** and **a'** domains and/or differences in their domain structures.

RNase A Refolding, Dithiol Oxidation and Disulfide Reduction Activities of GmPDIL-2 and GmPDIM Mutants and Domain Fragments

When GmPDIM and GmPDIL-2 were present simultaneously, the refolding rate of denatured RNase A was stimulated synergistically. To analyze molecular mechanisms of this synergistic effect, variants of GmPDIL-2 and GmPDIM, are mutated active sites or lack some

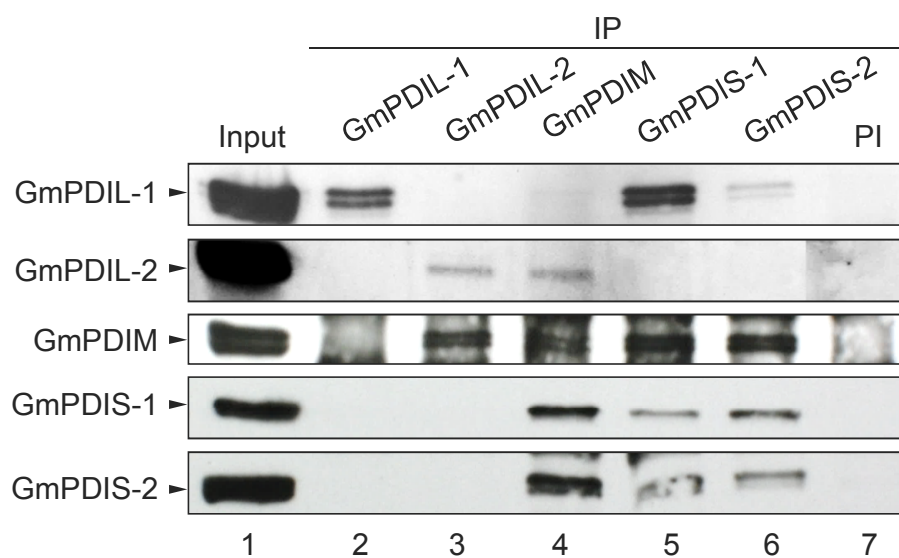


Figure 3-1. Detection of ER oxidoreductase complexes.

IP from the cotyledon extract was carried out with each anti-ER oxidoreductase serum or PI. Immunoprecipitates and the cotyledon extract (lane 1) were analyzed by western blot with each anti-ER oxidoreductase serum. IP, immunoprecipitation; PI, preimmune serum.

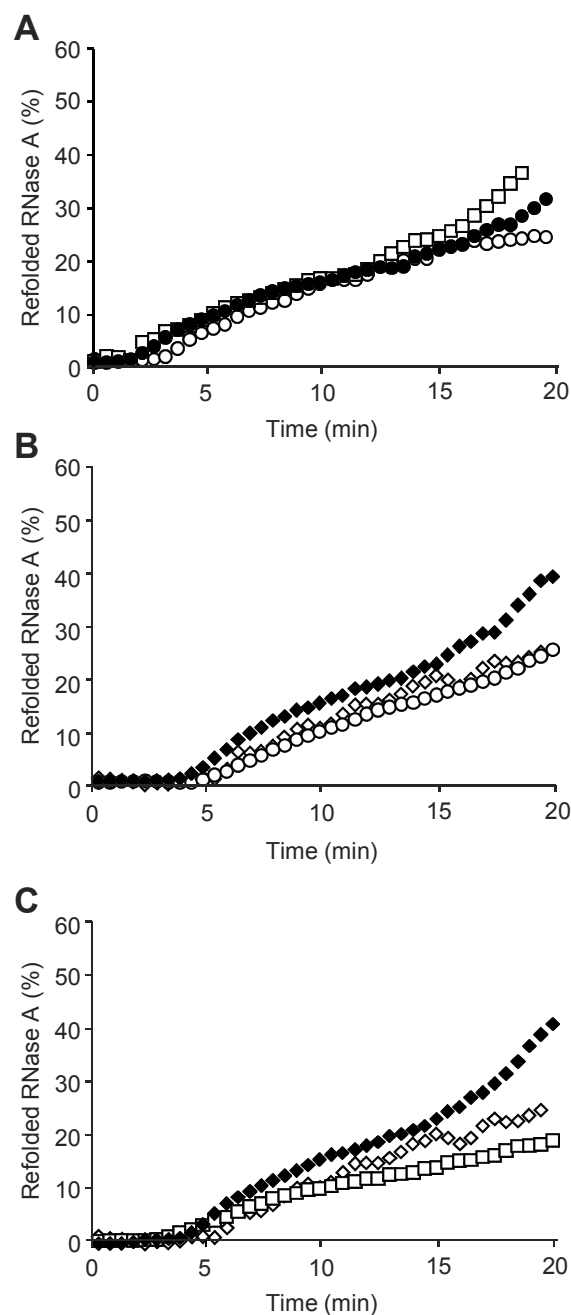


Figure 3-2. Refolding of RNase A by multiple soybean ER oxidoreductases.

(A) Reduced and denatured RNase A (8 μM) was incubated with 3 μM GmPDIS-1 (white circles), 3 μM GmPDIS-2 (white squares), or 3 μM GmPDIS-1 and 3 μM GmPDIS-2 (black circles) in the presence of 1 μM GmERO1a, and recovered RNase A activity was assayed.

(B) Reduced and denatured RNase A (8 μM) was incubated with 3 μM GmPDIS-1 (circles), 3 μM GmPDIM (white diamonds), or 3 μM GmPDIS-1 and 3 μM GmPDIM (black diamonds) in the presence of 1 μM GmERO1a.

(C) Reduced and denatured RNase A (8 μM) was incubated with 3 μM GmPDIS-2 (white squares), 3 μM GmPDIM (white diamonds), or 3 μM GmPDIS-2 and 3 μM GmPDIM (black diamonds) in the presence of 1 μM GmERO1a.

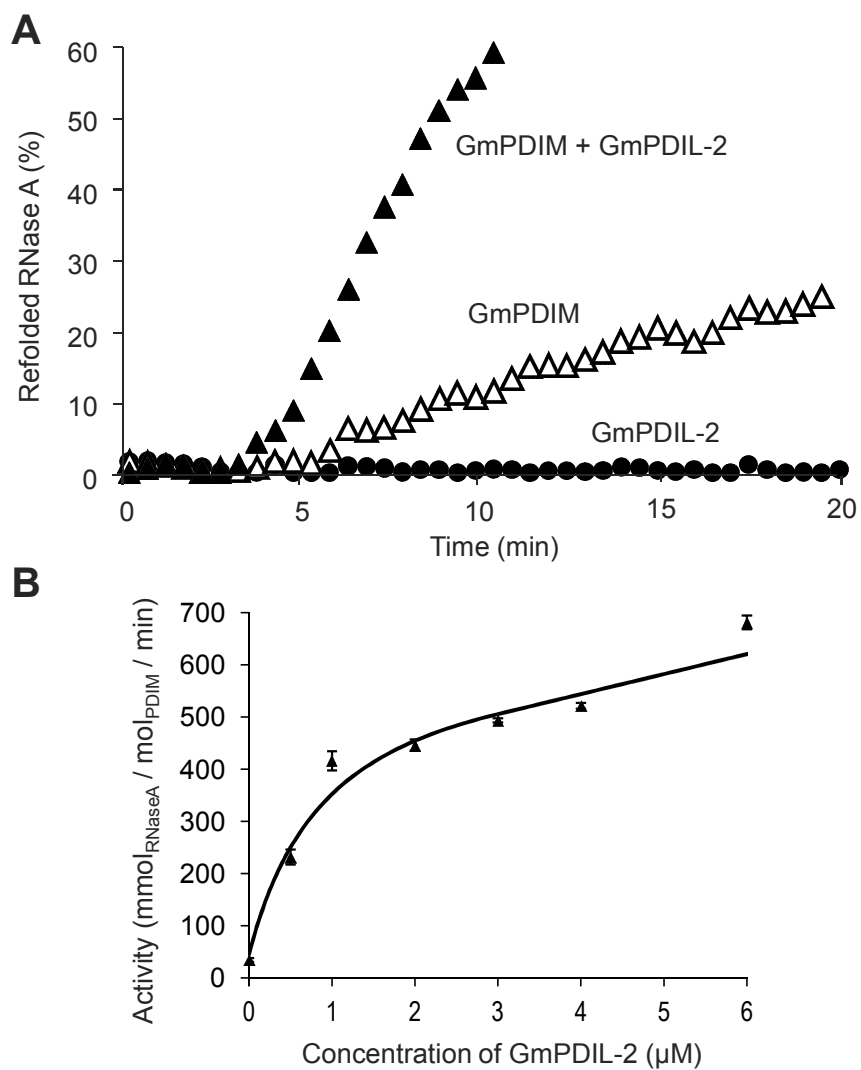


Figure 3-3. Effects of the addition of GmPDIL-2 to GmPDIM on the refolding of RNase A.

(A) Reduced and denatured RNase A (8 μM) was incubated with 3 μM ER oxidoreductases in the presence of 1 μM GmERO1a.

(B) GmPDIL-2 concentration dependence of the refolding activity in the presence of 1 μM GmERO1a and 1 μM GmPDIM. Data are represented as mean ± SE of $n = 3$.

of domains, were prepared and characterized (Figure 3-4A, B and Figure 3-5A, B). Active-site mutants and domain fragments of GmPDIL-2 and GmPDIM were generated and purified (Figure 3-4C and Figure 3-5C). All recombinant proteins were expressed in the soluble form. Wild-type GmPDIL-2 and its mutants and domain fragments eluted as monomers from a gel filtration column (data not shown). Wild-type GmPDIM eluted as a dimer from a gel filtration column, whereas its domain fragments eluted as monomers, suggesting that the whole domain structure is necessary for dimerization of GmPDIM (data not shown). Mutation of the active-site cysteines to alanines had no effect on dimer formation. To determine whether the recombinant proteins were folded, far-UV CD spectra were obtained. All recombinant proteins yielded CD spectra typical of a folded protein (Figure 3-4D–F and Figure 3-5D, E).

The RNase A-oxidative refolding activity of the active-site cysteine mutants of GmPDIL-2 was measured, in the presence of glutathione redox buffer (Figure 3-6, black bars). The active-site mutant C101/104/440/443A, in which both cysteine residues at the two active sites were replaced with alanines, demonstrated no oxidative refolding activity. Removal of the active site in either the **a'** (C440/443A) or **a** (C101/104A) domains reduced the activity to 47% or 68%, respectively, indicating that the two active sites function independently in oxidative refolding and that the **a'** domain active site is more active than the **a** domain active site. Whereas the **a'** domain active site of yeast PDI is also more active than the **a** domain active site, the reverse is the case with human PDI (Lyles and Gilber, 1994; Westphal *et al.*, 1999).

Dithiol oxidation activity was assayed with a short synthetic peptide containing two cysteine residues (Figure 3-6, hatch-pattern bars). Although removal of the active site in the **a'** domain reduced the activity to 41%, removal of the active site in the **a** domain resulted in no decrease in dithiol oxidation activity, suggesting that disulfide bonds are predominantly introduced into the substrate by the **a'** domain. The disulfide reduction activities determined

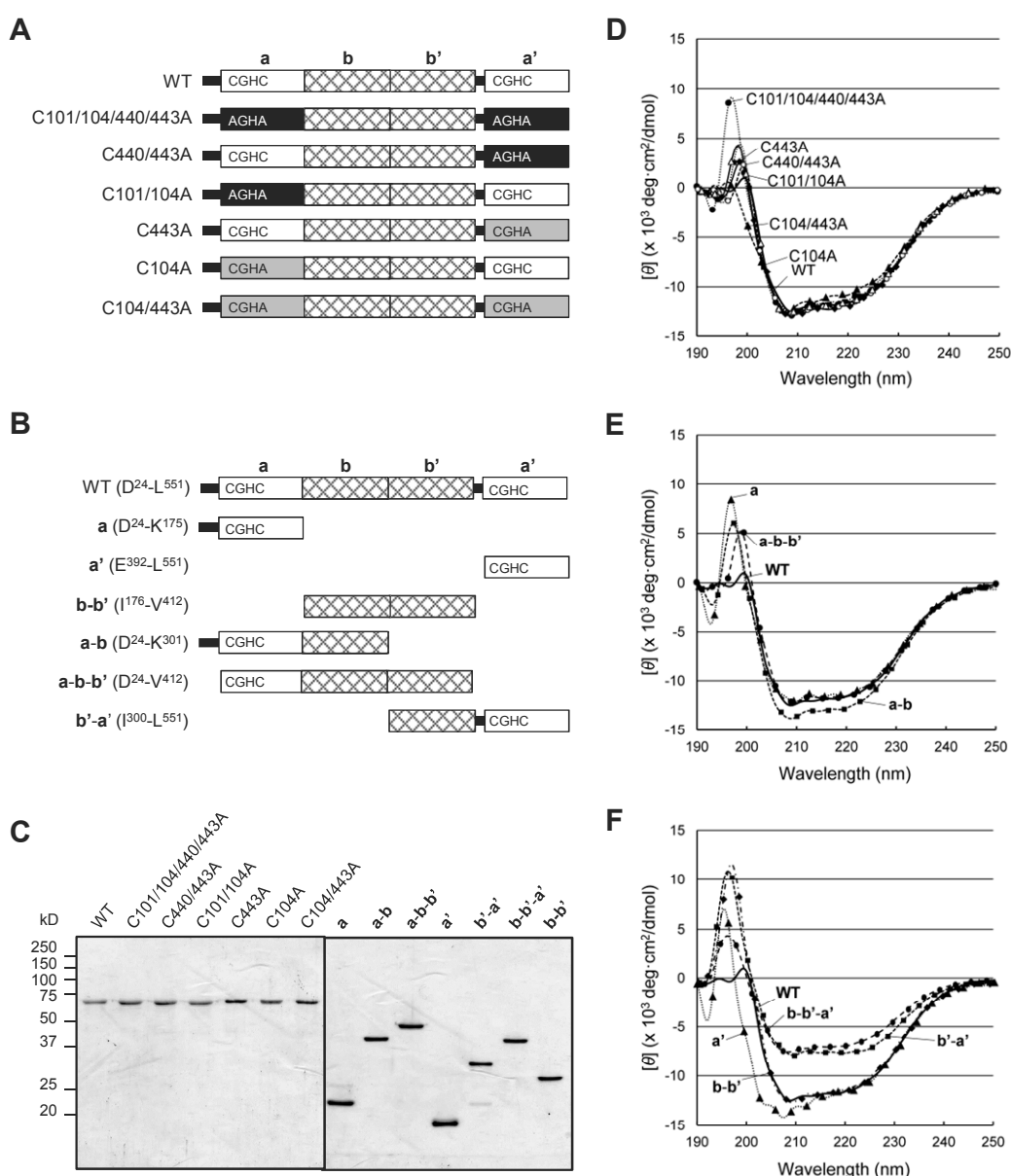


Figure 3-4. Expression and purification of wild-type GmPDIL-2 and its variants.

(A) Schematic representation of wild-type GmPDIL-2 (WT) and its active-site mutants. Boxes represent domains. “CGHC”, “AGHA” and “CGHA” represent the amino acid sequences of the active sites in the a and a’ domains. Numbers in the name of mutants represent Cys residues mutated to Ala.

(B) Schematic representation of wild-type GmPDIL-2 (WT) and GmPDIL-2 domain fragments.

(C) SDS-PAGE of purified wild-type GmPDIL-2 (WT) and its variants.

(D) CD spectra of purified recombinant GmPDIL-2 and each of its active-site mutants. Wild type, bold straight line with no symbol; C440/443A, open circle; C101/104A, open triangle; C101/104/440/443A, filled circle; C443A, diamond; C104A, filled square; and C104/443A, filled triangle.

(E) CD spectra of purified recombinant GmPDIL-2 and each of its domain fragments. Wild type, bold straight line with no symbol; a, filled triangle; a-b, filled square; a-b-b’, filled circle.

(F) CD spectra of purified recombinant GmPDIL-2 and each of its domain fragments. Wild type, bold straight line with no symbol; a’, filled triangle; b’-a’, filled square; b-b’-a’, filled circle; and b-b’, diamond.

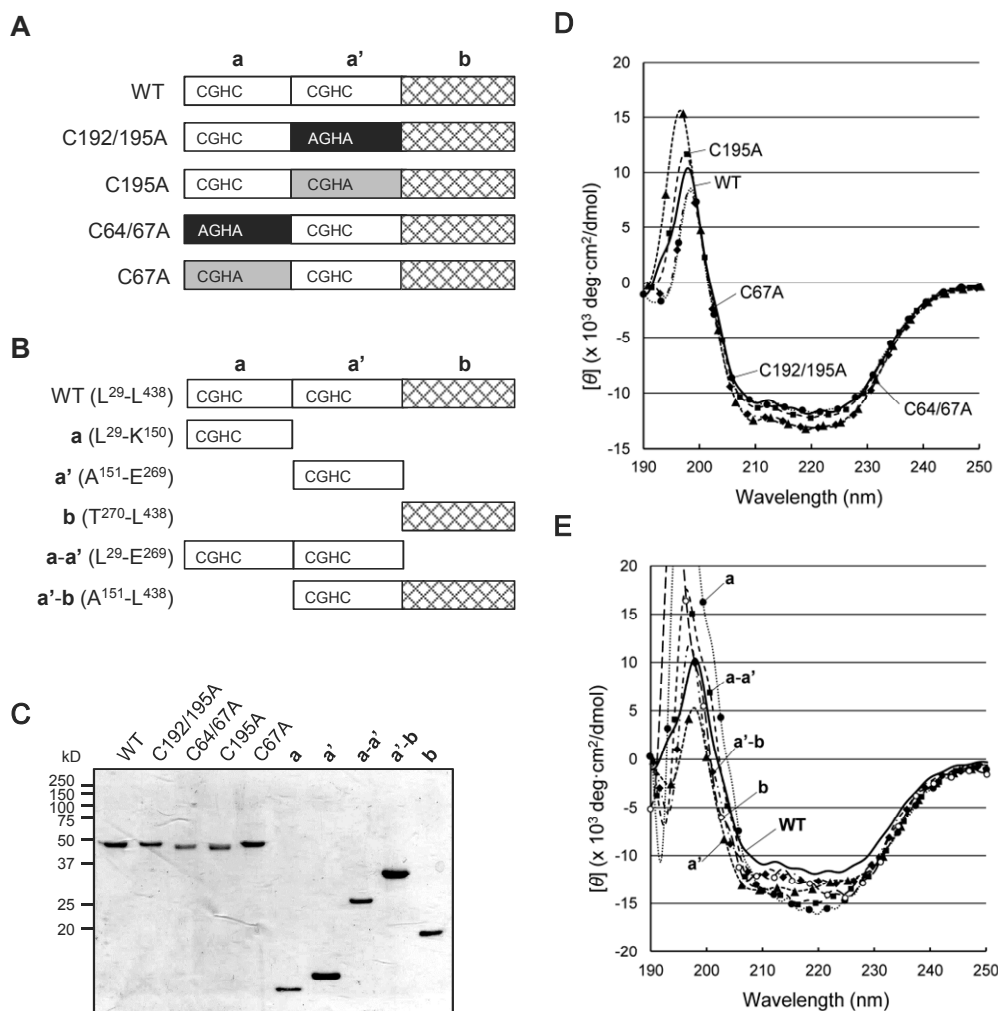


Figure 3-5. Expression and purification of wild-type GmPDIM and its variants.

(A) Schematic representation of wild-type GmPDIM (WT) and GmPDIM domain fragments. Boxes represent domains. “CGHC”, “AGHA” and “CGHA” represent the amino acid sequences of the active sites in the a and a' domains.

(B) Schematic representation of wild-type GmPDIM (WT) and its mutants. Numbers in the name of mutants represent Cys residues mutated to Ala.

(C) SDS-PAGE of purified WT GmPDIM and its variants.

(D) CD spectra of purified recombinant GmPDIM and each of its active-site mutants. Wild type, bold straight line with no symbol; C192/195A, filled circle; C64/67A, filled triangle; C195A, filled square; and C67A, diamond.

(E) CD spectra of purified recombinant GmPDIM and each of its domain fragments. Wild type, bold straight line with no symbol; a, filled circle; a', filled triangle; a-a', filled square; a'-b, diamond; and b, open circle.

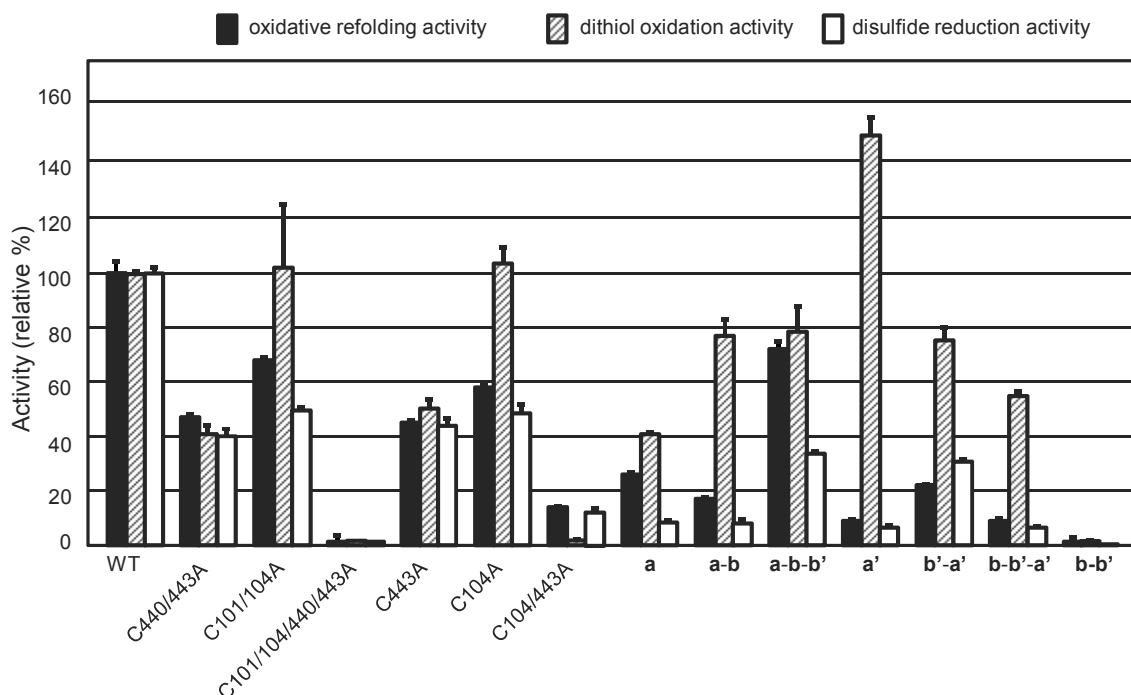


Figure 3-6. Oxidative refolding, oxidation, and reduction activities of wild-type and variants of GmPDIL-2.

Oxidative refolding of denatured and reduced RNase A (73.5 μM) in glutathione redox buffer (2 mM glutathione/0.5 mM glutathione disulfide) was assessed by monitoring the hydrolysis of cCMP (305.7 μM) in the presence of wild-type (WT) GmPDIL-2 and its variants (4.2-14.6 μM , enzyme activity-concentration relationships fit a linear in this range). The specific activity of WT GmPDIL-2 (259 $\text{mmol}_{\text{RNase A}}/\text{mol}/\text{min}$) was taken as 100%. Dithiol oxidation activity was assayed using a synthetic peptide. The specific activity of WT GmPDIL-2 [23.3 s^{-1} ($10^3 \times 1/\text{oxidation half-time}$)] was taken as 100%. Disulfide reduction activity was assayed using insulin. The specific activity of WT GmPDIL-2 (23.7×10^{-3} absorbance unit/mol/min) was taken as 100%. Data are represented as mean $\pm$ SE of $n = 3-6$

by reduction of the insulin disulfide bonds by C440/443A and C101/104A were 40% and 50% of that of wild-type GmPDIL-2, respectively (Figure 3-6, open bars), demonstrating that both the **a** and **a'** domains of GmPDIL-2 can catalyze the reduction.

Refolding with disulfide bond formation catalyzed by ER oxidoreductases was accompanied by formation and rearrangement of intramolecular disulfide bonds (Figure 3-7). There are two possible rearranging pathways, direct isomerization (pathway 1) and reduction-oxidation cycles (pathway 2) (Hatahet and Ruddock, 2009). Mutation of the C-terminal cysteine residue in an active site Cys-Gly-His-Cys to alanine was thought to cause defects in the dithiol oxidation and disulfide reduction activities but not in direct isomerase activity. As expected, the dithiol oxidation and disulfide reduction activities of C443A and C104A were almost same as those of C440/443A and C101/104A, demonstrating the loss of these activities in the mutated **a'** or **a** domains, respectively. The refolding activities of C443A and C104A were also similar to those of C440/443A and C101/104A. These results suggest that the reduction-oxidation cycles pathway plays a pivotal role in the RNase A-refolding activity of GmPDIL-2, whereas the direct isomerization pathway appears to play no role in the refolding activity of GmPDIL-2. However, C104/443A showed 14% of the refolding activity of wild-type GmPDIL-2, despite lacking thiol-oxidation activity. This result demonstrated that GmPDIL-2 rearranges the incorrect disulfides introduced non-enzymatically in the denatured RNaseA with the isomerization activity. Darby and Creighton reported that replacing the C-terminal cysteine residue with alanine diminishes the intrinsic reactivity of the N-terminal cysteine residues in the human PDI active sites toward oxidized glutathione by about 50% (Darby and Creighton, 1995). If replacement of the C-terminal cysteine residue with alanine in GmPDIL-2 had a similar effect on the reactivity of N-terminal cysteine residue, the low refolding activities of the active-site mutants can be partly explained by decreased reactivity of the N-terminal cysteine residue.

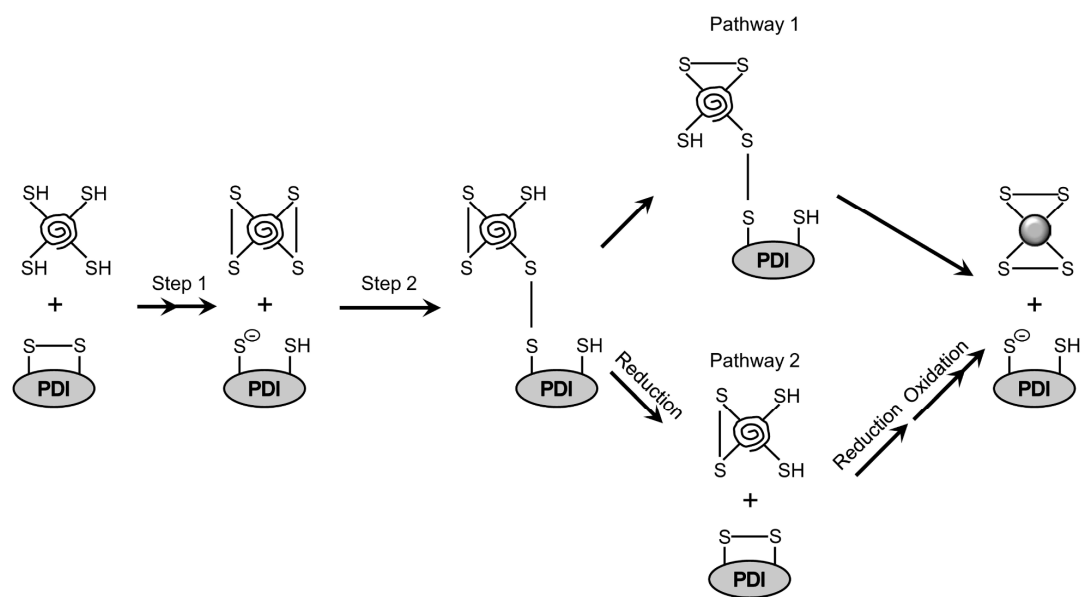


Figure 3-7. Folding activity of ER oxidoreductases.

Disulfide bonds in a substrate protein are formed by the oxidized form of an ER oxidoreductase (PDI) (step 1). These disulfide bonds are formed between cysteine residues in incorrect combinations. The mixed disulfide between the PDI protein and substrate protein is formed as an intermediate (step 2). Rearranging of the disulfide bonds to the correct combination is catalyzed by direct isomerization (pathway 1) or by reduction-oxidation cycles (pathway 2).

The refolding activities of the **a** and **a'** fragments were very low, at only 26% and 9% of the wild-type GmPDIL-2 activity. Therefore, the **b** and **b'** domains are essential for full refolding activity. The refolding activity of the **a** and **a'** domains was increased to 72% and 22%, respectively, by fusion of the **b-b'** domains to the **a** fragment and fusion of the **b'** domain to the **a'** fragment. These results suggest that both the **b** and **b'** domains are important for full activity. The **b** and the **b'** domains may play an important role in substrate binding.

The **b'** domain of human PDI has been identified as a primary substrate binding domain, and the **b'** domain and the subsequent x-linker region have been shown to assume at least two different conformations in solution (Klappa *et al.*, 1998; Nguyen *et al.*, 2008). A crystal structure of yeast PDI has been solved. According to yeast PDI crystal structure, the **b** and the **b'** domains of yeast PDI form a U-shaped hydrophobic pocket for substrate binding (Tian *et al.*, 2006). According to Fungus and human PDI structures, conformations of these PDIs are controlled by the redox states of active site cysteine residues in the **a** and **a'** domain (Nakasako *et al.*, 2010; Wang *et al.*, 2013). It remains unclear whether GmPDIL-2 is regulated by each redox mechanism. Fusion of the **b** domain to the **a** fragment or the **b-b'** domains to the **a'** fragment did not increase refolding activity. That is, fusing the **b** domain to the terminus of the fragment had a negative effect or no positive effect on the refolding activity of the **a** or **a'** domains. As the CD spectrum of the **a-b** fragment was similar to that of wild-type GmPDIL-2 (Figure 3-4C), the change in the conformation of the **a-b** fragment, but not secondary structure, may have caused the reduction in refolding activity.

The dithiol oxidation activity of the **a** fragment was 41% of that of GmPDIL-2. Fusion of the **b** and the **b-b'** domains to the **a** fragment resulted in an increase in the dithiol oxidation activity to 78% of that of GmPDIL-2, suggesting that the **b** domain is important for the oxidation activity of the **a** domain. The dithiol oxidation activity of the **a'** fragment was 152% of that of wild-type GmPDIL-2, indicating that one, two or all three of the **a**, **b** and **b'** domains attenuate the dithiol oxidation activity of the **a'** domain. The underlying mechanism

is unclear, however. The disulfide reduction activities of the **a** and the **a'** domains were 8 and 7%, respectively, of the activity of wild-type GmPDIL-2. Fusion of the **b-b'** domains to the **a** fragment and the **b'** domain to the **a'** fragment increased the reduction activity to about 30% of that of wild-type GmPDIL-2. Taken together, these results suggest that the **b'** domain plays an important role in the disulfide reduction activity of GmPDIL-2. In the case of human PDI, the most significant increase in disulfide reduction activity reportedly occurs after fusion of the **b'** domain to the **a-b** fragment (Darby *et al.*, 1998).

Replacing the C-terminal (C192/195A) or the N-terminal active-site cysteine residues (C64/67A) with alanines decreased the RNase A oxidative refolding activity to 64% or 80% of the activity of wild-type GmPDIM, respectively, indicating that the two active sites function independently in oxidative refolding (Figure 3-8, black bars). Mutant C195A, in which the C-terminal cysteine residue of the active-site of the **a'** domain was replaced with alanine, demonstrated refolding activity equivalent to that of C192/195A. This result suggests that the **a'** domain completely rearranges the disulfide bonds of denatured RNaseA through reduction-oxidation cycles. In contrast to C195A, mutant C67A, in which the C-terminal cysteine residue of the **a** domain was replaced with alanine, exhibited refolding activity equivalent to that of wild-type GmPDIM. Thus, disulfide rearranging of denatured RNaseA by the **a** domain is accomplished solely through direct isomerization rather than reduction-oxidation cycles. Although the dithiol oxidation (Figure 3-8, hatch-pattern bars) and disulfide reduction activities of C67A (Figure 3-8, white bars) were lower than those of wild-type GmPDIM, these reactions were not the rate-limiting steps for RNase A refolding. The refolding activities of the **a**, **a'** and **a-a'** fragments were 25%, 20%, and 62% of the GmPDIM activity. Addition of the **b** domain to the **a'** fragment increased the activity to 73% of the GmPDIM activity. These results indicate that the **b** domain is important for refolding activity and probably functions in substrate binding. The function of the **b** domain of P5, a mammalian ortholog of GmPDIM, has not been elucidated to date. ER oxidoreductases show

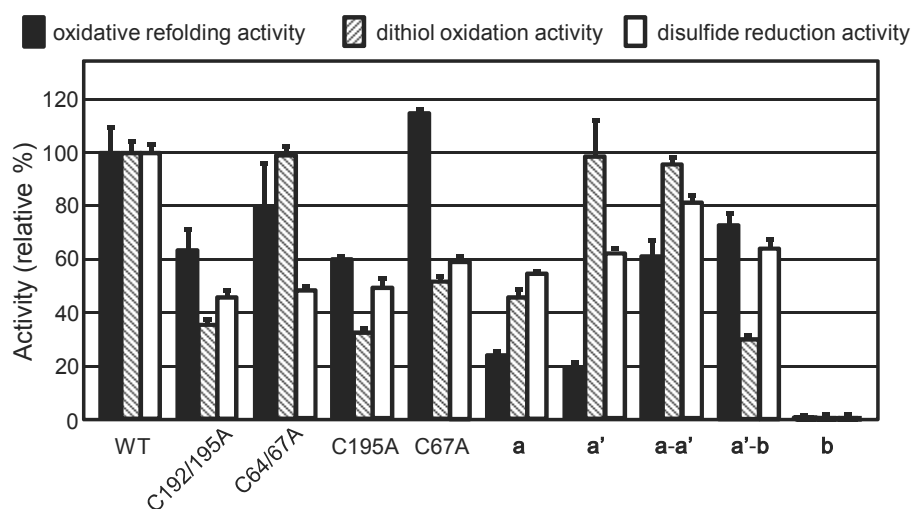


Figure 3-8. Oxidative refolding, oxidation, and reduction activities of wild-type and variants of GmPDIM.

Oxidative refolding activity was assessed using reduced and denatured RNase A as described in Figure 3-6. The specific activity of wild-type (WT) GmPDIM ($64 \text{ mmol}_{\text{RNase A}}/\text{mol}/\text{min}$) was taken as 100%. Dithiol oxidation activity was assayed using a synthetic peptide. The specific activity of WT GmPDIM [$27.6 \text{ s}^{-1} (10^3 \times 1/\text{oxidation half-time})$] was taken as 100%. Disulfide reduction activity was assayed using insulin. The specific activity of WT GmPDIM ($5.9 \times 10^{-3} \text{ absorbance unit}/\text{mol}/\text{min}$) was taken as 100%. Data are represented as mean $\pm$ SE of $n = 3-6$.

distinct substrate specificity; P5 is targeted to ER chaperone BiP client proteins (Jessop *et al.*, 2009). The dithiol oxidation activity of C192/195A contrasted markedly with that of C64/67A. The activity of C192/195A was 36% of the GmPDIM activity, whereas the activity of C64/67A was almost the same as that of GmPDIM. These results suggest that the **a'** domain has higher disulfide oxidative activity than the **a** domain. This conclusion is supported by the observation that **a'** fragments exhibited higher dithiol oxidation activity (97% of the GmPDIM activity) than the **a** fragment (45%). These results also suggest that the **a'** domain is predominantly involved in substrate disulfide formation. However, for unknown reasons, fusion of the **b** domain to the **a'** fragment resulted in a decrease in dithiol oxidation activity. The disulfide reduction activities of C192/195A and C64/67A were around half of the GmPDIM activity. The disulfide reduction activities of the **a**, **a'** and **a-a'** fragments were 54 %, 62%, and 82% of the GmPDIM activity. Addition of the **b** domain to the **a'** or **a-a'** fragments had only a minimal effect on the reduction activity of the **a'** or **a-a'** domains.

Comparison of GmPDIL-2 and GmPDIM

The oxidative refolding specific activity of GmPDIL-2 toward denatured RNase A was 4 times higher than that of GmPDIM (Table 3-3). The refolding activities of the **a** and **a'** fragments of GmPDIL-2 were higher than those of PDIM (i.e., the ratio of the activities of the **a** and **a'** fragments of GmPDIL-2 and the **a** and **a'** fragments of GmPDIM was 1:0.45:0.36:0.2). The higher oxidative refolding activities of the **a** and the **a'** domains of GmPDIL-2 may partly explain the higher oxidative refolding activity of GmPDIL-2. In addition, the domain structure of GmPDIL-2, consisting of **a**, **b**, **b'** and **a'** domains, contributes to the protein's higher oxidative refolding activity.

The oxidative refolding activity of GmPDIL-2 and GmPDIM depends on the dithiol oxidation, disulfide reduction and direct isomerase activities of the **a** and **a'** domains. The thiol oxidation activity of GmPDIL-2 was similar to that of GmPDIM (Table 3-3). The ratio

Table 3-3. Comparison of the oxidative refolding, oxidation and reduction activities of wild-type GmPDIL-2, GmPDIM and their variants.

		oxidative refolding activity (mmol _{RNase A} /min/mol)	dithiol oxidation activity (s ⁻¹ [$\times 10^3$ /oxidation half-time])	disulfide reduction activity ($\times 10^{-3}$ A_{340nm} /min/mol)
PDIL-2	WT	258.7 $\pm$ 5.3	23.3 $\pm$ 0.3	23.67 $\pm$ 0.49
	a	66.5 $\pm$ 6.0	9.6 $\pm$ 0.1	1.95 $\pm$ 0.04
	a'	23.6 $\pm$ 1.2	35.6 $\pm$ 1.3	1.55 $\pm$ 0.03
PDIM	WT	64.0 $\pm$ 5.4	27.7 $\pm$ 1.0	5.93 $\pm$ 0.12
	a	15.8 $\pm$ 1.1	12.3 $\pm$ 1.0	3.24 $\pm$ 0.05
	a'	12.6 $\pm$ 1.3	26.9 $\pm$ 3.8	3.67 $\pm$ 0.04

Oxidative refolding of denatured and reduced RNase A (73.5 μ M) in glutathione redox buffer (2 mM GSH/0.5 mM GSSG) was assessed in the presence of wild-type (WT) or mutated GmPDIL-2 and GmPDIM and their variants (4.2-14.6 μ M, enzyme activity-concentration relationships fit a linear in this range). Dithiol oxidation activity was assayed using a synthetic peptide. Disulfide reduction activity was assayed using insulin. Each value is represented as mean $\pm$ SE of $n = 3-6$.

of the activities of the **a** or **a'** fragment of GmPDIL-2 to the **a** or **a'** fragment of GmPDIM was also similar (1:1.3 and 3.7:2.8). The disulfide reduction activity of GmPDIL-2 was 4 times higher than that of GmPDIM (Table 3-3). However, the ratio of the disulfide reduction activities of the **a** and **a'** fragments of GmPDIL-2 and the **a** and **a'** fragments of GmPDIM was 1:0.8:1.6:2.5. These data indicate that the **b** and **b'** domains of GmPDIL-2 enhance its disulfide reduction activity, whereas the **b** domain of GmPDIM has only a small effect on the enzyme's activity. In addition, the reason for the higher refolding activities of the **a** and the **a'** domains of GmPDIL-2 is unknown, but it is thought that the higher refolding activities are related to factors other than these domains' oxidation and reduction activities. The **a** fragment of human PDI acts as a "place holder" for substrate folding, which is a rate-limiting step in disulfide bond formation (Kosuri *et al.*, 2012). The **a** and the **a'** domains of GmPDIL-2 may more adequately serve as place holders.

GmPDIL-2 has high activities as a folding enzyme and reduction, and almost the same activity as a oxidation compared to GmPDIM. On the other hand, GmPDIL-2 was not oxidized directly by GmERO1a (Figure 2-3 and Figure 2-6). Since reduction-oxidation cycles need a reduced form of ER oxidoreductase (Figure 3-7), GmPDIL-2 may mainly catalyze rearrangement of disulfide bonds introduced in substrates by other ER oxidoreductase. It is likely that GmPDIM initially form disulfide bonds in a substrate and GmPDIL-2 rearrange them by reduction-oxidation cycles.

Although GmPDIL-2 was not oxidized by GmERO1a, GmPDIL-2 showed the dithiol oxidation activity equal to that of GmPDIM, and the higher disulfide reduction activity than that of GmPDIM in the presence of glutathione buffer. Therefore, if GmPDIL-2 also works as an oxidase *in vivo*, GmPDIL-2 have to accept oxidizing equivalent from GmERO1 indirectly. Next, how the domains and active sites of GmPDIM and GmPDIL-2 contribute to cooperative oxidative folding was analyzed.

The Active Sites of GmPDIL-2 Are Essential to Their Cooperative Activity with GmPDIM

The acceleration of oxidative refolding of RNase A by the cooperation of GmPDIL-2 and GmPDIM depends on the catalytic cysteine residues in the **a** and **a'** domains of GmPDIL-2. When four catalytic cysteine residues were replaced with alanine [GmPDIL-2(C101/104/440/443A)], the acceleration of oxidative refolding was eliminated (Figure 3-9). Replacing two catalytic cysteine residues in either the **a** or **a'** domain with alanine [GmPDIL-2(C440/443A) or GmPDIL-2(C101/104A)] caused a decrease in oxidative refolding activity to 55% or 69% of wild-type levels, respectively. In mutants in which the C-terminal catalytic cysteine residue in the **a'** and/or **a** domain was replaced by alanine [GmPDIL-2(C104/443A), GmPDIL-2(C443A), and GmPDIL-2(C104A)], oxidative refolding was slower than that of GmPDIL-2(C440/443A) or GmPDIL-2(C101/104A). The effects of **a**, **a'**, **a-b**, **a-b-b'**, **b-b'-a'**, and **b'-a'** fragments on the acceleration of oxidative refolding were all very low (Figure 3-9). One reason for the small effects of the domain fragments on the acceleration of oxidative refolding, other than the **a-b-b'** fragment, may be their low oxidative refolding activities and disulfide reduction activities seen in the presence of glutathione redox buffer without GmERO1a (Figure 3-6). Although the **a-b-b'** fragment have comparable oxidative refolding activity to wild-type, the **b'-a'** fragment rather effective than the **a-b-b'** fragment (Figure 3-9), suggesting that another factor, the association of GmPDIL-2 and GmPDIM, is also important for the cooperation.

The association of GmPDIL-2 and GmPDIM was confirmed by far-western blot analysis (Figure 3-10A and B). To identify the relevant domains for the binding of GmPDIM and GmPDIL-2, various domain fragments of GmPDIL-2 and GmPDIM were subjected to far-western blot analysis. Only the **b'-a'** fragment of GmPDIL-2 bound to GmPDIM with affinity comparable to wild-type GmPDIL-2 (Figure 3-10A). The **a-a'** fragment of GmPDIM bound with high affinity to GmPDIL-2 (Figure 3-10B). Taken together, the binding sites of

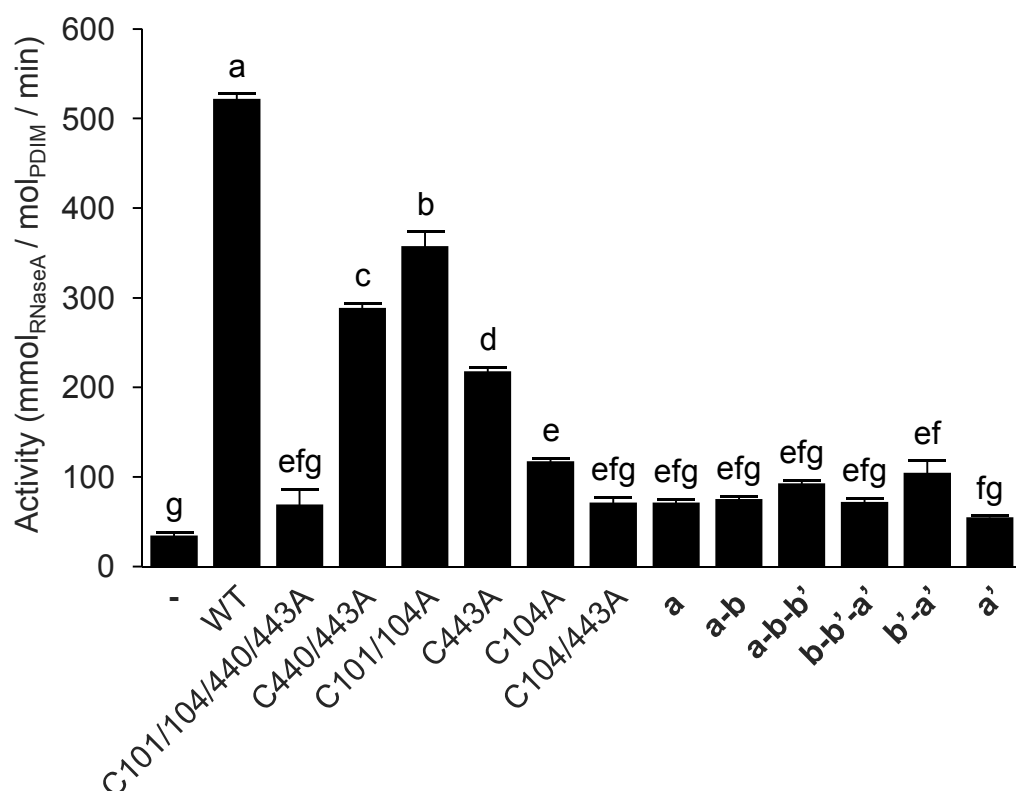


Figure 3-9. Acceleration of refolding by GmPDIL-2 mutants and GmPDIL-2 domain fragments in the GmERO1a/GmPDIM system.

Reduced and denatured RNase A (8 μ M) was incubated with 1 μ M GmPDIM and 4 μ M wild-type GmPDIL-2 (WT), its mutants, or domain fragments in the presence of 1 μ M GmERO1a. Data are represented as mean $\pm$ SE of $n = 3-5$. Bars with the same letters are not significantly different at $P < 0.05$ by Tukey-Kramer test.

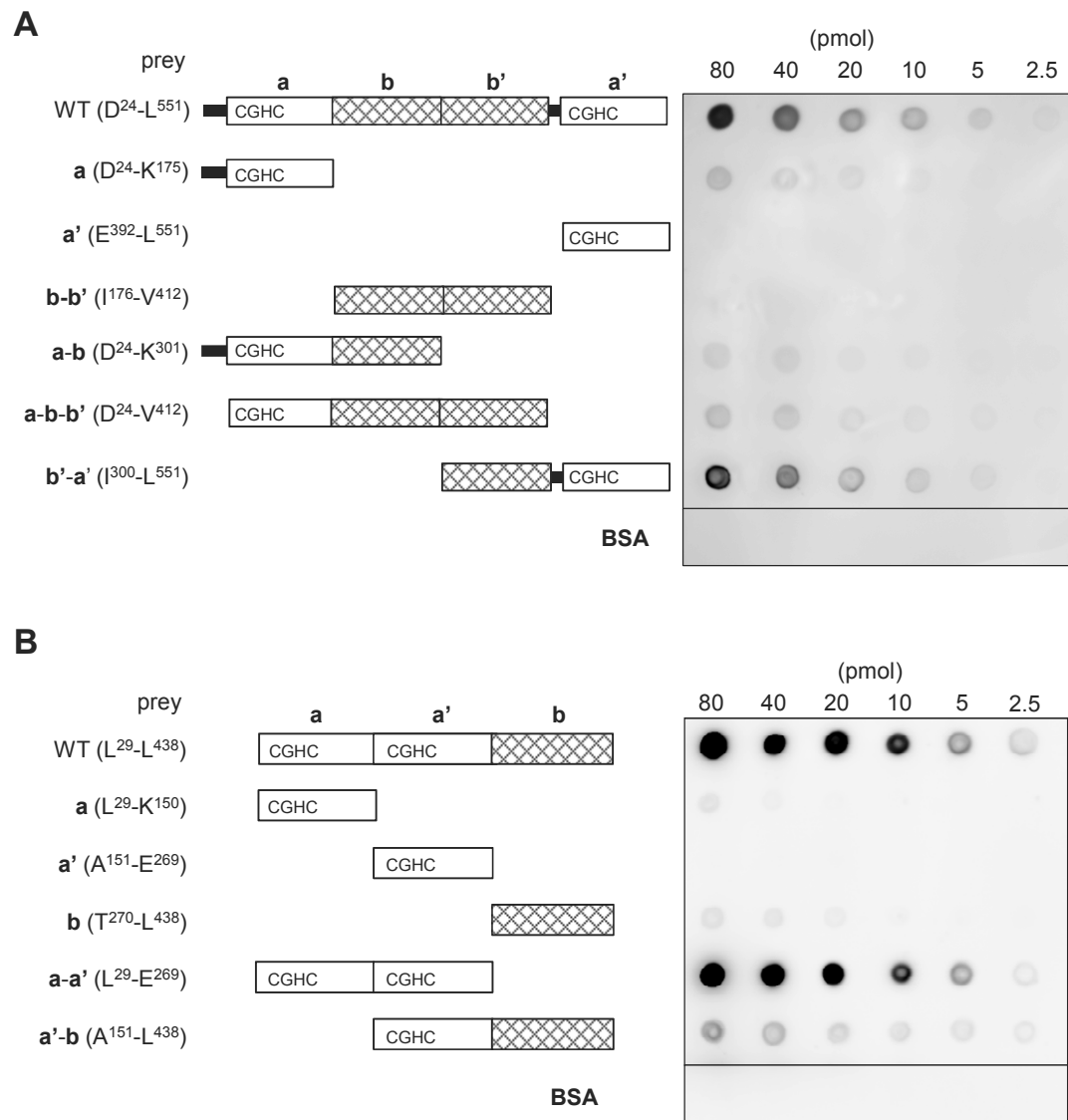


Figure 3-10. Far-western blot analysis of the association of GmPDIL-2 and GmPDIM.

(A) Far-western blot analysis of the association of GmPDIL-2 (WT) or GmPDIL-2 domain fragment with GmPDIM. Indicated amounts of GmPDIL-2, each GmPDIL-2 domain fragment, or BSA (prey) were dot-blotted and incubated with GmPDIM. Bound GmPDIM was immunostained.

(B) For GmPDIM (WT), each GmPDIM domain fragment or BSA (prey) was dot-blotted and incubated with GmPDIL-2. Bound GmPDIL-2 was immunostained. BSA, bovine serum albumin; WT, wild type.

GmPDIL-2 and GmPDIM are composed of the **b'** and **a'** domains of GmPDIL-2 and the **a** and **a'** domains of GmPDIM.

Oxidative refolding by ER oxidoreductases is achieved through the formation of non-native disulfide bonds in substrate proteins and their subsequent rearrangement to native bonds as seen in Figure 2-5A. Therefore, it was predicted that GmPDIL-2 would stimulate the formation and/or rearrangement of substrate disulfide bonds. The addition of GmPDIL-2 to GmPDIM induced more rapid conversion of "D_{red}" into "D_{ox}" and "N" than did GmPDIM alone (Figure 3-11A and B). Mutations in the active sites of GmPDIL-2 caused retardation in the conversion into both "D_{ox}" and "N" (Figure 3-11C–E). The rate of disulfide bond formation during the first 10 min of the addition of GmPDIL-2 to GmPDIM was twice that of GmPDIM alone (Figure 3-12), suggesting that GmPDIL-2 stimulates the oxidation of GmPDIM by GmERO1a. Mutant GmPDIL-2(C101/104/440/443A) was devoid of the acceleration effect on refolding. Since GmPDIL-2(C440/443A) and GmPDIL-2(C101/104A) showed about half the acceleration effect of the wild type, the active sites of the **a** and **a'** domain appeared to act independently. The amount of native disulfide bonds formed during the reaction was calculated from the amount of refolded RNase A. The formation rate of native disulfide bonds during the cooperation of GmPDIL-2 and GmPDIM (Figure 3-12, red curve) was four-fold higher than GmPDIM alone (Figure 3-12, blue curve), suggesting that GmPDIL-2 accelerates both disulfide bond formation and the rearrangement accompanied with folding.

GmPDIL-2 Accelerates the Oxidation of GmPDIM by GmERO1a

From the results shown in Figure 3-11 and Figure 3-12, it was suggested that GmPDIL-2 accelerates the oxidation of GmPDIM by GmERO1a. To confirm this possibility, oxidation of GmPDIM by GmERO1a was measured in the presence of GmPDIL-2. In this experiment, GSH was used as a substrate. Addition of GmPDIL-2 caused an increase in O₂

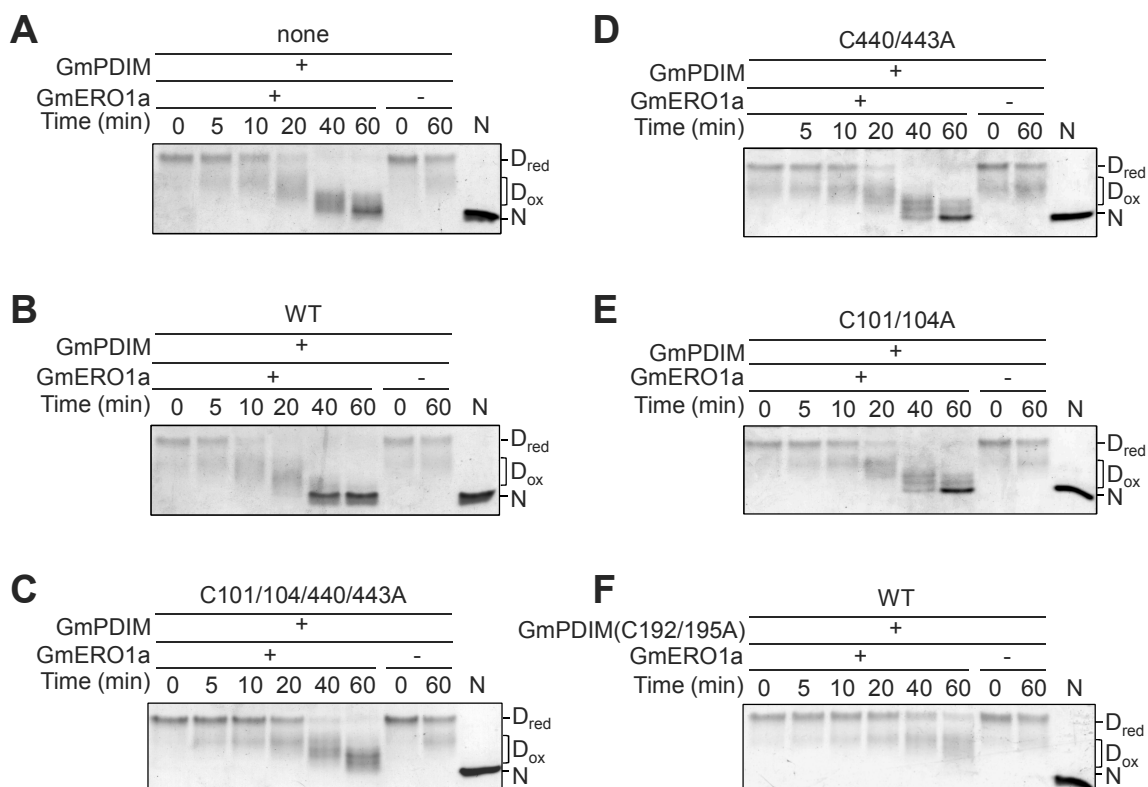


Figure 3-11. Disulfide bond formation in reduced and denatured RNase A during cooperative refolding.

(A–E) Disulfide bond formation in reduced and denatured RNase A (8 μ M) during cooperative refolding by GmPDIM (1 μ M) and GmPDIL-2 or GmPDIL-2 mutants (3 μ M) in the presence of GmERO1a (1 μ M) was analyzed by nonreducing SDS-PAGE. D_{red} , reduced and denatured RNase A; D_{ox} , denatured RNase A with nonnative disulfides; N, native RNase A.

(F) As in A, GmPDIM(C192/195A) (1 μ M) and GmPDIL-2 (3 μ M) were incubated.

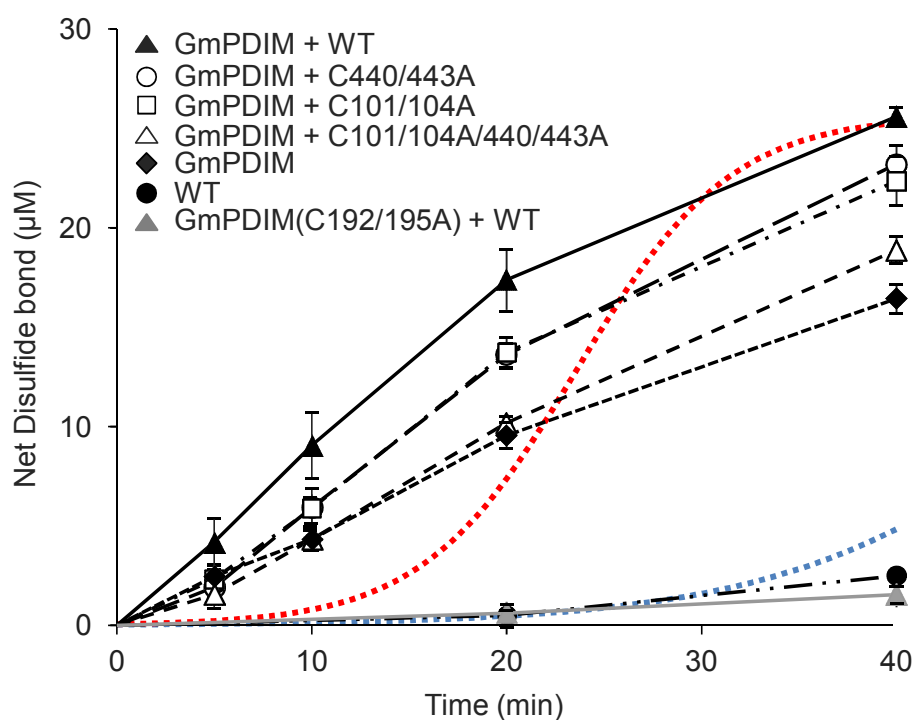


Figure 3-12. Disulfide bond formation in RNase A over time.

Reduced and denatured RNase A was incubated with indicated combinations of proteins as in Figure 3-11. The amounts of free thiol groups in the reaction mixtures were measured and net disulfide bonds were calculated by subtracting the free disulfide bonds from total Cys residues. Red and blue dotted lines show the amounts of native disulfide bonds formed in RNase A, which were calculated from the amounts of refolded RNase A. Data are represented as mean $\pm$ SE of $n = 3$. WT, wild type GmPDIL-2.

consumption rate to 130% of that without GmPDIL-2 (Figure 3-13A). The acceleration effect was lost upon mutation of the active site of the **a'**; GmPDIL-2(C440/443A) and GmPDIL-2(C101/104/440/443A) showed no acceleration effects. The acceleration effect of GmPDIL-2(C101/104A) was lower than that of wild-type GmPDIL-2. Similar effects by GmPDIL-2 and its active-site mutations were observed in experiments coupled with glutathione disulfide reduction and NADPH (Figure 3-13B–D). Fragments **a** and **a'** showed an acceleration effect of one-half that of the wild type. Fragment **b–b'** showed no effect. The attachment of the **b**, **b'**, or **b–b'** domain to the **a** or **a'** domain had no positive effect.

GmERO1a Preferentially Oxidizes the Active Site in the **a' Domain of GmPDIM; GmPDIM Oxidizes Both Active Sites in GmPDIL-2**

From the results shown in Figure 3-13, I envisioned a pathway by which GmPDIM, oxidized by GmERO1, transfers disulfide bonds to the active sites of GmPDIL-2. To test this hypothesis, I determined which active site of GmPDIM is oxidized by GmERO1a using active-center mutants of GmPDIM (Figure 3-14, black bars). Both GmPDIM(C192/195A) and GmPDIM(C195A) were negligibly oxidized by GmERO1a. Therefore, no disulfide bond formation in reduced and denatured RNase A by GmPDIM(C192/195A) and GmPDIL-2 was detected (Figure 3-11F and Figure 3-12). In contrast to GmPDIM(C192/195A) and GmPDIM(C195A), GmPDIM(C64/67A) and GmPDIM(C67A) were oxidized by GmERO1a, although at a slower rate than wild-type GmPDIM. These findings suggest that GmERO1a specifically oxidizes the active site in the **a'** domain of GmPDIM. This finding was corroborated by the fact that the **a'** fragment, but not the **a** fragment, was oxidized at 74% of the oxidation rate of wild-type GmPDIM by GmERO1a. The oxidation rate of the **a'–b** fragment was slightly slower than that of the **a'** fragment, and the **a–a'** fragment was oxidized at almost same rate as wild-type GmPDIM, suggesting that the **b** domain does not contribute to oxidation by GmERO1a. Association of GmPDIM and GmERO1a was detected

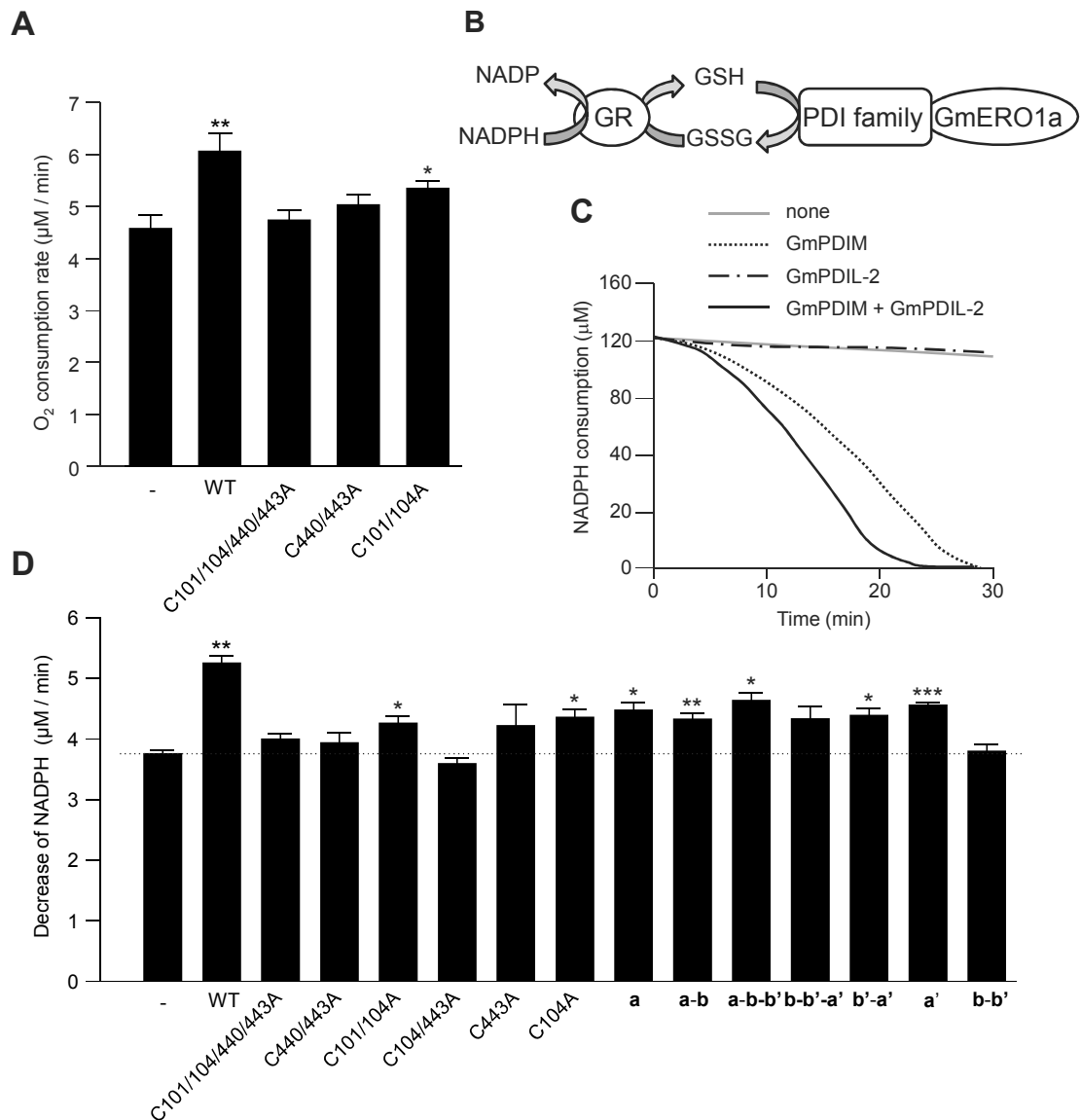


Figure 3-13. GmPDIL-2 accelerates the oxidation of GmPDIM by GmERO1a.

(A) Oxygen consumption was monitored during incubation of 2 μM GmERO1a and 3 μM GmPDIM without or with 3 μM GmPDIL-2 or its mutants in the presence of 10 mM GSH. Data are represented as mean ± SE of $n = 6$. *, $P < 0.05$; **, $P < 0.01$ compared with the reaction without GmPDIL-2 (-; Welch's t test).

(B) Schematic representation of the coupling reaction of oxidation by GmERO1a and the reduction of GSSG by GR in the presence of GSH and NADPH. GR, glutathione-disulfide reductase; PDI family, ER oxidoreductases.

(C) NADPH consumption over time in the presence of 1 μM GmERO1a and 3 μM GmPDIM, 3 μM GmPDIL-2, or 3 μM GmPDIM and 3 μM GmPDIL-2 in the presence of 3 mM GSH, 120 μM NADPH, and 1 U/mL GR.

(D) Decrease in NADPH in the presence of indicated combinations of proteins as in C. Data are represented as mean ± SE of $n = 10$ (without GmPDIL-2); $n = 3-4$ (with wild-type GmPDIL-2 and its mutants). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the reaction without GmPDIL-2 (-; Welch's t test).

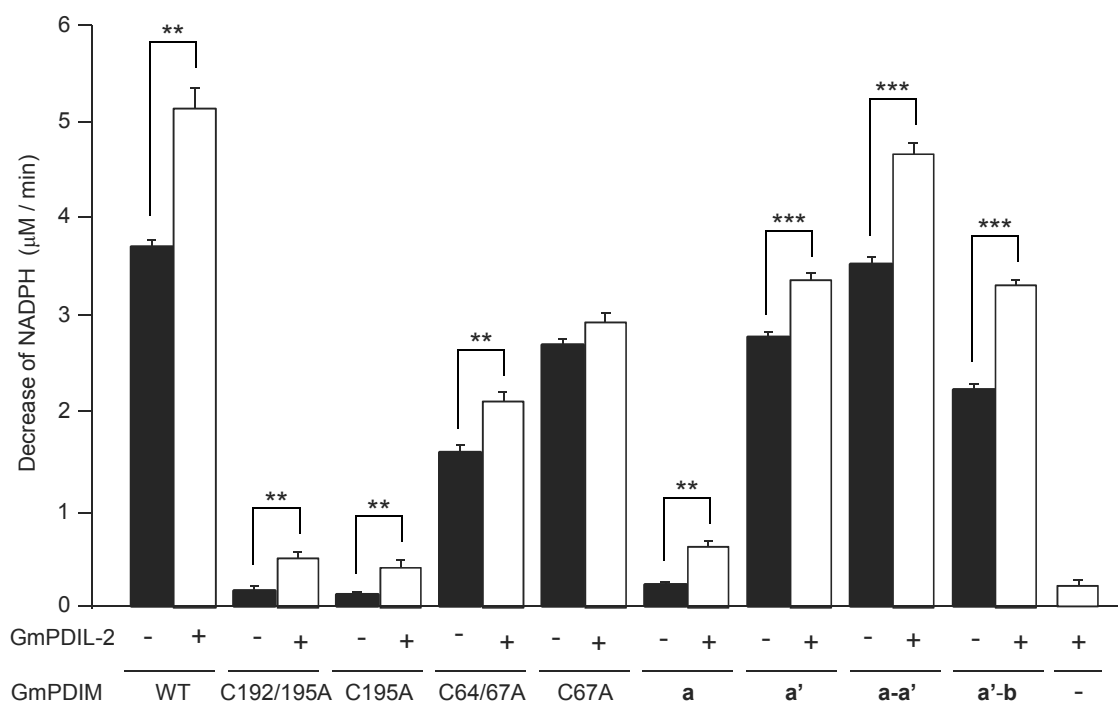


Figure 3-14. Oxidation of GmPDIM (WT), its mutants, or its domain fragments by GmERO1a.

Oxidation of GmPDIM (WT), its mutants, or its domain fragments by GmERO1a without or with GmPDIL-2 was measured as in Figure 3-13C. Data are represented as mean $\pm$ SE of $n = 3-6$. **, $P < 0.01$; ***, $P < 0.001$ (Welch's t test). WT, wild-type GmPDIM.

by far-western blot analysis (Figure 3-15). The **a**, **a'**, or **b** fragment of GmPDIM hardly associated with GmERO1a. However, either the **a-a'** or **a'-b** fragment associated with GmERO1a at an affinity comparable to wild-type GmPDIM. Taken together, the **a** domain of GmPDIM is thought to be involved in not only the association with GmERO1a but also the oxidation of GmPDIM by GmERO1a. Oxidation of GmPDIM(C64/67A) by GmERO1a was slightly accelerated by GmPDIL-2 (Figure 3-14, white bar). On the other hand, oxidation of GmPDIM(C67A) was not accelerated by GmPDIL-2. Since the active site in the **a** domain of GmPDIM(C67A) generates a mixed disulfide bond difficult to cleave, it is likely that the **a** domain of GmPDIM transfer a disulfide bond to the active site of GmPDIL-2.

To confirm that the disulfide bond in the active site of the **a'** domain of GmPDIM formed by GmERO1a is transferred to the active site of GmPDIL-2 via the active site of the **a** domain of GmPDIM, I analyzed mixed disulfide complexes of the active-site mutants of GmPDIM and GmPDIL-2. When GmPDIM(C67A) and GmERO1a were incubated with GmPDIL-2(C104/443A), two types of mixed disulfide complexes (L2-M¹ and L2-M²) of GmPDIM(C67A) and GmPDIL-2(C104/443A), and the GmPDIM(C67A) dimer (M₂) were detected (Figure 3-16A). When GmPDIM(C67A) and GmERO1a were incubated with GmPDIL-2(C104A) or GmPDIL-2(C443A), only one mixed disulfide complex, L2-M² (Figure 3-16B) or L2-M¹, (Figure 3-16C) was formed. Neither the mixed disulfide complex nor the GmPDIM(C67A) dimer was generated in the absence of GmERO1a (Figure 3-16D). When wild-type GmPDIM and GmERO1a were incubated with GmPDIL-2(C104/443A), only a few mixed disulfide complexes were detected (Figure 3-16E). In addition, no mixed disulfide complexes were formed from GmPDIM(C195A) (Figure 3-16F). From these results, L2-M¹ and L2-M² were deduced to be mixed disulfide complexes, which were linked by disulfide bonds between Cys67 of GmPDIM and either Cys443 or Cys104 of GmPDIL-2.

The results shown in Figure 3-14 suggest that a disulfide bond introduced into the active site of the **a'** domain of GmPDIM is transferred to the active site of the **a** domain of

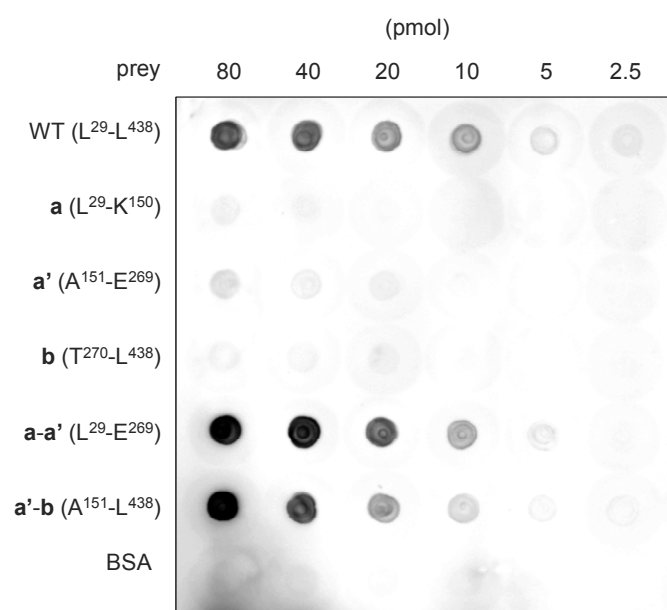


Figure 3-15. Far-western-blot analysis of the association of GmPDIM or its domain fragment with GmERO1a.

Indicated amounts of GmPDIM (WT), each domain fragment or BSA (prey) was dot-blotted and incubated with GmERO1a. Bound GmERO1a was immunostained. BSA, bovine serum albumin; WT, wild type.

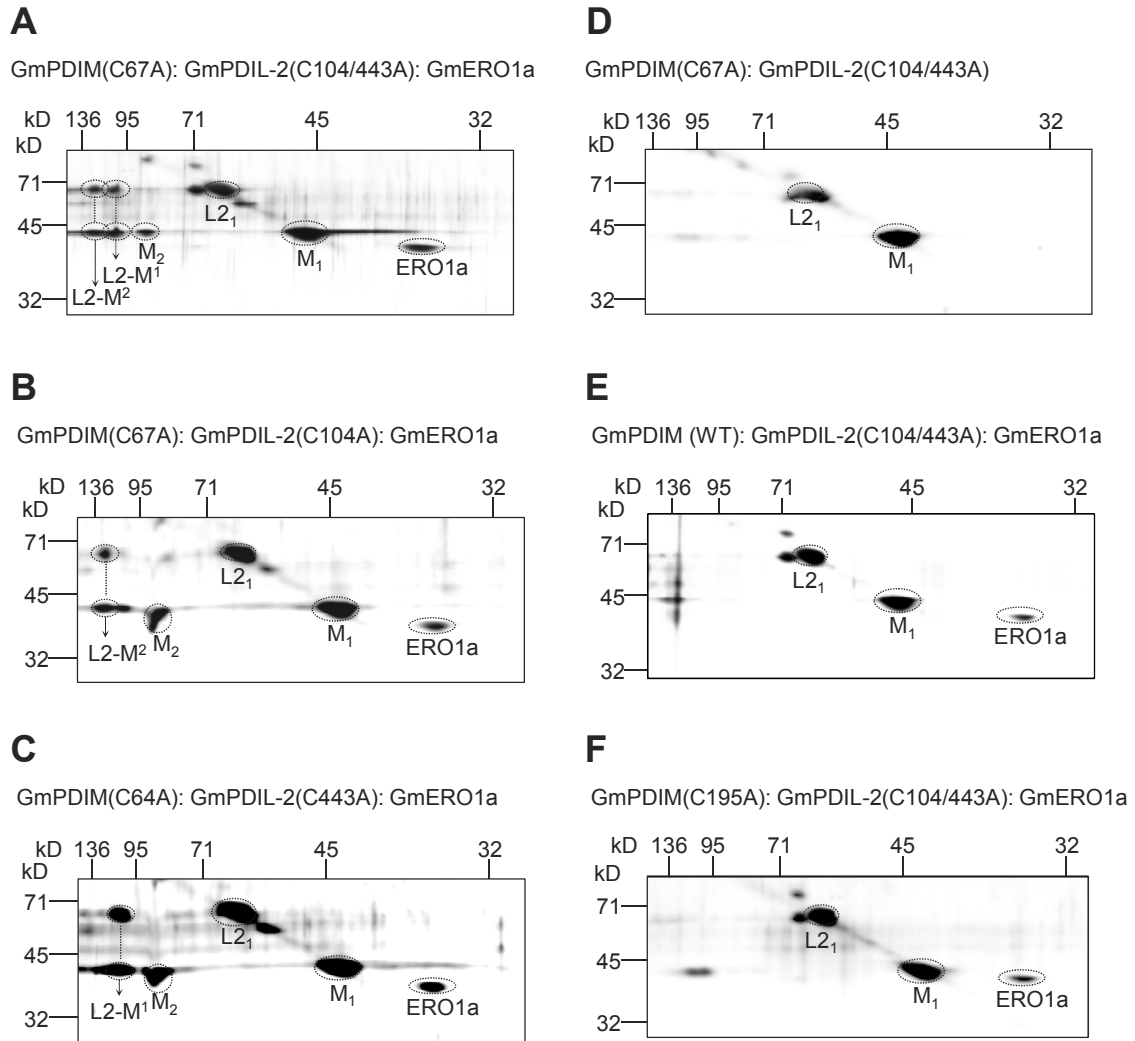


Figure 3-16. The mixed disulfide of the GmPDIM and GmPDIL-2 mutants.

(A) GmPDIM(C67A) (6 μ M), 3 μ M GmPDIL-2(C104/443A), and 1 μ M GmERO1a were incubated at 25°C for 60 min and separated by two-dimensional electrophoresis. M₁, GmPDIM monomer; L2₁ GmPDIL-2 monomer; M₂, GmPDIM dimer; L2M¹ and L2M², mixed disulfide complex of GmPDIL-2 mutants and GmPDIM(C64A).

(B–F) Indicated combinations of proteins were incubated and analyzed as described in A.

GmPDIM. Then the oxidized GmPDIM can oxidize the active site of either the **a** or **a'** domain of GmPDIL-2 (Figure 3-16A-F). The direction of a redox reaction between functional groups depends on their redox potentials, i.e., a functional group with higher redox potential oxidizes a functional group with lower redox potential. To obtain the redox potentials of GmPDIM and GmPDIL-2, their redox equilibrium constants were determined. Redox equilibrium constants of GmPDIM and GmPDIL-2 were 2.0 mM and 3.4 mM, respectively (Figure 3-17A and D). Since these are the mean values of two active sites, redox equilibrium constants of the redox-inactive mutants of GmPDIM and GmPDIL-2 were determined (Figure 3-17B, C, E, and F). GmPDIM(C192/195A) showed the lowest redox equilibrium constant ($K_{eq} = 0.7$) among the mutant proteins. The reduction potential E_0' values calculated from each redox equilibrium constant of GmPDIM(C192/195A), GmPDIM(C64/67A), GmPDIL-2(C440/443A), and GmPDIL-2(C101/104A) were -147 mV, -166 mV, -169 mV, and -163 mV, respectively. These values suggest that the **a** domain of GmPDIM has a tendency to oxidize both the active sites of the **a** and **a'** domains of GmPDIL-2.

Far-western blot analysis of the active-site cysteine mutants supports this idea. All active-site mutations of GmPDIL-2 decreased bound GmPDIM (Figure 3-18A). On the other hand, the mutations in the active site **a'** domain of GmPDIM had no effect on affinity for GmPDIL-2, and interestingly, GmPDIM(C64/67A) and GmPDIM(C67A), mutated in the active site of the **a** domain, showed much lower affinity for GmPDIL-2 compare to wild-type GmPDIM (Figure 3-18B). These results indicated that the active sites of the **a** and **a'** domains of GmPDIL-2, and the **a** domain of GmPDIM, involved in intermolecular disulfide transfer, also important for binding.

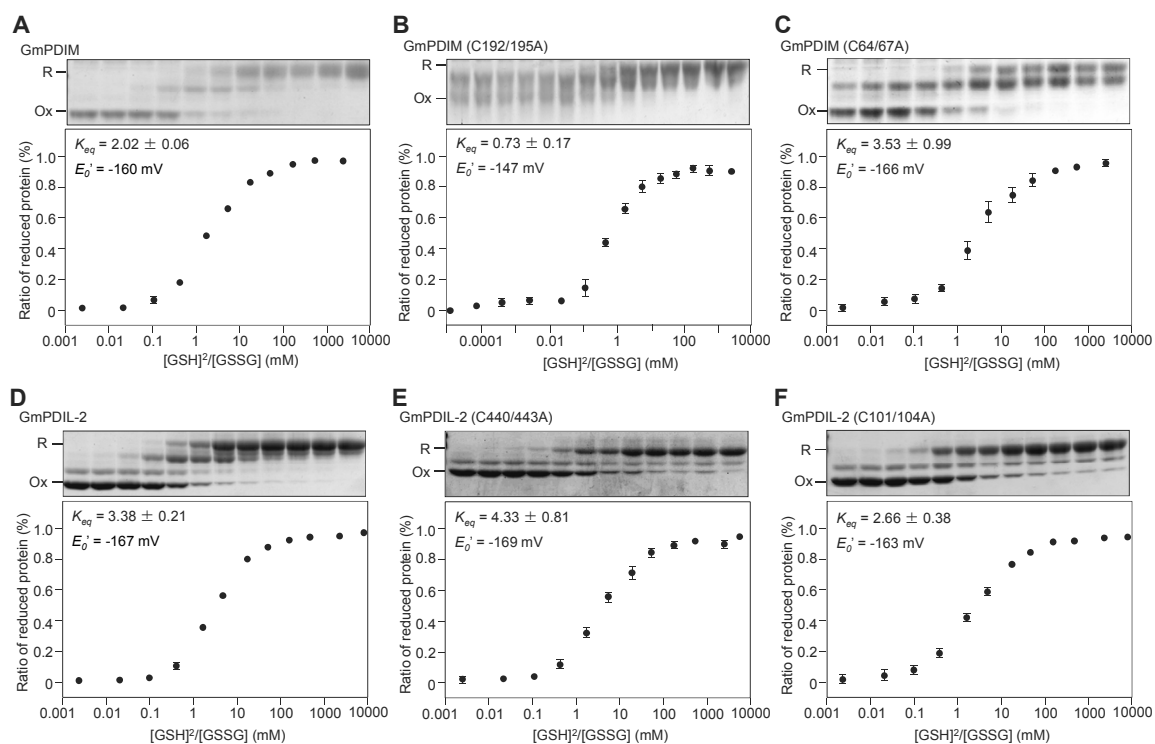


Figure 3-17. Assay of redox equilibrium constants of GmPDIM, GmPDIL-2, and their mutants.

(A–F) Coomassie Brilliant Blue stainings of the SDS-polyacrylamide gels are shown above the redox graphs. Data are represented as mean $\pm$ SE of $n = 3$.

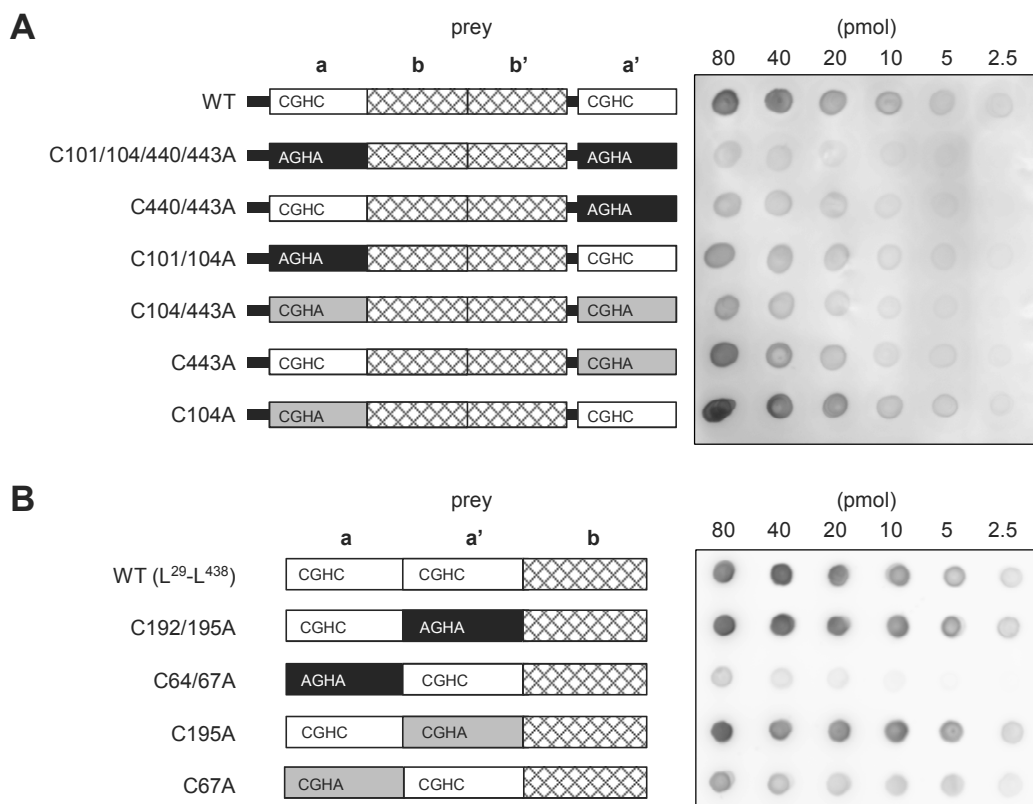


Figure 3-18. Far-western blot analysis of the active-site mutants of GmPDIL-2 and GmPDIM.

(A) Far-western blot analysis of the association of GmPDIL-2 (WT) or GmPDIL-2 active-site mutants with GmPDIM. Indicated amounts of GmPDIL-2 or each GmPDIL-2 active-site mutant were dot-blotted and incubated with GmPDIM. Bound GmPDIM was immunostained.

(B) Far-western blot analysis of the association of GmPDIM (WT) or GmPDIM active-site mutants with GmPDIL-2. Indicated amounts of GmPDIM or each GmPDIM active-site mutant were dot-blotted and incubated with GmPDIL-2. Bound GmPDIL-2 was immunostained. WT, wild type.

Two Molecular Mechanisms Achieve Efficient Cooperative Oxidative Folding by GmPDIM and GmPDIL-2

Among the soybean ER oxidoreductases examined in this study, only GmPDIL-2 was hardly oxidized by GmERO1a. The K_{eq} values of the active sites in the **a** and **a'** domains of GmPDIL-2 were 4.33 and 2.66, respectively. Since these values are similar to that of the **a'** domain of GmPDIM ($K_{eq} = 3.53$), which was preferentially oxidized by GmERO1a, the lack of oxidation by GmERO1a is not due to the reduction potential of the active sites of GmPDIL-2. One reason may be the low affinity between GmERO1a and GmPDIL-2 (Figure 2-7).

The association of GmPDIL-2 and GmPDIM was found *in vivo* (Figure 3-1). GmPDIL-2 can associate with GmPDIM to cooperatively fold denatured RNase A *in vitro* (Figure 3-10 and Figure 3-18). Oxidative folding proceeds via two steps: (Step 1) introduction of transient, nonnative disulfide bonds and (Step 2) their isomerization into native disulfide bonds. The oxidative folding activity of GmPDIM was lower than that of GmPDIL-2 in the presence of glutathione buffer without GmERO1a (Figure 3-6 and Figure 3-8). Therefore, there may be a pathway in which GmPDIM forms nonnative disulfide bonds in the substrate and then GmPDIL-2 folds and rearranges these disulfide bonds into native bonds (Figure 3-19A).

In addition to synergistic effects on folding, the addition of GmPDIL-2 to GmPDIM accelerated the formation of disulfide bonds in RNase A. GmPDIL-2 may increase the net substrates for GmPDIM by reducing nonnative disulfide bonds and stimulating folding. However, even when GSH was used as a substrate, GmPDIL-2 increased the rate of oxidation of GmPDIM by GmERO1, suggesting that increasing the rate of oxidation does not depend on substrate folding only. I found that the active site of the **a** domain of GmPDIM oxidized the active site of either the **a** or **a'** domain of GmPDIL-2 (Figure 3-16, A–F). The increase in the oxidation rate of GmPDIM by GmERO1a may be explained by the transfer of disulfide

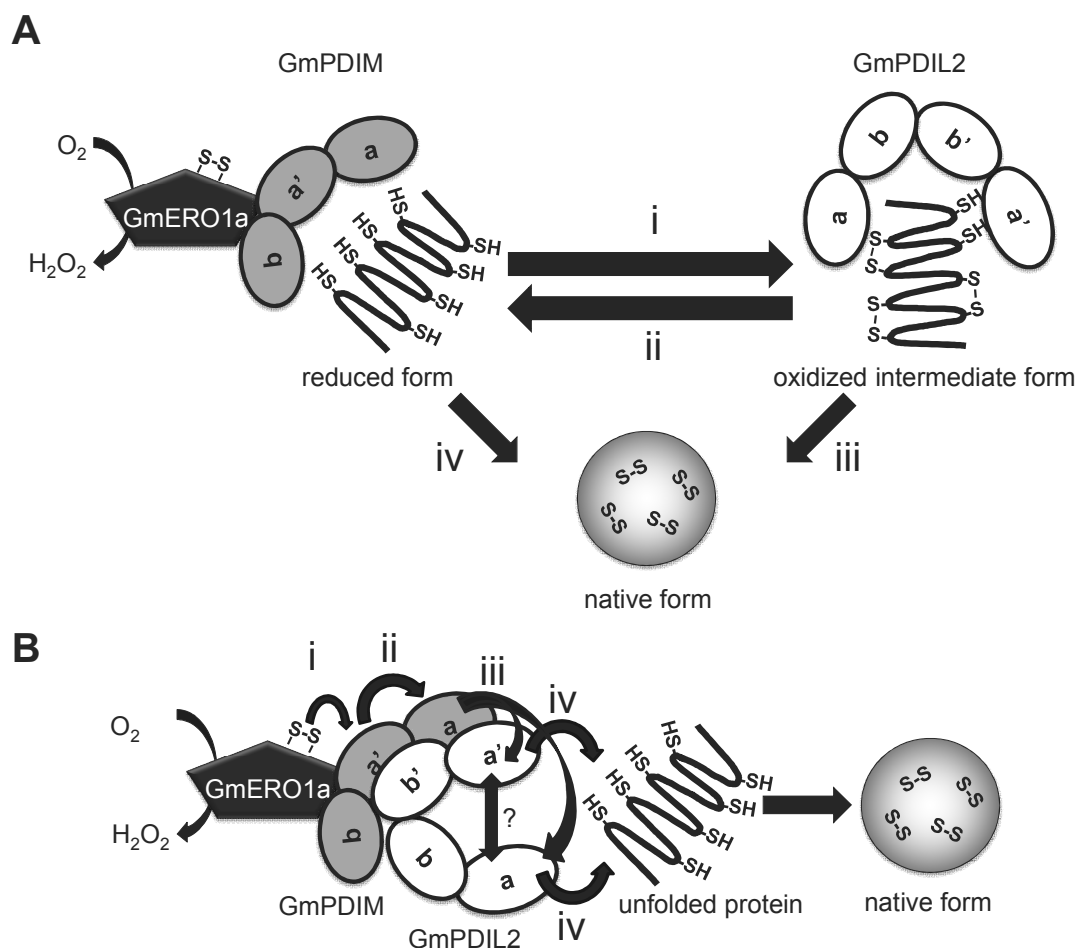


Figure 3-19. Models of cooperation of GmPDIM and GmPDIL-2.

(A) Role-sharing model of GmPDIM and GmPDIL-2. GmPDIM oxidized by GmERO1a introduces transient disulfide bonds in an unfolded substrate protein (i). Transient disulfide bonds in the substrate protein are reduced by the reduced form of GmPDIL-2 (ii). Reduced thiols are rearranged into native disulfide bonds, mainly by GmPDIL-2 (iii), but partially by GmPDIM (iv).

(B) Model of oxidative relays from GmERO1a to GmPDIM, GmPDIL-2, and substrate. GmERO1a oxidizes the active site in the **a'** domain of GmPDIM (i). The formed disulfide bond is transferred sequentially from the **a'** domain of GmPDIM to the **a** domain of GmPDIM (ii), either the **a** or **a'** domain of GmPDIL-2 (iii), and substrate (iv). Transferring of a disulfide bond between the **a** and **a'** domains of GmPDIL-2 is unclear. Both **a** and **a'** domains of GmPDIM and both **b'** and **a'** domains of GmPDIL-2 are essential for the association of GmPDIM and GmPDIL-2.

bonds from GmPDIM to the associated GmPDIL-2, in addition to substrate proteins. The absolute concentration of glutathione in the ER is unknown; however, a consensus exists that the total glutathione concentration in the ER is around 9 to 10 mM and that the ratio of $[GSH]^2/[GSSG]$ in the ER is around 3:1 (Hatahet and Ruddock, 2009). From these values, the reduction potential for ER glutathione is estimated as -191 mV. Under these redox conditions in the ER, the active sites of GmPDIM and GmPDIL-2 would be largely reduced. This means that GmPDIM, once oxidized by GmERO1a, would oxidize GmPDIL-2.

Using the active-center mutants, a model of the pathway of disulfide bond transfer from GmPDIM to GmPDIL-2 can be constructed (Figure 3-19B). First, GmERO1a entirely oxidizes the **a'** domain of GmPDIM (i). The preference of GmERO1a for the **a'** domain of GmPDIM is probably due to the reduction potential and the affinity between GmERO1a and the **a'** domain of GmPDIM. Since the reduction potential of the **a'** domain was lower than that of the **a** domain, the **a'** domain will be more easily oxidized by GmERO1a. Then, the oxidized GmPDIM transfers a disulfide bond to GSH or GmPDIL-2 mainly from the **a** domain, because the oxidation of GmPDIM(C64/67A) and GmPDIM(C67A) was significantly lower than that of wild-type GmPDIM (Figure 3-14). This finding suggests that the disulfide bond transferred to the **a'** domain of GmPDIM from GmERO1a is intramolecularly transferred to the **a** domain (ii). The oxidation of the **a** domain by the **a'** domain is thermodynamically contradictory, as the apparent redox equilibrium constant of the **a** domain of GmPDIM(C192/195A) (0.73) was lower than that of the **a'** domain of GmPDIM(C64/67A) (3.53). If the **a'** domain of GmPDIM is oxidized, the redox equilibrium constant of the **a** and/or **a'** domain may be altered by conformational changes such that the **a** domain becomes susceptible to oxidation by the **a'** domain. In human PDI, the **a'** domain was first oxidized by Ero1 α , which then oxidizes the **a** domain, and the reduced **a'** domain is oxidized by Ero1 α . During the sequential transfer of disulfide bonds, PDI dynamically changes its structure from a closed form to an open form (Nakasako *et al.*, 2010; Serve *et al.*,

2010; Wang *et al.*, 2013). Subsequently, disulfide bonds are transferred from the GmPDIM to either the **a** or **a'** domain of GmPDIL-2 (iii) and from the oxidized GmPDIL-2 to a protein substrate (iv).

Human PDI has been shown to oxidize other ER oxidoreductases, such as ERp47, ERp57, and P5 (Araki *et al.*, 2013; Oka *et al.*, 2015). Therefore, Ero1 α and PDI are thought to constitute a regulatory hub for the regulation of the redox states of ER oxidoreductases. In contrast, multiple soybean ER oxidoreductases are able to be oxidized by GmERO1a. Therefore, in plant, it is thought that Ero1 and ER oxidoreductases constitute a robust network for disulfide bond transfer and the regulation of redox homeostasis in the ER. The relay of oxidizing equivalents from one ER oxidoreductase to another may play an essential role for cooperative oxidative folding by multiple ER oxidoreductases.

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SUMMARY AND CONCLUSIONS

CHAPTER 1

The cDNA-encoded soybean Ero1 orthologs GmERO1a and GmERO1b were cloned. GmERO1 proteins were shown to be ER-localized membrane proteins modified with one or more high-mannose type N-glycan(s). GmERO1 proteins were expressed ubiquitously in soybean tissues. The expression levels of GmERO1 proteins and their mRNAs were high when seed storage proteins such as β -conglycinin and glycinin were synthesized in the cotyledon. These results suggest that GmERO1 proteins are involved in the oxidative folding of seed storage proteins.

CHAPTER 2

An active recombinant GmERO1a expression system was established in *Escherichia coli*. GmERO1a was found to oxidize multiple soybean ER oxidoreductases, GmPDIL-1, GmPDIM, GmPDIS-1, and GmPDIS-2, in contrast to mammalian Ero1, which has a high specificity for protein disulfide isomerase. On the other hand, GmPDIL-2 was hardly oxidized by GmERO1a. This substrate specificity of GmERO1a is partly explained by the affinity between GmERO1a and ER oxidoreductases.

CHAPTER 3

GmPDIM was found to be associated *in vivo* and *in vitro* with GmPDIL-2. GmPDIL-2 synergistically accelerated oxidative refolding by GmPDIM and GmERO1a. In this process, GmERO1a preferentially oxidizes the active site in the **a'** domain of GmPDIM. The disulfide bond was shown to be transferred to the active site of the **a** domain of GmPDIM and then to the active sites of the **a** or **a'** domain of GmPDIL-2. GmPDIL-2 also accelerates rearrangement of disulfide bonds by reduction-oxidation cycles. Taken together, our data

show that two soybean ER oxidoreductases and GmERO1a achieve cooperative oxidative folding by sophisticated molecular mechanisms: GmPDIM introduces transient disulfide bonds into substrates, and GmPDIL-2 rearranges these disulfide bonds to native ones. In addition, GmPDIL-2 also introduces disulfide bonds accepted from GmPDIM into substrates. This is the first demonstration in plants of a relay of disulfide bonds produced by GmERO1a from one ER oxidoreductase to another. The findings of this study may explain why higher eukaryotes have multiple ER oxidoreductases. It is hoped that these findings will contribute to a general understanding of oxidative protein folding in the plant ER, and thereby lead to the wide application of soybean as an expression system.

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