We have isolated a protein-disulfide isomerase (PDI) from *Oldenlandia affinis* (OaPDI), a coffee family (Rubiaceae) plant that accumulates knotted circular proteins called cyclotides. The novel plant PDI appears to be involved in the biosynthesis of cyclotides, since it co-expresses and interacts with the cyclotide precursor protein Oak1. OaPDI exhibits similar isomerase activity but greater chaperone activity than human PDI. Since domain c of OaPDI is predicted to have a neutral pI, we conclude that this domain does not have to be acidic in nature for PDI to be a functional chaperone. Its redox potential of \(-157 \pm 4\) mV supports a role as a functional oxidoreductase in the plant. The mechanism of enzyme-assisted folding of plant cyclotides was investigated by comparing the folding of kalata B1 derivatives in the presence and absence of OaPDI. OaPDI dramatically enhanced the correct oxidative folding of kalata B1 at physiological pH. A detailed investigation of folding intermediates suggested that disulfide isomerization is an important role of the new plant PDI and is an essential step in the production of insecticidal cyclotides.

Cyclotides have a characteristic surface-exposed patch of hydrophobic residues that accounts for their late elution on reverse-phase HPLC and membrane binding properties (15). In general, the exposure of a hydrophobic patch on a protein surface is energetically unfavorable, thus requiring special conditions to facilitate its formation. In the case of cyclotides, the hydrophobic patch is locked in place by the disulfide bonds of the cyclic cystine knot motif once it is formed, but the driving force for initial formation of the hydrophobic patch is not known. In *vivo*, a hydrophobic co-solvent, such as 50% 2-propanol in aqueous buffer, markedly enhances the folding efficiency of cyclotides (16, 17), but the mechanism of folding *in vivo* has until now remained unknown. Fig. 1 summarizes the current understanding of cyclotide biosynthesis.

In this study, we isolated a novel cDNA clone encoding a protein-disulfide isomerase from the cyclotide-producing plant *O. affinis*. After having established a transcriptional co-expression and biomolecular interaction of the cyclotide precursor Oak1 and the novel PDI (OaPDI), we compared enzyme activities of the recombinant protein with that of recombinant human PDI proteins and related the results to unique primary sequence features of the plant PDI. We examined the role of OaPDI in oxidative folding of the prototypic cyclotide kalata B1 and a synthetic linear analogue. This is the first time that the oxidative folding of a cyclic cystine knot protein has been studied using PDI. The results have application in the large scale production of native and modified cyclotides *in vitro* and *in vivo*, particularly when exploiting their potential as molecular scaffolds for pharmaceutical applications. This study more...
**Mechanism and Function of a Novel Plant PDI**

**FIGURE 1. Biosynthesis and structures of cyclotides.** The prototypic cyclotide kalata B1 is synthesized in *O. affinis* as part of a precursor protein, Oak1 (~11 kDa), comprising an ER target signal, the N-terminal pro-region, an N-terminal repeat (NTR), the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor proteins contain up to three mature cyclotide domains. Processing of the precursor involves oxidative folding by an oxidoreductase enzyme to form three disulfide bonds, excision of the mature cyclotide domain, and a C-terminal propeptide (CTPP) (9). Other cyclotide precursor prote
teins were stored at 4 °C and used within 7 days of purification. Purity and molecular mass were confirmed using SDS-PAGE and/or MS.

**Liquid Chromatography-MS Analysis**—MS analysis was performed on an HP series 1100 liquid chromatograph coupled to a Micromass LCT mass spectrometer (Waters) equipped with an electro spray ionization source. Samples were subjected to a gradient of 90% acetonitrile with 0.1% formic acid over 0.1% formic acid (25–75% over 30 min at 0.3 ml min−1). Mass spectra were acquired over a mass range of 800–1800 Da with a capillary voltage of 3.5 kV and a cone voltage of 30 V.

**Reverse Transcription PCR**—Total RNA was extracted from O. affinis seedling (2 weeks old), leaf, flower, and root and were reverse transcribed (minus RT for controls) to yield cDNA. Gene-specific primers were designed for Oak1 (5′-ACTGATGTCGCAGGAAGAT-TTATGGGACATCAACATCACA-3′) and OakPDI (5′-GCTGAGGAATGTGGTGAGGAG-3′ and 5′-CCACAAGACATCCAAAGCC-3′), and the housekeeping gene O. affinis GAPDH (5′-AATCAGGAACGTCTCGTTGCTA-3′ and 5′-GGTAGTGCAACTGGCATTGG-3′). Expression levels were analyzed by PCR using 25–35 cycles. Samples were separated on an agarose gel, and band intensities were quantified.

**Antibody Production and Western Blotting**—A partial OakPDI protein comprising domains b’ and a’ was expressed in the same manner as full-length OakPDI, and the protein was purified from insoluble inclusion bodies using FastBreak reagent (Promega) according to the manufacturer’s instructions, metal affinity chromatography (as described above), and RP-HPLC (Solvent A: Milli-Q water with 0.05% trifluoroacetic acid; Solvent B: 90% acetonitrile with 0.05% trifluoroacetic acid). Puriﬁed protein was used to raise polyclonal antibodies in rabbits. The IgG fractions in the preimmune and immune serum were quantiﬁed.

**Production of Recombinant Oak1 and Biomolecular Interaction of OakPDI and Oak1**—The coding region of Oak1 cDNA minus the ER signal sequence was ampliﬁed by PCR with the primers 5′-CGGGATCTTTGGATCTGAGCTT-3′ and 5′-GGTAGTGCAACTGGCATTGG-3′ and cloned into the pSET-A plasmid (Invitrogen) using BamHI and HindIII restriction endonucleases. The resulting plasmid, incorporating an N-terminal His6 tag, was transformed into E. coli BL21(DE3) cells (Stratagene). The cells were grown and induced (at 30 °C) and lysed as described above for OakPDI to yield soluble recombinant Oak1 protein, which was puriﬁed to homogeneity using metal afﬁnity and size exclusion chromatography as described above for OakPDI. Puriﬁcation was monitored by SDS-PAGE and MS. Surface plasmon resonance was performed with the BIACORE biosensor system 3000 (Biacore) as reported earlier (22). Oak1 protein was prepared in HBS buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20 (v/v)) at 5, 10, and 20 μM, containing 2 mM reduced glutathione. Data analysis was performed using BIA evaluation software (version 3.2).

**Isomerase and Chaperone Assays**—The isomerase activity of OakPDI was determined according to the method of Lambert and Freedman (23). In this method, the reshufﬂing of disulﬁde bonds in insulin (Sigma) catalyzed by GSH is associated with the reduction of GSSG to GSH that is mediated by NADPH (Sigma) and glutathione reductase. The associated conversion of NADPH to NADP+ was monitored by change of absorbance at 340 nm. Chaperone activity, as determined by the prevention of aggregation of denatured rhodanese (Sigma), which contains no disulﬁde bonds, was measured according to Martin et al. (24). The aggregation of denatured rhodanese was investigated by monitoring the increase in absorbance at 320 nm. Puriﬁed hPDI and hP5 were used as controls. Isomerase and chaperone activities were expressed as percentages by normalizing the absorbance values at time points of 10 and 15 min, respectively, to the corresponding value for hPDI (set to 100%).

**Determination of Redox State and Circular Dichroism Spectroscopy**—The presence of free thiols in puriﬁed OakPDI (2–4 μM) was determined using the method of Ellman (25). Circular dichroism spectra were obtained on a Jasco J-810 spectropolarimeter. Spectra of native OakPDI (0.48 μM) were recorded at 25 °C in 1 mM KH2PO4, pH 7.0, and heat-denatured (30 min incubation at 25 °C prior to measurement) and heat-denatured (3 min incubation at 95 °C prior to measurement). Secondary protein structure contents were predicted using k2d (available on the World Wide Web).

**Fluorescence Spectroscopy and Redox Potential Measurements of OakPDI**—Fluorescence emission spectra of OakPDI (0.5 μM) were recorded at 25 °C on a Jasco FP-770 spectrophotometer in 50 mM KH2PO4, pH 7.0, 1 mM EDTA. OakPDI was oxidized by adding 0.5 mM GSSG and reduced by adding 1 mM dithiothreitol for 30 min. The redox potential of OakPDI was measured using a redox equilibrium of 1.7 μM protein and different ratios of reduced to oxidized glutathione ([GSH]/[GSSG]) from 1 to 6 × 10−7 M by keeping [GSSG] constant at 0.5 mM and varying [GSH] from 0.08 to 10 mM. All buffer components were prepared in degassed 50 mM KH2PO4, KOH, pH 7.0, 1 mM EDTA and allowed to reach equilibrium for 12–16 h at 25 °C. After excitation at 280 nm, the ﬂuorescent emission was measured on a PerkinElmer Life Sciences LS50B spectrophotometer for 30 s at 338 nm. The equilibrium constant Keq was determined (26), and the redox potential of OakPDI was calculated using the Nernst equation at standard conditions (E′o[GSSG]/[GSH] = −240 mV, pH 7.0, 25 °C).

**Extraction, Synthesis, and Purification of Cyclotides**—Native kalata B1 was isolated from O. affinis as reported earlier (5) and puriﬁed by RP-HPLC. Oxidized kalata B1 (6 mg ml−1) was reduced by incubating in 0.1 mM NH4HCO3, pH 8.5, with 0.25–1.0 mM dithiothreitol at 25 °C for 3 h with agitation. Linear kalata B1 was assembled on phenylacetamidomethyl resin by manual solid-phase peptide synthesis using an in situ neutralization/HBTU protocol for Boc chemistry as previously described (27). Purity of peptides was conﬁrmed by RP-HPLC and MS. Concentrations were determined by A280 measurement (εkalata B1 = 6410 liters mol−1 cm−1).
Mechanism and Function of a Novel Plant PDI

A

| Domain a | b | b' | linker x | a' | c |
|----------|---|----|----------|----|---|
| hPDI    | CGHCK |   |   | CGHCK | KDEL |
| OaPDI   | CGHCQ |   |   | CGHCQ | KDEL |

B

| Amino Acid Sequence |
|---------------------|
| hPDI                |
| OaPDI               |
| AtPDI               |
| yPDI                |
**Mechanism and Function of a Novel Plant PDI**

Oxidative Folding of Linear and Reduced Kalata B1—Oxidative folding was performed at 25 °C in folding buffer containing 0.05 m Tris-HCl, 1.4 mm EDTA, pH 7.5, 2 mm GSH. Aliquots were removed from the different reactions at various time intervals between 1 min and 24 h, quenched with 4% trifluoroacetic acid, and analyzed by RP-HPLC. PDI-assisted folding was initially tested using bovine PDI (4 μM) (Sigma) to optimize experimental conditions. Deoxyribonuclease (bovine, type I; Sigma) and lysozyme C (chicken egg white; Sigma) were used as control proteins. Kinetic analysis of oxidative folding was carried out with final concentrations of 50 μM reduced peptides and 3.9 μM OaPDI (molar ratio of peptide/enzyme ~ 13:1).

**Mass Spectrometric Analysis of Oxidative Folding**—MS analysis of folding intermediates was carried out by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Voyager-DE STR Biospectrometry Work station operated in positive ion reflector mode. RP-HPLC-derived peptide (80 μM) fractions were lyophilized and alkylated by incubation with 140 mM iodoacetamide, 0.1 M NH₄HCO₃ (pH 8.5) at 65 °C for 1 min. The reaction was quenched with 4% trifluoroacetic acid.

**NMR Analysis of Linear and Cyclic Kalata B1**—A large scale folding experiment (50 μM reduced peptide in 0.1 M NH₄HCO₃, 2-propanol (1:1, v/v), pH 8.5, 2 mM GSH) was carried out to produce peptide for NMR analysis. Following a 24-h incubation, the mixture was subjected to RP-HPLC, and the native oxidized peak was collected and freeze-dried, yielding ~1.5 mg of peptide. All NMR spectra were recorded on a Bruker ARX 600 spectrometer (298 K, pH ~ 3), processed using Topspin (Bruker) software, and assigned as reported earlier (28).

**Large Scale OaPDI-assisted Folding of Cyclic Kalata B1 and Larval Antifeedant Assays**—Reduced cyclic kalata B1 (50 μM) was folded at 25 °C in folding buffer containing OaPDI (3.8 μM) for 24 h. Folded peptide was purified by RP-HPLC and analyzed by MS and one-dimensional NMR. Antifeedant activity was determined using an adaptation of a previously reported method (9). Briefly, third instar Helicoverpa armigera larvae, which were raised on an artificial haricot bean diet, were starved for 3 h and transferred to diets supplemented with native kalata B1 (0.21%, w/v), OaPDI folded kalata B1 (0.21%, w/v), and a casein control. After 16 h, the larvae were weighed, and the amount of frass produced and diet remaining was recorded.

**RESULTS**

Cyclotides are structurally unique proteins with a topologically complex arrangement of disulfide bonds, suggesting that auxiliary proteins may be important in their correct folding in the cell. Relatively few plant PDI systems are known, and none has been characterized with respect to their role in folding plant defense proteins. Thus, we isolated cDNA sequences encoding PDI from the Rubiaceae plant O. affinis using a PCR approach with oligonucleotides based on conserved regions in known PDI proteins from higher eukaryotes. Transcriptional analysis and surface plasmon resonance studies suggest co-expression and biomolecular interaction of the novel OaPDI and the cyclotide precursor Oak1. Oxidative folding of the prototypic cyclotide kalata B1 and a synthetic linear analogue of this macrocyclic peptide was further studied in vitro using RP-HPLC, MS, and NMR. We examined the folding of cyclotide precursors in the presence of PDI to better understand the mechanism of PDI and the biosynthetic pathway of plant cyclotides. We further characterized this novel plant PDI by comparing its chaperone and isomerase activities to human PDI proteins and here for the first time report the electrochemical redox potential of a plant PDI protein.

**Isolation and Primary Sequence of the Novel Plant PDI**—Clones from a screen of an O. affinis cDNA library were sequenced, and the deduced mature OaPDI sequence (Fig. 2) was used for BLASTp similarity analysis. The highest score hits were for other plant PDI and PDI-like sequences, including thale cress (Arabidopsis thaliana; Swissprot accession codes NP_851234, AAM65262/Q8LAM5, NP_191056), rice (Oryza sativa; BAD38565/Q671X6), African oil palm (Elaeis guineensis; AAO26314/Q84XU4), and corn (Zea mays; AAX09962/Q5EUD9) PDIs. The mature OaPDI sequence, without the ER signal, comprises 491 amino acids and to a large extent shows typical primary structure features of a PDI. The two highly conserved TRX motifs, domain boundaries, and interdomain distances are comparable with those of hPDI (Swissprot P07237), as outlined in Fig. 2. However, the new PDI is different from most known PDIs in several respects. First, it contains a Gln residue instead of a Lys following both active site motifs, and second, the sequence of its C-terminal domain c has a high predicted pI (6.6) in contrast to an acidic (low pI) domain c in other known PDIs.

**Expression of OaPDI and Interaction of OaPDI and Oak1**—Transcriptional co-expression of OaPDI and the Oak1 precursor protein were established using reverse transcription-PCR. Both transcripts co-express in a variety of plant tissues, and their transcriptional regulation within those tissues coincides (Fig. 3A) (i.e. OaPDI and Oak1 are expressed at a similar level) (standardized relative to a housekeeping gene GAPDH). OaPDI was detected by Western blot in O. affinis extract. Similar PDI proteins are expressed in other cyclotide-producing (V. odorata) and non-cyclotide-producing plants (N. benthamiana), but no related proteins were detected in a human cell extracts control (Fig. 3B).

**FIGURE 2. Domain structure and alignment of PDI proteins.** A, the domain structures of HPD, OaPDI, and hP5. OaPDI has a typical PDI domain structure (i.e. a-b-a'-b'-x-a'-c). Domain boundaries were identified by sequence alignment to the human and yeast PDI sequences. All domains are highlighted by color: active site domains a (magenta) and a’ (red) for PDI and a’’ (gray) and a’ (gray) for hP5, respectively; domains b (blue) and b’ (yellow) and C-terminal extension domains (green) of PDI proteins. Thioredoxin motifs are indicated as TRX 1 and 2, respectively. The C-terminal extension domain of hP5 has not been fully characterized yet and is named as other. Interdomain linkers are indicated as black lines. The endoplasmic reticulum signal KDEL is shown using gray color. The endoplasmic reticulum signal KDEL is shown using gray color.
Mechanism and Function of a Novel Plant PDI

The dissociation constant \( K_{eq} \), which defines the strength of this interaction, was measured to be 7.2 μM.

Recombinant Production and Biochemical Characterization of OaPDI—For biochemical in vitro analysis, mature OaPDI protein was produced recombinantly in E. coli cells as a His fusion protein and purified using a metal affinity column and size exclusion chromatography (Fig. 4, A and C). The purified product was characterized by MS and has a molecular mass of 55,914 ± 18 Da. The oxidation state of the PDI was determined, and the number of free thiols was ∼0.7 per molecule of PDI on average. OaPDI contains four cysteines, which are located in the active site motifs and, unlike mammalian PDIs, does not contain any non-active site cysteines. Therefore, the isolated protein was predominantly oxidized with its active site cysteines in the disulfide form.

The secondary structures of the reduced, native, and heat-denatured OaPDI were examined by circular dichroism. Spectra of native and reduced OaPDI exhibited a typical α-helical structure (Fig. 4D), and the calculated α-helical content was >31% for both native and reduced proteins, confirming a well defined secondary structure. Heat denaturation dramatically changed the circular dichroism spectra and suggested a random coil structure of the denatured protein (Fig. 4D). Having identified and purified the novel PDI and showed that it was folded into a defined structure, we then characterized it functionally.

Isomerase and Chaperone Activities and Redox Potential of OaPDI—Isomerase activity of OaPDI was measured using a well established assay based on the correct reshuffling of disulfide bonds in insulin (23). Human PDI, human P5 (hp5) (Fig. 2A), and mutants of these proteins have been studied extensively (19, 20, 29). Human PDI and hp5 were both purified to homogeneity (Fig. 4B) and provided a basis for comparison with the new plant PDI. Specifically, we compared the isomerase and chaperone activity of OaPDI with hp5. By contrast, hp5, a redox-active member of the PDI family, has significantly lower chaperone activity of OaPDI with hPDI. By contrast, hp5, a redox-active member of the PDI family, has significantly lower chaperone activity than hp5 and was used as control. As is clear from Fig. 4E, OaPDI and hp5 have about 70 and 29% of the isomerase activity of hp5, respectively. Chaperone activity was determined in an assay measuring inhibition of aggregation of rhodanase. Interestingly, OaPDI had increased (140%) chaperone activity relative to hp5 and hp5 had reduced activity (65%), as deduced from Fig. 4F.

The electrochemical potential of OaPDI was measured using the redox equilibrium between OaPDI and glutathione (Fig. 5, A and B). OaPDI was allowed to reach redox equilibrium with a redox pair of known potential, namely reduced and oxidized glutathione. The equilibrium of this redox reaction yielded an average equilibrium constant of \( K_{eq} = 1.6 \mu \text{M} \), and the redox potential of OaPDI was calculated using the Nernst equation to be \(-157 ± 4 \text{ mV} \), which is similar to the known redox potential of PDI values from other higher organisms (Fig. 5C).

These studies established that the new plant PDI is functional in chaperone and isomerase assays and comparable in its redox potential with other known PDI proteins. Hence, it was of interest to determine if it has a role in the folding of the major protein products of O. affinis, namely cyclotides. Cyclotides are processed from larger precursor proteins, but it has not yet been established whether they are oxidized prior to cyclization.

After having established the protein presence in plant extracts and co-expression of OaPDI and Oak1 at the transcriptional level, we analyzed their biomolecular interaction by surface plasmon resonance. Biacore sensograms (Fig. 3C) showed a typical concentration-dependent protein-protein interaction.

![Figure 3](image-url)
we examined the effects of PDI on both linear and cyclic forms of the prototypic cyclotide kalata B1.

Kinetic Analysis of Oxidative Folding of Cyclotides Using OaPDI—Reduced linear and cyclic kalata B1 peptides were prepared, and their purity and molecular masses were confirmed by MS and RP-HPLC. Initially, the folding was studied using bovine PDI to optimize the concentration of enzyme and substrates for folding assays. Following these initial studies, a Tris/EDTA buffer at physiological pH (7.5) was chosen for a detailed analysis of OaPDI-assisted folding of linear and cyclic kalata B1 (Fig. 6, A and B), and it was clearly established that the PDI has a profound influence on folding. As shown in Table 1, the folding rate was greater for linear kalata B1 than for cyclic kalata B1, but the latter folded to the correct disulfide-bonded product with a greater yield. The difference between the yields obtained in the presence and absence of PDI in the same folding buffer was striking (Fig. 6C). Without OaPDI, neither linear (0.6% yield) nor cyclic kalata B1 (1.1% yield) were effectively oxidized into the native disulfide conformation under physiologically relevant conditions. In the presence of OaPDI, the yields of correctly folded linear and cyclic kalata B1 peptides after 24 h were 6 and 29%, respectively. As a further control and to examine the potential effects of molecular crowding, we undertook folding studies in the presence of lysozyme and deoxyribonuclease as examples of Cys-containing proteins with no PDI function. In the presence of these proteins and in the absence of PDI, the yields of native folded peptides were insignificant relative to PDI-assisted folding, and therefore the role of PDI as purely a molecular crowding agent was excluded.

To confirm the native fold and disulfide connectivity of the oxidized cyclotides, a large scale folding reaction in 50% 2-propanol, a hydrophobic solvent that has been previously shown to assist folding yields of cyclotides in vitro (27), was carried out to obtain enough linear and cyclic kalata B1 for 1H NMR spectro-

or cyclized prior to oxidation. Correctly folded cyclotides can be made in vitro by synthetic strategies using either order of these events (16), although it seems likely that oxidation of a linear precursor occurs prior to cyclization in vivo. Therefore,
**Mechanism and Function of a Novel Plant PDI**

**FIGURE 5.** Fluorescent spectroscopy and electrochemical redox potential of OaPDI. A, fluorescent emission scan from 300 to 400 nm after excitation at 280 nm for oxidized (dotted line) and reduced OaPDI (solid line). The emission at 338 nm had the greatest difference between oxidized and reduced protein (as indicated by a scale bar). B, graph of the redox equilibrium between OaPDI and the glutathione redox pair (GSH/GSSG). Two independent measurements at various ratios of reduced to oxidized glutathione ([GSH]/[GSSG]) are plotted versus the fraction of reduced OaPDI. The equilibrium constant \( K_{eq} = 1.6 \mu M \) for this redox reaction was obtained from a best fit sigmoidal curve \( (r = 0.973) \) as the midpoint where the curvature changes. C, comparison of the redox potential of several oxidoreductases, such as thioredoxin (TRX1), bacterial DsbA, the two cysteine pairs of DsbB (P1 and P2), DsbC, DsbD, mammalian PDI (mPDI); A1 and A2) (38). The redox potential for OaPDI was calculated using the Nernst equation with the \( K_{eq} \) obtained as described above. DTT, dithiothreitol.

A comparison of the H\(_n\) backbone chemical shifts of these peptides with native kalata B1 (Fig. 6D) confirmed that both were correctly folded based on published chemical shifts (28). Furthermore, the retention times of linear and cyclic kalata B1 were identical regardless of whether folding was OaPDI- or solvent-assisted, confirming that the three-dimensional structures of the peptides folded with OaPDI or in the presence of 2-propanol were identical.

The integrity of OaPDI-produced kalata B1 was verified biologically by comparing its activity as an insecticidal agent to native plant-extracted kalata B1. *Helicoverpa armigera* larvae were fed with native kalata B1, OaPDI-folded kalata B1, and a casein control diet. The plant-extracted and OaPDI-produced peptides had identical antifeedant effects on the larvae relative to the noninsecticidal control protein (Fig. 6E).

**Characterization of Folding Intermediates**—We next determined the disulfide content of intermediates in the folding pathway in an attempt to see if the PDI-assisted mechanism was similar to that reported previously for *in vitro* folding in the presence of 50% 2-propanol and to see how PDI-assisted folding in physiological buffer differs from nonenzymatic folding. The quenched mixtures were separated by RP-HPLC, and major peaks were collected for alkylation and MS analysis. The intermediates identified in the presence of OaPDI are summarized in Table 2. PDI-assisted folding pathways for both peptides appeared more complex with respect to the number of intermediate species and the accumulation of Ila but were generally similar to that seen for hydrophobic solvent-assisted folding (16, 17). To further study the function of the new PDI, folding intermediates in physiological buffer in the absence of correct connectivity. This isomerization step is necessary to produce fully functional insecticidal cyclotides. The results provide a valuable insight into the mechanism of PDI but more importantly provide the first evidence for oxidative folding of cyclic cystine knot proteins using PDI as a catalyst.

PDIs from all organisms contain a conserved TRX active site motif (CXXC) in which the central dipeptide plays a critical role in oxidoreductase activity. A distinguishing feature of OaPDI is that it contains a Gln residue following both active site motifs, in contrast to hPDI, which contains a Lys at the corresponding position in both domains. Gln occurs only very rarely at this position among known PDIs, and the Lys reportedly is important for isomerase activity of hPDI (29). The PDI-like protein hP5, which contains a Gln instead of the Lys in domain a, has greatly compromised isomerase activity. Replacing Gln with Lys in the active site of hP5 increases the isomerase activity, and conversely a Lys to Gln exchange in the Lys in domain a, is usually very acidic (low pI) in PDI proteins from other organisms, but its exact role is unclear. On one hand, domain c of human PDI has been suggested as not critical for chaperone activity, but expression of the protein is expressed in a plant that produces circular defense proteins. OaPDI and cyclotide precursor Oak1 transcripts are co-expressed, and the two proteins show a strong biomolecular interaction, suggesting an *in vivo* interaction. We recombinantly produced the new PDI and showed that it is important for the oxidative folding of linear and cyclic forms of the prototypic cyclotide kalata B1 in *vitro*. OaPDI significantly increases the yield of correctly disulfide-bonded species in *vitro* and is able to shuffle nonnative disulfide bonds into their correct connectivity. This isomerization step is necessary to produce fully functional insecticidal cyclotides. The results provide a valuable insight into the mechanism of PDI but more importantly provide the first evidence for oxidative folding of cyclic cystine knot proteins using PDI as a catalyst.
activity on the basis of structural and activity studies of deletion mutants (30). On the other hand, domain c has been suggested to stabilize the overall structure of PDI under denaturing conditions (31). This suggestion is supported by the yeast PDI structure, which contains an α-helix in domain c that stabilizes the structure of domain a (32). The potential importance of the acidic nature of domain c has not previously been established, but in this study we have provided the first activity data for a PDI containing a nonacidic domain c. OaPDI exhibits greater chaperone activity than hPDI, so it is clear that domain c does not have to be acidic for PDI to be a functional chaperone.

Other sequence features of OaPDI are similar to known PDIs, in particular its domain structure and redox potential. Specifically, the PDI sequence from O. affinis has similar domain boundaries and interdomain distances to yeast, plant, and human PDI proteins. Not surprisingly, OaPDI is more similar to other plant PDIs than to human PDI, consistent with the observation that polyclonal raised antibodies to OaPDI also detect similar proteins in other plants. There are at least 22 different PDIs present in the ER of A. thaliana (33), and O. affinis plants may also express other PDI and PDI-like proteins, since we have isolated additional sequences showing similarity to PDI-like proteins. Furthermore, OaPDI has a redox potential of \(-157 \pm 4\) mV, which is similar to reported redox potentials for mammalian PDI proteins (34–36). Yeast PDI, a very potent isomerase, compared with mammalian PDIs (37), has a more negative redox potential (\(-188\) and \(-152\) mV for the cysteines in domain a and domain a', respectively (38)). Although the biological behavior of dithiol-disulfide oxidoreductases cannot solely be deduced from their *in vitro* redox potential, this value is often a valuable indicator for *in vivo* function of a novel oxidoreductase. Hence, by analogy and with the known function of other PDI proteins, OaPDI is likely to have a role in the folding of cellular proteins in the plant O.

4 C. W. Gruber and D. J. Craik, unpublished observations.
Mechanism and Function of a Novel Plant PDI

TABLE 2
Folding intermediates in OaPDI-assisted cyclotide folding

Retention times and relative abundances (relative area of peptide species of total area under peak) were taken from the quantitative folding experiments. Molecular mass is shown as monoisotopic [M + H], unless mass was not determined (ND). Retention times and relative abundances were taken from the quantitative folding experiments. Expected masses [M + H] for alkylated peptides are as follows: linear reduced (0SS), 3257.2 Da; linear one-disulfide intermediates (1SS), 3141.2 Da; linear two-disulfide intermediates (2SS), 3025.2 Da; and linear native oxidized or misfolded (3SS) 2909.2 Da; cyclic reduced (0SS) 3141.2 Da; cyclic one-disulfide intermediates (1SS), 3123.2 Da; cyclic two-disulfide intermediates (2SS), 3007.2 Da; and cyclic native oxidized or misfolded (3SS), 2891.2 Da. Folding conditions were as follows: 80 mM Tris-HCl, 1.4 mM EDTA, 2 mM reduced glutathione, pH 7.5, 25 °C, 6.2 mM purified OaPDI.

| Folding time | Peptide species | Retention time | Abundance | Molecular mass | Disulfide species |
|--------------|-----------------|----------------|-----------|----------------|------------------|
|              |                 | min            | %         | Da             |                  |
| Linear kalata B1 + OaPDI |                 |                |           |                |                  |
| 1            | Reduced         | 39.0           | 30        | 3257.6         | 0SS              |
|              | Intermediate Iα | 37.6           | 34        | 3141.6         | 1SS              |
|              | Intermediate Iβ | 35.1           | 7.5       | 3141.2         | 1SS              |
|              | Intermediate Iγ | 36.3           | 6.1       | 3141.1         | 1SS              |
| 3            | Intermediate Iα | 37.4           | 16        | 3141.4         | 1SS              |
|              | Intermediate Iβ | 40.5           | 9.6       | 3025.2         | 2SS              |
|              | Intermediate Iγ | 33.1           | 7.9       | 3025.4         | 2SS              |
|              | Intermediate Iδ | 33.8           | 8.4       | 3025.7         | 2SS              |
|              | Misfolded IIβ   | 38.7           | 16        | 2909.5         | 3SS              |
|              | Native           | 57.4           | <4.0      | ND             | 3SS              |
| 24           | Misfolded IIα   | 39.1           | 25        | 2909.2         | 3SS              |
|              | Misfolded IIβ   | 38.4           | 28        | ND             | 3SS              |
|              | Native           | 57.5           | 8.0       | 2909.4         | 3SS              |

Cyclic kalata B1 + OaPDI

| Folding time | Peptide species | Retention time | Abundance | Molecular mass | Disulfide species |
|--------------|-----------------|----------------|-----------|----------------|------------------|
| 1            | Reduced         | 22.6           | 56        | 3239.7         | 0SS              |
|              | Intermediate Iα | 22.2           | 12        | 3123.6         | 1SS              |
|              | Intermediate Iβ | 23.3           | 4.4       | 3123.8         | 1SS              |
| 3            | Reduced         | 22.6           | 36        | 3239.4         | 0SS              |
|              | Intermediate Iα | 22.2           | 21        | 3123.1         | 1SS              |
|              | Intermediate Iδ | 21.9           | 7.3       | 3123.5         | 1SS              |
|              | Intermediate Iε | 34.1           | <4.0      | 3007.6         | 2SS              |
| 24           | Intermediate Iα | 34.1           | <4.0      | 3007.4         | 2SS              |
|              | Intermediate Iε | 21.4           | 12        | 3007.6         | 2SS              |
|              | Misfolded IIIa  | 22.7           | 12        | 2891.7         | 3SS              |
|              | Misfolded IIIb  | 24.1           | 16        | 2891.6         | 3SS              |
|              | Native           | 40.6           | 33        | 2891.2         | 3SS              |

TABLE 3
Folding intermediates after 24 h of folding without OaPDI

Abundance describes the relative area of peptide species of total area under peak. Molecular mass is shown as monoisotopic [M + H], unless mass was not determined (ND). Up to three peptide species were found to co-elute in the same peak, for which their masses and number of disulfide bonds is shown. Folding conditions were as for Table 2 without OaPDI.

| Retention time | Abundance | Mass | Disulfide species |
|----------------|-----------|------|------------------|
| min            | %         | Da   |                  |
| Linear kalata B1 |          |      |                  |
| 30.5           | 4.2       | 2908.6/3024.7 | 3SS/2SS |
| 31.7           | 3.5       | 3022.9/2908.5 | 2SS/3SS |
| 32.5           | 2.3       | 2908.7/3024.7 | 3SS/2SS |
| 33.3           | 8.6       | 3024.8/2908.8 | 2SS/3SS |
| 34.0           | 11.0      | 3025.4/2909.3 | 3SS/2SS |
| 35.0           | 6.3       | 2908.9/3024.9 | 3SS/2SS |
| 36.3           | 5.4       | 2908.8/3024.9 | 3SS/2SS |
| 37.1           | 6.3       | 2908.9/3024.8 | 3SS/2SS |
| 38.0           | 14.2      | 2908.8/3024.9/3140.9 | 3SS/2SS/1SS |
| 38.9           | 3.0       | 2908.9 | 3SS |
| 39.6           | 7.1       | 2909.1 | 3SS |
| 41.4           | 14.0      | 2909.0/3025.0 | 3SS/2SS |
| 58.3           | 1.7       | 2908.6 |                  |

Cyclic kalata B1

| Retention time | Abundance | Mass | Disulfide species |
|----------------|-----------|------|------------------|
| 19.1           | 10.7      | 3023.2 | 2SS |
| 19.7           | 9.5       | 3022.9/2890.8 | 2SS/3SS |
| 20.3           | 6.8       | 3006.8/2890.8 | 2SS/3SS |
| 20.9           | 15.9      | 3006.9 | 2SS |
| 21.5           | 7.2       | 3006.8/2890.8/3122.8 | 2SS/3SS/1SS |
| 22.0           | 8.0       | 2890.8/3006.8 | 3SS/2SS |
| 22.8–23.1      | 8.8       | 2890.8/3006.8 | 3SS/2SS |
| 24.4           | 1.9       | 2890.8 | 3SS |
| 33.1           | 2.3       | 3006.9 | 2SS |
| 39.5           | 11.2      | 2891.0 | 3SS |

affinis, from where it derives. One of the most important families of proteins in this plant are cyclotides, circular cystine knot defense proteins, so we hypothesized that OaPDI might assist in their folding.

Oxidative folding of linear and cyclic kalata B1 was monitored in aqueous buffer at neutral pH with and without OaPDI. Very little correctly folded peptide was obtained after 24 h without the enzyme. On the other hand, when the folding was catalyzed by OaPDI, the yield increased by ~10- and ~26-fold for linear and cyclic kalata B1, respectively. This increased folding yield is specific for PDI, since lysozyme and deoxyribonuclease controls did not induce any folding of the peptides. Interestingly, bovine PDI also facilitates folding of cyclotides. This is not unexpected, since bovine PDI has also been shown to facilitate folding of other nonbovine disulfide-rich peptides, including examples from cone snails and scorpions (39, 40). The final yield of correctly folded cyclic peptide in the presence of OaPDI was about 4-fold greater than that of the linear peptide, although the folding rate was about 3-fold greater for linear kalata B1. These results are consistent with surface plasmon resonance studies in which we found that the linear reduced peptides interact with OaPDI with 3–4-fold greater affinity (Kd = 7.2 μM) than cyclic kalata B1 (Kd = 28 μM).

The folding rate of the linear and cyclic peptides is determined by a combination of oxidation and isomerization events. Intrinsically, the unfolded cyclic peptide has a lower entropy...
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Mechanism and Function of a Novel Plant PDI

To obtain more information about the role of OaPDI in the oxidative folding of cyclotides, we examined the major intermediate species on folding pathways in the presence and absence of PDI. On the basis of molecular masses, retention times, and HPLC elution profiles of the major peaks, mostly nonnative 3SS species and some co-eluting 2SS species were found for both linear and cyclic kalata B1 (Tables 2 and 3). Fig. 7 summarizes the OaPDI-assisted folding pathways of the linear and cyclic peptides in the form of a folding funnel diagram (41, 42).

Without PDI, the folding still proceeds to the initial stage of nonspecific disulfide bond formation by oxidation (“packing”) to the point where mostly oxidized and partially oxidized (3SS and 2SS) peptides are present, but it appears that they are trapped and are not able to effectively undergo disulfide reshuffling (“consolidation”) to the native species, resulting in a lower yield of correctly oxidized peptides. In short, oxidation proceeds without the enzyme, but the shuffling of misfolded or partially folded intermediates into their native disulfide connectivity is significantly reduced. This reinforces the notion that isomerization is a major function of this new plant PDI.

In vitro, a co-solvent, such as 2-propanol, creates a hydrophobic environment that enhances the folding efficiency of kalata B1 (16, 17). The similarities in intermediates under different conditions of oxidative folding can be explained on the basis that cyclotides find a similar hydrophobic environment in the presence of an enzyme (OaPDI) as they do with 2-propanol. The recently solved crystal structure of yeast PDI (32) revealed a large hydrophobic area primarily in domain β'. Unfolded cyclotides are likely to be attracted to this hydrophobic area, allowing an interaction with the active site. Formation of the correct disulfide bonds would then be driven by interactions based on the amino acid sequence of the polypeptide. In the presence of PDI, the disulfide bonds are formed by dithiol-disulfide exchange between the active site CXXC motif and the peptide thiolestes.

The current model for the production of cyclotides in planta is that the disulfide bonds in the precursor molecule are oxidized within the ER and that processing and cyclization occurs further downstream in the secretory pathway. A processing enzyme, which is presumed to be localized in plant vacuoles could recognize a conserved Asn/Asp residue at the C-terminal end of the mature cyclotide sequence and a conserved tripeptide motif that flanks both sides of the mature peptide in the precursor protein to cleave and cyclize the mature peptide (9). This implies that oxidative folding occurs prior to cyclization in the ER. Hence, the potential entropic advantage provided by the cyclic backbone may not be realized for in vivo oxidative folding, supporting the need for an enzyme, such as PDI, to provide a catalytic surface to drive the folding process. In vivo, the longer precursor protein with its conserved N-terminal repeat region (43), could be a better substrate due to an extended area for interaction with the PDI binding surface. This is consistent with data observed for in vitro folding of conotoxins, disulfide-rich peptides of similar size to the cyclotide, for which conserved elements of the precursor protein are thought to provide improved binding properties for PDI (44).

In conclusion, in the current study, we have examined the oxidative folding of cyclotides with the aid of a newly discov-
Mechanism and Function of a Novel Plant PDI

erated PDI from *O. affinis*. We have shown that OaPDI and the Oak1 precursor co-express on a transcriptional level and show a strong biomolecular interaction, which together suggest an *in vivo* interaction of both proteins. OaPDI significantly enhances the yield of correctly disulfide-bonded species *in vitro* under physiological conditions. The plant PDI has lower isomerase but greater chaperone activity than hPDI, which is probably due to distinct sequence variations near the active site motif and in the C-terminal domain. In aqueous buffer, cyclotides do not acquire their native fold without enzyme and instead accumulate trapped nonnative species. From the available data, we propose that a major function of OaPDI is to shuffle nonnative disulfide bonds into their correct connectivity to produce fully functional insecticidal cyclotides as confirmed by larval antifeedant assays. The folding pathway of kalata B1 peptides with OaPDI is similar to that in the presence of the hydrophobic solvent 2-propanol. This leads to the hypothesis that hydrophobic patches on PDI provide a binding mechanism to assist in the formation of the native disulfide bonds in the cyclotide kalata B1.

The findings reported here are potentially important for biotechnological applications. One limiting factor for the overproduction of proteins *in vitro* and *in vivo* is the formation of the correct disulfide connectivity, and there is a need for effective catalysts to form disulfide-rich peptides. OaPDI could potentially become an important tool for the production of cyclotides *in vitro* and *in vivo*. Cyclotides have a range of biological activities that make them attractive candidates for drug development and agricultural applications as well as novel molecular scaffolds (14). To increase the rate and yield of the folding process, OaPDI could be used as an additive in the final refolding step for cyclotides made either synthetically or in transgenic plants by OaPDI co-expression.

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