Barley 2-cysteine peroxiredoxin (2-Cys Prx) was analyzed for peroxide reduction, quaternary structure, thylakoid attachment, and function as well as in vivo occurrence of the inactivated form, with emphasis on the role of specific amino acid residues. Data presented show the following. 1) 2-Cys Prx has a broad substrate specificity and reduces even complex lipid peroxides such as phosphatidylcholine dilineoyl hydroperoxide, although at low rates. 2) 2-Cys Prx partly becomes irreversibly oxidized by peroxide substrates during the catalytic cycle in a concentration-dependent manner, particularly by bulky hydroperoxides. 3) Using dithiothreitol and thioredoxin (Trx) as reductants, amino acids were identified that are important for peroxide reduction (Cys64, Arg140, and Arg189), regeneration by Trx (Cys185), and conformation changes from dimer to oligomer (Thr96, Trp99, and Trp189). 4) Oligomerization decreased the rate of Trx-dependent peroxide detoxification. 5) Comparison of PrxWT, W99L, and W189L using static and time-resolved LIF techniques demonstrated the contributions of the tryptophan residues and yielded information about their local environment. Data indicated protein dynamics in the catalytic site and the carboxyl terminus during the reduction-oxidation cycle. 6) Reduced and inactivated barley 2-Cys Prx oligomerized and attached to the thylakoid membrane in isolated chloroplasts. The in vivo relevance of inactivation was shown in leaves subjected to cold and wilting stress and during senescence. Based on these results, it is hypothesized that in addition to its function in peroxide detoxification, 2-Cys Prx may play a role as a structural redox sensor in chloroplasts.

Peroxiredoxins (Prxs) constitute a family of peroxidases found in all biological kingdoms from bacteria to plant and animal. Prxs are heme-free peroxidases that reduce alkyl hydroperoxides and hydrogen peroxide. During the reaction cycle, the cysteine residue in the active site (Cys64) is oxidized to sulfinic acid, whereas hydrogen peroxide, peroxynitrite, and a broad range of alkyl hydroperoxides are reduced to water, nitrite, or the corresponding alcohol (1–3). The oxidized cysteine residue is regenerated via intra- or intermolecular disulfide formation and electron transfer from donors such as thioredoxin, glutaredoxin, or glutathione (4, 5). The regeneration mechanism is specific for each of the four types of peroxiredoxins, namely 1-Cys Prx, type II Prx, Prx Q, and 2-Cys Prx (5). The various peroxiredoxins reveal distinct tissue distribution and transcriptional regulation, structural and biochemical properties suggesting specific biological tasks (5–7). Evidence exists that Prxs are involved in redox signaling and affect protein phosphorylation, transcriptional regulation, and apoptosis in animals (4).

Crystal structure analysis groups Prxs into the thioredoxin superfamily (8). Type II Prxs (PRDX5) act as monomers (8). 1-Cys Prxs (HORF6) are monomeric too but form homodimers at protein concentrations of >1 mg/ml (9). 2-Cys Prxs have a second Cys at the C terminus (Cys185), which reacts with Cys64 of the other subunit to form a disulfide bridge. Crystal structures of reduced (TryP; Crithida fasciculata), oxidized (HBP23; rat), and overoxidized (TPxB; human) 2-Cys Prx homologues showed that reduction of the disulfide bridge induces a conformational change in the quaternary structure from a dimeric to a decameric doughnut-shaped form (10–12). Although oligomerization seems to be a general property of 2-Cys Prx, the in vivo parameters inducing oligomerization and its function are unknown. Low or high ionic strength, acidic pH, and reducing conditions are reported to promote oligomerization but differ in their effects upon various 2-Cys Prx (13).

In plants, the 2-Cys Prx is a nuclear encoded chloroplast protein. Analysis of transgenic Arabidopsis plants with decreased 2-Cys Prx levels showed impaired photosynthesis and increased oxidative damage of chloroplast proteins during early plant development (14, 15). Regeneration of oxidized plant 2-Cys Prx is coupled to the photosynthetic electron transport chain via the electron donors Trx m and Trx f (16, 17). In addition to thioredoxin, reduction by a chloroplastic drought-induced 32-kDa stress protein has been reported (18). The redox potential of −315 mV (16) places reductive regeneration of 2-Cys Prx between Calvin cycle activation and redox switching of the malate valve (16). Apparently, in chloroplasts, the 2-Cys Prx detoxifies peroxides depending on the redox state of photosynthesis.

Adaptation to the changing redox environment of chloroplasts might have imposed specific features on plant 2-Cys Prx such as catalytic activity, subcellular localization, and interacting partners. The present study on plant 2-Cys Prx function in the context of photosynthesis focuses on three major aspects: 1) the substrate specificity, 2) the reaction mechanism, and 3) the

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role of inactivation. To address these questions, a set of amino acid substitution mutants was designed and analyzed biochemically and physico-chemically. It will be shown that 2-Cys Prx reduces a wide range of peroxides including complex lipid hydroperoxides, which also inactivate the enzyme in a substrate- and concentration-dependent manner. An in vivo relevance of the oxidative inactivation is shown for stress conditions. Based on the observation that oxidatively inhibited 2-Cys Prx changed its quaternary structure and attached to the thylakoid membrane, it is hypothesized that 2-Cys Prx, besides its antioxidative function, might be a structural redox sensor of chloroplasts.

EXPERIMENTAL PROCEDURES

Plant Growth—Barley (Hordeum vulgare var. Gerbel) was grown in soil under controlled environmental conditions with a 14-h light period at a photosynthetic active radiation of 100 μmol quanta m⁻² s⁻¹ and 25 °C and 10 h of darkness at 20 °C. For stress treatments, 12-day-old barley seedlings were grown for 48 h under the following conditions: 1) salt stress by irrigation with 500 mM NaCl; 2) cold stress at 4 °C. Barley seedlings were grown for 48 h under the following conditions: 1) salt stress by irrigation with 500 mM NaCl; 2) cold stress at 4 °C; 3) wilting stress without watering; or 4) control condition as described above. Site-directed Mutagenesis of 2-Cys Prx—The pQE-30 vector-based expression system (Qiagen, Hilden, Germany) was used to express Prxwt, and the variants heterologously as His-tagged fusion proteins in E. coli M15[pREP4]. Site-directed mutagenesis was performed with mutant primers in two subsequent polymerase chain reactions with Pfu polymerase (Table I) (Stratagene, La Jolla, CA) according to Montemartini et al. (19) using the Prxwt expression vector described previously (16) as template. The mutations were verified by sequencing (MWG Biotech, Ebersberg, Germany).

Culture Growth and Purification of His-tagged Proteins—1-Liter LB medium containing 50 μg/ml ampicillin was inoculated with 10 ml of a noninduced overnight bacteria culture and was grown to A595 = 0.7 at 37 °C. Expression of recombinant protein was induced by adding isopropyl-1-thio-β-d-galactopyranoside to a final concentration of 0.4 mM. After 4 h, the cell pellet obtained by centrifugation at 5000 rpm for 30 min was stored overnight at −20 °C. For protein purification, the cells were resuspended in 5% (with respect to culture volume) of lysis buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10 mM ascorbate, 0.5 mg/ml lysozyme, pH 8.0 (NaOH), and shaken at 4 °C for 60 min. The cell debris was removed by spinning at 15,000 rpm at 4 °C for 30 min. The supernatant was loaded onto the nickel-nitrilotriacetic acid column (2 ml/1 liter of culture), previously equilibrated with 20 mM imidazole buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0 (NaOH), followed by 20 volumes of buffer A supplemented with 20% (v/v) glycerol. The protein was eluted with 250 mM imidazole in buffer A. The protein-containing fractions, as assessed by measuring A₂₈₀nm, were pooled and dialyzed three times against a 25-fold volume of 40 mM K-P, (pH 7.0) for 4 h. The enzyme concentrations were determined from the molar absorption coefficient calculated with a

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**Table I**

| Mutation | Mutation primer |
|----------|-----------------|
| pQE-30 promoter | 5'-ggcgtatacagggccctgcttgg-3' |
| pQE-30 reverse | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx forward | 5'-ggcgtatacagggccctgcttgg-3' |
| 2-Cys Prx reverse | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Cys64 → SerS | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Cys64 → SerA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Cys185 → SerS | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Cys185 → SerA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Trp149 → LeuS | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Trp149 → LeuA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Trp99 → LeuA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Trp99 → LeuS | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Thr66 → AlaA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Thr66 → AlaS | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Arg140 → GlnA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Arg140 → GlnS | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Arg140 → AlaA | 5'-cattataaggcccatcaaggg-3' |
| 2-Cys Prx Arg140 → AlaS | 5'-cattataaggcccatcaaggg-3' |
g. The supernatant was taken as stroma fraction, and the sedimented thylakoids were washed twice with K-P buffer (pH 7.5) prior to resuspension in the same buffer. Volumes equivalent to 10 µg of stroma and thylakoid proteins were analyzed by SDS-PAGE followed by Western blot using the Lumilight detection system (Roche Applied Science) and the BAS1 antibody specific to barley 2-Cys Prx (22).

Detection of Inactivated 2-Cys Prx in Vivo—Leaf tissue was ground with sand in a buffer containing 75 mM Tris-HCl, pH 7.5, and 50 mM NaCl. 8 µg of total protein from controls and stressed plant material were loaded per lane under nonreducing conditions. Sample was separated by SDS-PAGE, and 2-Cys Prx was detected by Western blot analysis.

Time- and Wavelength-Resolved Measurements—Time- and wavelength-resolved measurements were performed using a short pulse laser system in conjunction with an intensified streak camera. The laser system (Spectra Physics) consisted of a Ti:sapphire laser (Tsunami) with additional regenerative and two linear amplification stages. After frequency doubling and mixing of the second harmonic with the fundamental wavelength, UV radiation tunable between 250 and 300 nm was obtained. The settings were as follows: wavelength, 280 nm; pulse length, 80 ps; bandwidth, ~0.7 cm⁻¹; pulse energy, ~50 µJ. Signals were collected with a spherical mirror, dispersed by an astigmatism-corrected spectrometer, and detected by a streak camera (Hamamatsu C2830). A more detailed description of the optical setup and the necessary calibration and evaluation procedures was given previously (23).

Excitation-Emission Spectra—Excitation-emission spectra were obtained using a custom-built setup employing a 75-watt xenon lamp as light source, two astigmatism-corrected spectrometers for signal processing, and a back-thinned CCD camera (Roper Scientific) for detection (25).

Sample Preparation for Laser and Excitation-Emission Spectroscopy—For time- and wavelength-resolved and for excitation-emission spectroscopy, 300 µl of a 20 µM Prx_wt (9.1 µM W99L), and 12.7 µM (W189L) protein solution was prepared in a 40 mM phosphate buffer, pH 7.0. Under reducing conditions, the buffer contained 10 mM DTT. For all measurements, samples were contained in quartz cuvettes of 5×5 mm (Hellma type 111.057-QS, suprasil, optical precision).

RESULTS

Plant 2-Cys Prx Reduces Complex Lipid Hydroperoxides—Substrate specificity of plant 2-Cys Prx has not been studied in detail up to now. Therefore, an activity assay was performed in vitro using the electron transport chain E. coli Trx, thioredoxin reductase, and NADPH (Fig. 1A). The oxidation of NADPH was monitored at 340 nm. From the initial velocity, the catalytic activity constants were calculated following subtraction of the background NADPH oxidation in the absence of the complete electron transfer chain (16). The highest activity of 15.7 ± 0.8 min⁻¹ was measured with t-BOOH as substrate, followed by 13.6 ± 1.2 min⁻¹ with H₂O₂. The reduction of cumene hydroperoxide with 10.7 ± 0.84 min⁻¹ was already less. The complex lipid substrates linoleic acid hydroperoxide (3.0 ± 0.1 min⁻¹) and phosphatidylcholine dilinoleyl hydroperoxide (0.88 ± 0.14 min⁻¹) were reduced with considerably lower rates (Fig. 1B).

Sequence Alignment Characterizes 2-Cys Prx as a Hydrophobic Protein with a Positively Charged C-terminal Tail—In order to identify interesting targets for site-directed mutagenesis of 2-Cys Prx, amino acid sequences of 2-Cys Prx from plants, animals, fungi, and bacteria were compared. Common to all 2-Cys Prx is the highly conserved active site around Cys64, the F-motif (PTPVCPEEI), and the hydrophobic region around Cys285-286 (VCPXXXW) (4, 26). In the comparison, the conservation of several hydrophobic amino acids attracted special attention; the phenylalanine residues Phe87, Phe54, Phe86, Phe60, Phe62, Phe71, Phe78 and Phe145 in the active site region, two tryptophan residues (Trp99 and Trp149), and other hydrophobic amino acids are found in all 2-Cys Prx (Fig. 2). The number of conserved charged residues is low in comparison. The two polar residues Thr61 and Thr66 in the active site and three positively charged residues (Arg140, Arg163, and Arg170) are conserved, indicating involvement in the catalytic turnover of hydroperoxides. Compared with 2-Cys Prx from other organisms, the C-terminal tail (aa 186-210, length of 25 aa) of the higher plant homologues contains four instead of two Pro residues and is shorter. In nuclear encoded 2-Cys Prx from higher plants and in the plastome-encoded homolog of the red algae Porphyra purpurea, the amino acid residues AA1 follow the last conserved motif (FKEY), whereas in animal 2-Cys Prx the C terminus is more polar [e.g. ending with QKVNQ in AOP1 (27) or SKHN in TPx (28)]. Between Cys185 and the FKEY motif most charged residues, three aspartate or glutamate and five lysine residues are conserved, still giving the plant C-terminal tail a polar and positively charged character similar to several nonplant 2-Cys Prx. The plant specific modification and the conserved positive charging raised the question of their specific function and were taken as the basis of the subsequent analysis by site-directed mutagenesis.

Generation of Prx_wt and Variants—Site-directed mutagenesis of the 2-Cys Prx was performed to address the reaction mechanism. For substitutions, amino acid residues with similar size or charge, respectively, were chosen to keep the effects on the structural stability minimal. All proteins were overexpressed in E. coli. The N-terminally His-tagged Prx_wt and variants were isolated and purified on nickel-nitritriacetic acid columns under native conditions with yields of 5–25 mg/liter of LB culture. The addition of 10 mM ascorbate to the lysis buffer reduced inactivation and proteolysis during purification. On SDS-PAGE gels, the C64S and C185S variants dissociated into monomers under denaturing nonreducing conditions, whereas the other mutants were homodimers like Prx_wt (data not shown).

Peroxidase Activity of the Variants—The peroxidase activity of Prx_wt and variants was determined using two different substrates, the small polar H₂O₂ and the alkyl hydroperoxide t-BOOH, in both the Trx-dependent assay (16) and the assay with DTT (29). The remaining peroxide was subsequently determined with ferrithiocyanate (30) (see Fig. 3). In the DTT assay, Prx_wt only showed half the activity observed in the Trx

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2 A. Brochkinke, R. Plessow, K. Kohse-Höinghaus, and C. Herrmann, submitted for publication.
assay, suggesting that Trx reduces the 2-Cys Prx more efficiently than DTT. As reported before, C64S had no activity (16, 19, 31). The variant C185S exhibited little activity in the Trx-based assay, but its reducing activity was 19-fold higher than measured for the PrxWT in the DTT assay. Apparently, Cys 185 is not essential for peroxidase activity but for reductive regeneration of 2-Cys Prx by Trx.

Exchange of the generally conserved Trp99 by the hydrophobic, nonaromatic Leu residue suppressed activity in all assays, implicating an important structural function. The C-terminally located Trp189 is specific for 2-Cys Prx. Trp 189 and the active site Thr66 were replaced by Leu and the small hydrophobic Ala; the variants showed 47% (W189L) and 60% (T66A) residual activity in the Trx-based assay with H2O2, respectively. However, the activity was 50% of that with t-BOOH. In the DTT assay, the activities were not significantly different from those in the Trx assay with H2O2 for the T66A variant but increased in the DTT-based assay by 64% for the W189L variant as compared with Trx-dependent regeneration.

Arg140 is conserved among all Prx, whereas Arg 163 and Arg 170 are specific for 2-Cys Prx. The hydrophilic, but neutral, Gln residue was chosen to substitute for Arg. In the Trx-dependent assay, R163Q and R170Q variants showed only slightly lower activities than the PrxWT, whereas the R140Q variant was inhibited by about 50% in the Trx assay. Interestingly, all Arg variants, especially Arg140 and Arg163, revealed strongly reduced activities in the DTT-dependent assay compared with the Trx assay. It can be concluded that these residues are important for peroxidase activity and not for regeneration.

**Thioredoxin Mediates the Reduction of the 2-Cys Prx—**2-Cys PrxWT and the variants W189L and T66A were incubated with a limiting amount of DTT (0.2 mM instead of 10 mM as used above) or with Trx and DTT (Fig. 4). After 5 min, the redox state was fixed with N-ethylmaleimide. The reduced Prx fraction dissociated under denaturing conditions to the monomer, which can be distinguished from the dimer by SDS-PAGE. Only a small portion of 2-Cys PrxWT was reduced by DTT alone, whereas the addition of Trx promoted the reduction in all Prx variants. The reduced fractions of W189L and T66A in the presence of DTT were slightly greater than PrxWT but smaller with additional Trx. It is concluded that the variants are more easily reduced by DTT, but regeneration with Trx is inhibited compared with PrxWT.

**Quaternary Structure of 2-Cys Prx—**The dimer is the minimal catalytic unit. Depending on ionic strength and redox state, Prx dimers arrange in oligomers and finally in decameric doughnut-shaped complexes. The influence of amino acid substitutions on quaternary structure was explored by size exclusion chromatography on a Superose 12 column (Fig. 5). The elution profile showed that the oxidized PrxWT almost exclusively existed as homodimer, whereas the two Cys variants aggregated to higher molecular mass complexes like the overoxidized PrxWT (not shown). Another peculiar change of quaternary structure was observed for the W189L variant, which to a minor extent eluted as dimer like PrxWT but predominantly existed as decamer as shown by peak elution in fractions 10 and 11 (Fig. 5).

**High Phosphate Concentrations Decrease Prx Activity in the Trx-dependent Assay—**Barley 2-Cys Prx aggregates at high phosphate concentrations (16), and Chauhan and Mande (32) suggested that the aggregated form of PrxWT exhibits higher activity toward peroxide substrates. On the other hand, an interaction model of TryP and the reductant tryparedoxin in Trypanosoma indicates that the interaction is mediated by electrostatic forces involving Arg92, Lys93, Lys94, and Glu 171 and probably the C-terminal tail (31, 33). Reductive regeneration of the decameric complex (as present at high salt) may be the limiting factor in the catalytic cycle. Disassembly may increase the efficiency of the interaction with Trx. The influence of the quaternary structure on Prx activity in the Trx-dependent assay was analyzed by increasing the phosphate concentration from 20 to 200 mM (Fig. 5C). The rate of H2O2 reduction decreased with increasing salt concentrations, thus with the transition from dimer to oligomer. It is concluded that Prx regeneration by Trx is negatively affected by Prx oligomerization.
Excitation-Emission Spectroscopy: Excitation Spectra—2-Cys Prx contains two Trp residues that can be used as intrinsic fluorescence probes to investigate structural dynamics of the protein. The position of both Trp residues appeared ideal, since from structural analysis of other Prx, Trp99 was predicted to be located close to the catalytic center, and Trp189 was predicted to be in the C-terminal region. Excitation spectra determined by excitation-emission spectroscopy did not show a strong deviation between the excitation maximum for the PrxWT and single Trp variants (data not shown). The excitation maxima are close to the absorption maximum of Trp in aqueous solution \((/H9261\text{abs-max}/H11005/280\text{nm})\) (34), in accordance with the fact that the positions of excitation maxima of Trp are usually independent of the local environment but depend sensitively on the polarity of the surrounding medium (35).

Laser-induced Fluorescence: Emission Spectra—The emission spectra determined by LIF measurements for Prx WT and W189L were similar under both oxidizing and reducing conditions \((/H9261\text{em-max}/H11015/336\text{nm}, \text{corresponding to Trp99 fluorescence})\). In contrast, \(/H9261\text{em-max}/H11015/328\text{nm}, \text{corresponding to Trp189 fluorescence}\) (Fig. 6). Pure Trp in aqueous solution has a \(/H9261\text{em-max}/H11005/350\text{nm}\). According to the classification by Burstein et al. (36), the residue Trp\textsuperscript{189} belongs to class I; \(/H9261\text{em-max}/H11005/330–332\text{nm}, \text{corresponding to a Trp located in the interior of the protein, shielded from the solvent.}\) In contrast, Trp\textsuperscript{99} does not fall clearly into class I or class II \((/H9261\text{em-max}/H11005/340–342\text{nm}, \text{protein surface Trp})\). Therefore, this residue is located closer to the outside of the protein but not completely exposed to the solvent.

Excitation-Emission Spectroscopy: Excitation Spectra—The emission spectra determined by LIF measurements for Prx\textsubscript{WT} and W189L were similar under both oxidizing and reducing conditions \((/H9261\text{em-max}/H11005/336\text{nm}, \text{corresponding to Trp}\textsuperscript{99} \text{fluorescence})\). In contrast, \(/H9261\text{em-max}/H11005/328\text{nm}, \text{corresponding to Trp}\textsuperscript{189} \text{fluorescence})\) (Fig. 6). Pure Trp in aqueous solution has a \(/H9261\text{em-max}/H11005/350\text{nm}\). According to the classification by Burstein et al. (36), the residue Trp\textsuperscript{189} belongs to class I; \(/H9261\text{em-max}/H11005/330–332\text{nm}, \text{corresponding to a Trp located in the interior of the protein, shielded from the solvent.}\) In contrast, Trp\textsuperscript{99} does not fall clearly into class I or class II \((/H9261\text{em-max}/H11005/340–342\text{nm}, \text{protein surface Trp})\). Therefore, this residue is located closer to the outside of the protein but not completely exposed to the solvent.

Emission spectra of Prx\textsubscript{WT} and its single-Trp mutants were compared under oxidizing and reducing conditions. Upon reduction, Prx\textsubscript{WT} showed an increase of emission in the blue-
shifted range of the spectrum, according to an increase of fluorescence from Trp 189. For W99L, a red shift of $\lambda_{em-max}$ of about 5 nm was observed following reduction, whereas the mutant W189L exhibited similar spectra under both conditions. The first two observations can be explained by a more open structure at the dimer interface due to the cleavage of disulfide bonds, making Trp189 more accessible to the solvent, explaining the shift of $\lambda_{em-max}$. At the same time, the quenching influence of neighboring amino acids on Trp 189 is diminished, resulting in an increased emission from Trp 189 in the Prx WT spectrum.

LIF: Fluorescence Lifetimes—Fig. 7 shows the fluorescence lifetimes determined for Prx WT and its Trp variants under oxidizing and reducing conditions. With exception of the mutant W189L, which revealed typical lifetimes for Trp within a protein (37–39), the averaged fluorescence lifetimes of all samples investigated were significantly lower than those of tryptophan in aqueous solution ($\tau_1 = 2610$ ps, $\tau_2 = 2940$ ps, $\tau_3 = 280$ ps; determined at emission maximum $\lambda_{em} = 350$ nm) (Table II). This means that the lifetimes of both fluorophores were significantly shorter than those of free, solvent-accessible tryptophans, although they are shielded in the protein matrix (which

![Graph showing fluorescence lifetimes for Prx WT, W99L (Trp189), and W189L (Trp99).](image)
usually leads to longer fluorescence lifetimes.

The smallest averaged lifetimes \( \tau \) were found for the fluorescence emission from \( \text{Prx}_{189} \) (\( \tau_{\text{Prx}_{189}} \)), which has a \( \tau \) of less than 1000 ps under oxidizing conditions. Since the 2-Cys Prx mutant W99L does not contain a second Trp residue as a potential acceptor for resonance energy transfer, the strongly reduced \( \tau_{\text{Prx}_{189}} \) (and also the earlier mentioned diminished fluorescence contribution from \( \text{Prx}_{189} \)) can only be due to quenching by neighboring amino acids. Therefore, resonance energy homotransfer from \( \text{Prx}_{189} \) to \( \text{Prx}_{99} \) in the \( \text{Prx}_{2WT} \) is unlikely. Possible quenchers of \( \text{Prx}_{189} \) fluorescence are the charged amino acids of the C terminus. Upon reduction, \( \tau_{\text{Prx}_{189}} \) increases due to a reduced contact with intrinsic quenchers.

The averaged \( \tau \) for \( \text{Prx}_{2WT} \) was longer than for the variant W99L but nevertheless decreased relative to free Trp, due to the diminished \( \tau \) from residue \( \text{Trp}_{189} \) contributing to the wild type spectrum. For \( \text{Prx}_{2WT} \), a change from oxidizing to reducing conditions resulted in a small increase of \( \tau \), suggesting that these Trp residues are more shielded from the solvent. According to Wood et al. (13), decameterization of bacterial 2-Cys Prx brings five dimers with their Trp-containing domains closer to each other, resulting in doughnut-shaped \( (\alpha \beta)_{5} \) structure. \( \tau_{\text{Trp}_{99}} \) of the mutant W189L is typical for a relatively exposed Trp residue. Upon reduction, the averaged fluorescence \( \tau \) increased from \( \tau_{m} = 2530 \) to 3410 ps, supporting the possibility that \( \text{Trp}^{*} \) moves from a rather solvent-exposed to a fairly impolar environment.

**LIF: Calculation of Wild Type Spectrum**—The time-dependent spectrum of \( \text{Prx}_{2WT} \) can be calculated by a combination of the spectra of both single Trp variants according to the following equation:

\[
\text{LIF}(\text{WT}) = \alpha \cdot \text{LIF(W99L)} + (1 - \alpha) \cdot \text{LIF(W189L)} \quad (\text{Eq. 1})
\]

Time-dependent fluorescence decay was measured for \( \text{Prx}_{2WT} \), W99L, and W189L. The spectra of the single Trp variants (normalized to 1) were used to calculate the spectrum of \( \text{Prx}_{2WT} \) (Fig. 8). The ratio of fluorescence emission originating from \( \text{Trp}_{189} \) given by \( \alpha \) is different for the spectra under oxidizing and reducing conditions. Both Trp residues contribute differently to the time-dependent fluorescence decay. Under oxidizing conditions, the contribution of \( \text{Trp}_{189} \) is fairly low and amounts to 34% of the calculated time-dependent fluorescence. Upon reduction of 2-Cys Prx, the emission from \( \text{Trp}_{189} \) gains more influence and contributes to about 55% of the calculated decay.

**Peroxides Inactivate Prx Depending on Substrate Size**—The Trx-dependent assay was performed with increasing concentrations of \( \text{H}_{2}\text{O}_{2} \). The initial velocity of peroxide reduction decreased with increasing \( \text{H}_{2}\text{O}_{2} \) concentration with a 50% inhibition at 1 mM \( \text{H}_{2}\text{O}_{2} \) (Fig. 9A). Low concentrations of \( \text{H}_{2}\text{O}_{2} \) (10–50 \( \mu \text{M} \)) had no inhibitory effect on peroxide reduction. During the reaction cycle, the intermediate sulfenic acid form of Prx can be further oxidized to sulfenic acid that cannot be regenerated by DTT or Trx and is the irreversibly damaged form. In SDS-PAGE separations, the overoxidized Prx can be detected as monomers, because the disulfide bridge formation between the subunits is not any longer possible. The amount of monomeric fraction of Prx increased with the amount of \( \text{H}_{2}\text{O}_{2} \), indicating increased levels of irreversibly oxidized Prx (data not shown; see Ref. 16). The decrease in dimerized Prx correlated with the inhibition of activity. W189L and T66R variants were also inactivated at increasing \( \text{H}_{2}\text{O}_{2} \) concentrations, however to a lesser extent than \( \text{Prx}_{2WT} \) (not shown). It should be noted that \( \text{Prx}_{2WT} \) as well as the variants showed monomer formation even in the control treatment without added peroxide substrate. Thus, reactions with other reactive oxygen species also mediated inactivation rather than small amounts of \( \text{H}_{2}\text{O}_{2} \).

The time-dependent activity assays with Trx have revealed that the initial velocity of peroxide reduction rapidly decreased when bulky hydroperoxide substrates were administered at standard concentrations of 25 \( \mu \text{M} \) (not shown). To explore that relationship, \( \text{Prx}_{2WT} \) was reduced either by DTT or in the Trx system and incubated with various hydroperoxide substrates (Fig. 9, B–E). After 1 h of incubation, the samples containing DTT were dialyzed against 40 mM phosphate buffer to remove the reducing agent. Samples treated with Trx were autoxidized following consumption of the low amount of added NADPH. The amount of Prx monomer increased in the following order: hydrogen peroxide < t-buty1-hydroperoxide < cumene hydroperoxide < linoleic acid hydroperoxide < phosphatidylcholine dinitrophenyl hydroperoxide in both the DTT and Trx assay. Thus, bulky hydroperoxide substrates promote inactivation of \( \text{Prx}_{2WT} \), independent of the regeneration mechanism.

### Table II

Results of time-resolved LIF measurements for wild type, W99L, and W189L 2-Cys Prx

|       | Oxidized | Reduced |
|-------|----------|---------|
|        | \( \tau_{1} \) | \( \Delta \tau_{1} \) | \( \tau_{2} \) | \( \Delta \tau_{2} \) | Prx | \( \Delta \tau_{m} \) |
| WT    | 3412     | 113     | 411    | 52    | 1981 | 84  |
| W99L  | 1819     | 64      | 325    | 55    | 934  | 59  |
| W189L | 3750     | 168     | 517    | 52    | 1454 | 153 |
|        | 102      | 3407    |        |       |
|        | 222      |         |        |       |

**FIG. 8.** Calculation (WTsim) and comparison of time-dependent fluorescence decay for \( \text{Prx}_{2WT} \) and its single tryptophan mutants.

### Example of Table II

| Time | \( \tau_{1} \) | \( \Delta \tau_{1} \) | \( \tau_{2} \) | \( \Delta \tau_{2} \) |
|------|----------------|-------------------|----------------|-------------------|
| Oxidized | 3412 | 113 | 411 | 52 |
| Reduced | 1819 | 64 | 325 | 55 |
| WT | 3750 | 168 | 517 | 52 |
| W99L | 102 | 3407 | 222 |

### Example of Equation

\[
\text{LIF}(\text{WT}) = \alpha \cdot \text{LIF(W99L)} + (1 - \alpha) \cdot \text{LIF(W189L)}
\]

**Note:** The table and equation are presented as part of a larger text discussing the reaction mechanism of plant 2-Cys peroxiredoxin.
W189L and T66A variants mostly showed the same dependence on substrate size (Fig. 9, D and E); only the portion of monomer was smaller, and the inactivation was weaker than in the experiment with Prx WT.

**Reduction and Inactivation Mediates Attachment to the Thylakoid Membrane**—Tissue fractionation demonstrated that 2-Cys Prx is post-translationally targeted to chloroplasts, where it was initially reported to reside in the stroma (22). Later, immunogold labeling indicated 72% thylakoid attachment at standard growth conditions (16). Incubation of chloroplasts with ascorbate led to attachment of Prx to the thylakoids (16). For analysis whether the membrane attachment is redox-regulated, isolated intact chloroplasts were incubated at room temperature for 1 h and, in the case of the DTT-containing samples, dialyzed against 40 mM potassium phosphate buffer. The proteins were separated by SDS-PAGE and visualized by silver staining. The appearance of the monomeric band indicates formation of the overoxidized sulfenic acid form.

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External ascorbate was added to maintain reducing conditions, which enabled the chloroplast to keep 2-Cys Prx reduced (16) and promoted attachment of Prx to thylakoids (Fig. 10). Increasing amounts of H$_2$O$_2$ up to 0.75 mM led to dissociation of Prx from the thylakoids. A further increase of H$_2$O$_2$ stimulated the attachment to the thylakoids, supporting the view that the sulfenic acid form of Prx behaves like the reduced form. The crystal structure of overoxidized human TPxB revealed a decameric structure (11) similar to the reduced bacterial AhpC (13). Because oxidation to the sulfenic acid form conserves the reduced conformation, inactivated Prx structurally mimics reduced Prx. By building oligomers and associating with the

---

**Fig. 9. Inactivation of recombinant 2-Cys Prx by peroxides.** *A*, rate of H$_2$O$_2$ reduction as a function of the H$_2$O$_2$ concentration as determined in the Trx-based assay. The bars represent mean values from three determinations ± S.D. *B*-E, irreversible oxidation of Prx WT (*B* and *C*), W190L (*D*), and T66A (*E*) in the presence of 25 μM peroxide substrates of increasing molecular mass in the standard DTT (*B*) or Trx assay (*B* and *C*) as indicated. The samples were incubated at room temperature for 1 h and, in the case of the DTT-containing samples, dialyzed against 40 mM potassium phosphate buffer. The proteins were separated by SDS-PAGE and visualized by silver staining. The appearance of the monomeric band indicates formation of the overoxidized sulfenic acid form.
In the Western blot on the right, chloroplasts subjected to the 10 mM H$_2$O$_2$ treatment were further analyzed for distribution between thylakoid (T) and stroma (S) fraction following washing of the thylakoids with 5 mM K-Pi-buffer and for dissociation of the thylakoid-associated 2-Cys Prx by washes with 100 mM NaCl, 100 mM potassium iodide, and 0.1% SDS, respectively.

Inactivated 2-Cys Prx Is Observed in Vivo under Stress—The last set of experiments was designed to answer the question whether the inactive sulfenic acid form of Prx also occurs in vivo. Under nonreducing conditions, initially reduced 2-Cys Prx oxidizes to the disulfide form in the presence of atmospheric O$_2$ (see Fig. 9) (16). In vitro experiments have shown that sulfenic acid derivatives of Prx are only formed when Prx is reduced by reductants like Trx on the one hand and simultaneously a high concentration of peroxides is present (i.e., a redox situation with both reducing and oxidizing activities) (40). Such apparently contradicting redox conditions are frequently found in vivo under stress conditions. In an experimental approach, barley seedlings were maintained for 2 days under control conditions or irrigated with 500 mM NaCl to induce salinity stress, subjected to cold treatment at 4 °C and continuous light, or grown without irrigation at high light to cause wilting. Furthermore, leaf tips with symptoms of senescence were harvested from plants grown under control conditions. By nonreducing SDS-PAGE and Western blotting, only homodimers were found in the control (Fig. 11). A small amount of the overoxidized form was detected as monomers in NaCl-treated plants. In extracts from cold- and water-stressed leaves as well as from senescing leaf tips, monomeric Prx was detected in significant amounts, which could not form disulfide bridges any more and thus represented the sulfinic acid form.

2-Cys Prx Content in Barley—To quantify the protein fraction in leaves represented by 2-Cys Prx, defined amounts of leaf extracts were analyzed by SDS-PAGE and Western blotting and compared with a calibration immunodetection obtained with recombinant PrxWT. 2-Cys Prx protein was estimated to represent 0.6 ± 0.2% of total soluble leaf protein (n = 3).

DISCUSSION

This paper shows that the plant 2-Cys Prx has a dynamic protein structure that undergoes major conformational changes during catalysis, forms supercomplexes, reversibly attaches to the thylakoid membrane, is subject to irreversible inhibition, and exhibits a broad peroxide substrate specificity. These characteristics give strong implications for its possible in vivo function in peroxide detoxification and redox signaling.

A Role of 2-Cys Prx in Signaling by Regulating the Intracellular Concentration of H$_2$O$_2$ and Lipid Peroxides—The present work reports for the first time a peroxidase activity of plant 2-Cys Prx with complex peroxides such as linoleic acid hydroperoxide and phosphatidylcholine dilinoleyl hydroperoxide, suggesting a physiological function as an antioxidant protecting the thylakoid membrane. Although predicted before (41) on the basis of studies with bacterial and trypanosomatid Prx (1, 2), proof had been requested for plant Prx (42). The reducing activity decreased with the bulk size of the alkyl residue. The enzymatic characteristics of barley 2-Cys Prx were as follows: $k_{\text{cat}} = 0.23$ [s$^{-1}$], $k_{\text{cat}}/K_m(\text{ROOH}) = 1.1 \times 10^5$ M$^{-1}$ s$^{-1}$, and $K_m < 2 \mu$m (16) (this paper). These values are different from those reported for 2-Cys Prx from other organisms (Leishmania donovani H6TXNPx: $k_{\text{cat}} = 7.2$ s$^{-1}$, $K_m = 30.5$ μM; C. fasciculata TXNP: $k_{\text{cat}} = \infty$, $K_m = \infty$; ROOH = \infty; and Helicobacter pylori AhpC: $k_{\text{cat}} = \infty$, $K_m = \infty$, ROOH = \infty) (4) and indicate a remarkable high substrate affinity of barley 2-Cys Prx. Rate constants of about 10$^5$ M$^{-1}$ s$^{-1}$ were lower than those known from the ascorbate peroxidases and superoxide dismutases (43) but are comparable with the Cys-type of glutathione peroxidases exclusively found in plants (5). Like the Trypanosomas Prx (2) and human Prx (44), chloroplast 2-Cys Prx is an abundant protein representing 0.6 ± 0.2% of total leaf protein. All of
these data support the view that plant 2-Cys Prx protects against oxidative stress as a low efficiency, high abundance peroxidase.

Influence of Amino Acid Substitutions on 2-Cys Prx Activity—As shown before for nonplant 2-Cys Prx, Cys64 is essential for peroxidase function (19, 29). Comparative analysis in a DTT- and Trx-based activity assay (Fig. 3) demonstrated that Cys185 is not important for peroxidase activity but is important for reduction by Trx. Apparently, in the C185S variant, Cys64 was oxidized to sulfenic acid and could be more efficiently (19-fold better) reduced by DTT than the disulfide bridge formed in PrxWT. For regeneration by Trx, the disulfide bond formation between the Cys64 and Cys185 of the two subunits of the dimeric enzyme is essential. Consequently, the C185S variant was as inactive as the C64S variant in the Trx assay.

The variants C64S and C185S mimic the reduced enzyme, and both variants oligomerized (Fig. 5A). Reduction mediated a conformational change from a dimeric to a oligomeric form as postulated before (13, 16). It is suggested that the oligomer is the active form and regeneration is rate-limiting, because the variant C185S was much more active in the DTT-based assay than the PrxWT.

Crystal structure of the reduced TryP (10) and also of overoxidized rat TpxB (11) indicates a catalytic triad between Arg140, Thr9251, and Cys185. Consistent with that model, substitution of Arg140 reduced activity in both the DTT- and Trx-based assay. Within the Prx family, Arg163 and Arg170 are specific for 2-Cys Prx. In the crystal structures, the homolog of Arg163 directs toward the catalytic center (11). Like Arg170, Arg163 was less important in the Trx-based assay than in the DTT-based assay, indicating that it is more involved in peroxide reduction than reductive regeneration of the enzyme.

In contrast, the variant W189L was more easily reduced by DTT. Regeneration with Trx was inhibited compared with PrxWT (Figs. 3 and 4), implicating that the oxidized conformation is stabilized by Trp189 (Fig. 4). This conclusion is supported by the fact that substitution of Trp189 by Leu partially mediates aggregation to a decamer form (Fig. 5). It has been shown previously that high phosphate concentrations induce 2-Cys Prx aggregation independent of disulfide-bridge formation (16). Because high phosphate concentrations reduce the Prx activity in the Trx-based assay (Fig. 5), it can be concluded that the dimeric 2-Cys Prx is more easily reduced by Trx than the aggregated form. In a converse manner, DTT regenerated the aggregated form with higher rates. Thus, variants with stabilized reduced structure should be more active in the DTT-based assay than in the Trx-based assay compared with PrxWT.

Comparison of the activity rates (Fig. 3) suggests that Trp189, Trp99, Cys185, and Thr9251 stabilize the dimeric conformation, whereas the variants in these aa tend to aggregate, as shown for W189L and C185S (Fig. 5).

Structural Changes in 2-Cys Prx Based on Fluorescence of Single-tryptophan Variants: Oxidized Structure—Emission spectra (Fig. 6) and fluorescence lifetimes (Fig. 7) obtained by LIF for PrxWT, W99L, and W189L variants indicate that the residue Trp99 is located on the outside of the protein but not fully exposed to the solvent. This result stands in good agreement with the crystal structure published for mammalian 2-Cys Prx (12), which shows Trp99 being located in a ß-helical region close to the surface of the protein. The exact position of Trp189 is unknown. However, because of its proximity to Cys185 that is involved in the formation of the disulfide bridge and essential for dimerization, it can be assumed that Trp189 is located close to or even at the interface of both dimer halves.

The highly similar emission spectra of PrxWT and W189L (Fig. 7) suggest that the main part of protein fluorescence originates from Trp99. This is uncommon, since usually Trp residues located in the interior of proteins exhibit a higher quantum yield than surface-located Trp (45). An explanation for this behavior is quenching through adjacent aa within the protein, for example by strong quenchers such as Cys, Met, and Glu (37) as well as Arg and Lys (46). Cys185 as well as the charged aa from the C terminus could quench the emission from Trp189, making Trp99 the more emissive residue. Since both PrxWT and the mutant W99L have similar fluorescence lifetimes, fluorescence resonance energy transfer from Trp189 to Trp99 (38) can be disregarded as an alternative explanation of the smaller contribution of Trp189 to the overall spectrum.

Mammalian and Trypanosoma 2-Cys Prx decamelize upon reduction and at high protein concentration, bringing Trp99 into closer contact with ß-helices from neighboring monomers (10, 11, 33). Analysis by size exclusion chromatography confirmed oligomerization of plant 2-Cys Prx (Fig. 5). Therefore, oligomerization should be accompanied with a change of fluorescence from both Trp99 (because of contact to oligomerization partners) and Trp189 (because of a different microenvironment at the dimer interface).

The red shift in the emission spectrum of Trp189 in W99L as well as the increase of the fluorescence lifetime (7) might be explained by a reduced contact of the dimer halves upon reduc-
tion. The intensity increase in the blue-shifted range of the emission spectrum of PrxWT supports this thesis. Because of the cleavage of disulfide bonds, Trp189 is more solvent-accessible, whereas, at the same time, the quenching influence of neighboring aa on Trp189 is diminished. This can also be visualized by the increasing contribution of Trp189 to the calculated time-dependent PrxWT spectra under oxidizing and reducing conditions (Fig. 7). The increase in average τ_Cys99 in variant W189L as well as the general increase in τ for PrxWT (Fig. 6) shows that Trp189 moves from a rather solvent-exposed outer position to a fairly nonpolar environment between the monomers upon reduction. This finding provides spectroscopic evidence for oligomerization upon reduction.

C-terminal Tail Function in the Reaction Mechanism—Based on biochemical and physico-chemical studies, the following scenario is proposed for the reaction mechanism. Reduced thioredoxin interacts with the positively charged C terminus, and Cys185 of the oxidized dimeric 2-Cys Prx induces a conformational change and regenerates the active site. By comparison of several crystal structures, Wood et al. (13) proposed that the Cα-loop forms an α-helical turn and packs against the back site of region I (Fig. 2) in the reduced form. Thereby, the peroxidative active pocket is formed, and the C-terminal tail moves toward the active site and stabilizes the reduced conformation. The residues Arg140 and Arg163 are arranged in the vicinity of Cys64. Together Cys64, Thr62, and Arg140 constitute the catalytic triad and decrease the pK_a value of the Cys64 residue in the active site. Consequently, the nucleophilic attack on the hydroperoxide is facilitated. The reaction might be promoted by stabilization of the formed sulfenic acid by Arg140 and the alchoolate ion by Arg163.

The Cα-loop is in a dynamic equilibrium between folded and locally unfolded states (13). The latter form is necessary to facilitate disulfide bridge formation, whereby a conformational change occurs. Oxidation leads to a higher mobility of region I, which is pulled away from the interface, disrupting interactions by the homologues of Phe59 and Phe61. The movement of Phe60 uncovers Trp99 (13), which in turn adopts a new location in a more exposed environment (Fig. 6), resulting in weakened interactions within the oligomer interface. The active site pocket collapses in the oxidized state, and the carbonyl-O of the Asp homologue to Asp60 and the γO of Thr62 and Thr66 are directed into the active site pocket for hydrogen bonding (13). Consequently, the T66A variant was more locked in the reduced form with a folded Cα-loop, and replacement of the Thr66 residue impedes disulfide bridge formation. LIF spectroscopy analysis shows that the Trp189 residue is transferred to the interior during oxidation and stabilizes the oxidized form, whereby the C-terminal tail is loosened and freed for interaction with Trx, and the reaction cycle can start again.

Inactivation Occurs in Vivo under Several Stress Conditions, Suggesting a Structural Redox Signal of Reduced and Inactivated Prx—The sulfenic acid intermediate formed regularly in the reaction cycle is hypersensitive to oxidation. Only the reduced form is prone to oxidation, whereas the oxidized form is stable. As a consequence, the sulfenic acid form accumulates as a function of turnover number in in vitro assays as well as in cell cultures that could be detected for nonplant 2-Cys Prx by matrix-assisted laser desorption-ionization measurements (40, 47). Accumulation during stress treatments (Fig. 12) demonstrated that the formation and accumulation of the oxidized form takes place in plants in vivo and results in membrane attachment (Fig. 10). More than 40% of the 2-Cys Prx was attached to the thylakoids under severe oxidative conditions in organello. In light of the percentage of chloroplast protein represented by 2-Cys Prx and assuming molecular dimensions of 2-Cys Prx as described before (13), the figure of 40% thylakoid association allows us to calculate a surface coverage of about 100 cm²/mg chlorophyll or, when calculating stoichiometries, about one monomer 2-Cys Prx per electron transport unit with 600 chlorophyll molecules/unit.

Barley PrxWT showed no difference in inactivation pattern between Trx and DTT reduction (Fig. 3), demonstrating that the regeneration mechanism does not influence the process of inactivation but was correlated with the hydroperoxide concentration and the substrate size and indirectly related to the reaction rate (Figs. 9 and 10). This indicates that overoxidation of the active site is an indicator of ineffective peroxide detoxification.

Trp189 that is conserved in all 2-Cys Prx plays a crucial role in inactivation. The enzymatic activity of W189L was much lower than that of PrxWT, but almost no inactivation occurred (Fig. 9). It is postulated that the variant is locked in a state resembling the reduced form. Although to a lesser extent, replacement of Thr66 by Ala reduced 2-Cys Prx peroxidase activity and overoxidation (Fig. 9). The aromatic site chains of the Phe residues in the F-motif and the Trp189 around Cys62 and Cys185 that are involved in the transition states of the catalytic cycle may be essential for a conformation change that is necessary for the inactivation reaction as well as for regeneration by Trx.

Based on the experiments with chloroplasts treated with H₂O₂ (Fig. 10), the accumulation of inactive membrane-associated oligomers of 2-Cys Prx is redox-dependent. It is hypothesized that 2-Cys Prx acts as a structural redox sensor at the thylakoid membrane. As shown previously (16), reduction of 2-Cys Prx is linked to efficient photosynthetic electron transport. The reduced form of 2-Cys Prx can only accumulate if the redox potential drops below −315 mV (16). It is suggested that the attachment of the reduced form to the thylakoid membrane signals high electron pressure in the photosynthetic electron transport chain. If light energy perception exceeds the electron sink capacities of photosynthesis, increasing amounts of reactive oxygen species are formed (e.g. via the Mehler reaction) (24), and 2-Cys Prx gets overoxidized. Prx oligomerizes (Fig. 10) and attaches like the reduced form to the thylakoid membranes. The quantitative comparison indicates (Fig. 10) that the attachment increases with electron pressure in the photosynthetic electron transport chain. Enzymatic regeneration is not possible for the overoxidized form. Therefore, its binding to the thylakoid membrane could be a long-term memory for photooxidative stress. Structurally, oxidatively inactive 2-Cys Prx resembles the reduced active form. Both forms bind to the thylakoid membrane, whereas intermittently oxidized form is soluble (Fig. 10). A possible influence of inactivated 2-Cys Prx on the photosynthetic activity and a possible role in the adaptation process to oxidative stress will have to be explored in future studies.

REFERENCES

1. Hillas, P. J., del Alba, F. S., Oyarzabal, J., Wilks, A., and de Montellano, P. R. O. (2000) J. Biol. Chem. 275, 18601–18609
2. Nogoecke, E., Grimmel, D. U., Kies, M., Kalicz, H. M., and Flohé, L. (1997) Biochim. Biophys. Acta 1378, 827–836
3. Bryk, R., Griffin, P., and Nathan, C. (2000) Nature 407, 211–215
4. Hofmann, R., Hecht, H.-J., and Flohé, L. (1999) Biol. Chem. 380, 347–364
5. Dietz, K. J. (2003) Annu. Rev. Plant Biol. 54, 93–107
6. Horling, F., Lankemeyer, P., König, J., Finkemeier, I., Kandlbinder, A., Baier, M., and Dietz, K. J. (2003) Plant Physiol. 131, 317–325
7. Park, S. G., Cha, M. K., Jeong, W., and Kim, I. H. (2000) J. Biol. Chem. 759, 317–325
8. Dedefo, J. P., Evrard, C., Clippe, A., Vander Stricht, D., Bernard, A., and Knoppe B. (2001) J. Mol. Biol. 311, 751–759
9. Choi, H. J., Kang, S. W., Yang, C. H., Rhee, S. G., and Ryu, S. E. (1998) Nat. Struct. Biol. 5, 400–406
10. Alphhey, M. S., Bond, C. S., Tetaud, K., Fairlamb, A. H., and Hunter, W. N. (2000) J. Mol. Biol. 300, 901–916
11. Schroeder, E., Littlechild, J. A., Lebedev, A. A., Errington, N., Vagin, A. A., and
Reaction Mechanism of Plant 2-Cys Peroxiredoxin

---

Isager, M. N. (2000) *Struct. Fold. Des.* 8, 605–615

12. Hirotsu, S., Abe, Y., Okada, K., Nagahara, N., Hori, H., Nishino, T., and Hakoshima, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 12333–12338

13. Wood, Z. A., Poulie, L. B., Hantgan, R. R., and Karplus, P. A. (2002) *Biochemistry* 41, 5483–5504

14. Baier, M., and Dietz, K. J. (1999) *Plant Physiol.* 119, 1407–1414

15. Baier, M., Noctor, G., Foyer, C. H., and Dietz, K. J. (2000) *Plant Physiol.* 124, 823–832

16. König, J., Baier, M., Horling, F., Kahmann, U., Harris, G., Schürmann, P., and Dietz, K. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 5738–5743

17. Moteshaki, K., Kondoh, A., Stumpf, M. T., and Hisabori, T. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 11224–11229

18. Broun, M., Cuine, S., Eymery, F., and Roy, P. (2002) *Plant Cell* 14, 1417–1432

19. Montemartini, M., Kalisz, H. M., Hecht, H. J., Steinert, P., and Flohe, L. (1999) *Eur. J. Biochem.* 264, 516–524

20. Yamamoto, H., Miyake, C., Dietz, K. J., Tomizawa, K. I., Murata, N., and Yokota, A. (1999) *FEBS Lett.* 447, 269–273

21. Maierino, M., Gregolin, C., and Uraini, F. (1990) *Methods Enzymol.* 186, 448–457

22. Baier, M., and Dietz, K. J. (1997) *Plant J.* 12, 179–189

23. Plessow, R., Brockhunke, A., Eimer, W., and Kohse-Huisingh, K. (2000) *J. Phys. Chem. B* 104, 3695–3704

24. Mehler, A. H., and Brown, A. H. (1951) *Arch. Biochem. Biophys.* 33, 65–77

25. Brockhunke, A., Plessow, R., Dittrich, P., and Kohse-Huisingh-K. (2000) *Appl. Phys. B* 71, 755–763

26. Baier, M., and Dietz, K. J. (1996) *Plant Mol. Biol.* 31, 553–564

27. Tsugita, K., Copeland, N. G., Jenkins, N. A., Ohnata, M. (1995) *Biochem. J.* 307, 377–381

28. Lim, Y-S., Cha, M-K., Yun, C-H., Kim, H-K., Kim, K., and Kim, I-H. (1994) *Biochem. Biophys. Res. Commun.* 199, 199–206

29. Chea, H. Z., Uhm, T. B., and Rhee, S. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 7022–7026

30. Thurman, R. G., Ley, H. G., and Schola, R. (1972) *Eur. J. Biochem.* 25, 420–430

31. Flohe, L., Budde, H., Bruns, K., Castro, H., Clos, J., Hofmann, B., Kanze, Kalavar, S., Krumme, D., Menge, U., Plank-Schumacher, K., Satajar, H., Wissing, J., Wylegalla, C., and Hecht, H. J. (2002) *Arch. Biochem. Biophys.* 397, 324–335

32. Chaushan, R., and Mande, S. C. (2001) *Biochem. J.* 354, 209–215

33. Hofmann, B., Budde, H., Bruns, K., Guererro, S. A., Kalisz, H. M., Menge, U., Montemartini, M., Nogoecke, K., Steinert, P., Wissing, J. B., Flohe, L., and Hecht, H. J. (2001) *Biol. Chem.* 382, 459–471

34. Teale, F. W. J., and Weber, G. (1957) *Biochem. J.* 65, 476–482

35. Lakowicz, J. R. (1999) in *Principles of Fluorescence Spectroscopy* (Lakowicz, J. R., ed) Plenum Press, New York

36. Burstein, E. A., Vedenkina, N. S., and Ikvova, M. N. (1973) *Photochem. Photobiot.* 18, 263–279

37. Wells, T. A., Nakazawa, K., Manabe, K., and Song, P. S. (1994) *Biochemistry* 33, 708–712

38. Efting M., Ramsay, G. D., Burns, L., Maki, A. H., Mann, C. J., Matthews, C. R., and Ghiron, C. A. (1993) *Biochemistry* 32, 9189–9198

39. Waylowski, Z., Kaszyczki, P., Drewio, M. (1996) *J. Protein Chem.* 15, 45–58

40. Yang, K. S., Kang, S. W., Woo, H. A., Hwang, S. C., Chae, H. Z., Kim, K., and Rhee, S. G. (2002) *J. Biol. Chem.* 277, 38029–38036

41. Baier, M., and Dietz, K. J. (1999) *Trends Plant Sci.* 4, 166–168

42. Feussner, I., and Wasternack, K. (2001) *Trends Plant Sci.* 6, 268–273

43. Asada K. (1999) *Annu. Rev. Plant Phys.* 50, 691–699

44. Rhee, S. G., Kang, S. W., Netto, L. E., Seo, M. S., and Stadtman, E. R. (1999) *Biofactors* 10, 207–209

45. Elzing, M. R., and Ghizon, C. A. (1976) *Biochemistry* 15, 672–680

46. Yi-Brunozozi, H. Y., Stephenson, O. M., and Beal, P. A. (2001) *J. Biol. Chem.* 276, 37827–37833

47. Rahioud, T., Heller, M., Gassner, F., Ludwig, S., Roy, C., Aebersold, R., Benahmed, M., Louisot, P., and Lunardi, J. (2002) *J. Biol. Chem.* 277, 19386–19401

48. Dietz, K. J., Horling, F., König, J., and Baier, M. (2002) *J. Exp. Bot.* 53, 1321–1329
Reaction Mechanism of Plant 2-Cys Peroxiredoxin: ROLE OF THE C TERMINUS AND THE QUATERNARY STRUCTURE
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