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Detoxification of peroxides in the chloroplast: What's the role of 2-Cys peroxiredoxins?

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Introduction

Peroxiredoxins are present in all organisms and reduce H_2O_2 and alkyl hydroperoxides. One group of peroxiredoxins, the 2-Cys peroxiredoxins (2-Cys Prx), was found to be localized in chloroplasts and have been shown to be required for normal leaf development and photosynthetic function (Baier and Dietz, 1999; Cheong et al., 1999; Baier et al., 2000). However, the way that they interact with other components of the antioxidant system is still unknown. Several questions remain to be answered, for example the enzyme's redox state in vivo, the electron donor for regeneration of the enzyme and the identity of the in vivo substrates. Some of these questions are addressed in this short communication (Fig. 1).



Fig. 1: Schematic representation of the functional context of the 2-Cys Prx, which indicates some of the open questions. The enzyme is known to reduce a wide range of peroxides and to concur with the ascorbate and glutathione linked antioxidant systems. Ascorbate and glutathione, which also regulate gene expression, could together with NAD(P)H- and thioredoxin-linked enzymatic pathways and the photosynthetic electron transport chain regulate enzyme activity.

Peroxiredoxins form a small gene family: The genome of *Arabidopsis thaliana* encodes 10 peroxiredoxins: one 1-Cys peroxiredoxin, two 2-Cys peroxiredoxins (2-CPs), 6 type II-peroxiredoxins and one peroxiredoxin Q (Dietz et al., 2001). They are characterized by one or two cysteine residues, respectively, located in conserved environments (Chae et al., 1994; Baier and Dietz, 1996). Peroxides oxidize the cysteine residue via a sulfenic acid intermediate, which subsequently reacts with a thiol under formation of a disulfide bond. In 2-Cys Prx, another conserved cysteine residue in the second subunit of the homodimeric enzyme represents the interacting thiol. In the last step of the catalytic cycle redox proteins like thioredoxins are postulated to reduce the cysteine residue and regenerate the enzyme.

The subcellular localisation and expression of the 2-Cys Prx, and the antisense phenotype: In barley, 2-Cys Prx was found in chloroplasts (Baier and Dietz, 1997) where it is part of the antioxidant defence system and protects the photosynthetic membrane from oxidative damage. Antisense suppression of 2-Cys Prx in *Arabidopsis thaliana* affected chloroplast structure and function, particularly during early leaf development (Baier and Dietz, 1999). Following antisense suppression of 2-Cys Prx, the burden of antioxidant defense was shifted to the ascorbate system. Expression and activity of ascorbate peroxidase and monodehydroascorbate reductase were increased and the ascorbate pool was more oxidized in the 2-Cys Prx antisense plants. Under the same conditions the glutathione pool was unaffected in size and redox poise (Baier et al., 2000).

Expression studies in barley (Baier and Dietz, 1996), *Arabidopsis thaliana* (Horling et al., unpublished data) and the submerse form of the liverwort *Riccia fluitans* (Horling et al., 2001) showed that in plants the 2-Cys Prx transcript amount is tightly linked to the redox poise of low molecular weight antioxidants. In *Riccia fluitans* addition of 20 mM ascorbate to the nutrient medium resulted in a strong reduction of transcript amount within 2 to 4 h (Horling et al., 2001). Together with the observation that in antisense plants the decrease of 2-Cys Prx function caused a depletion of the ascorbate pool, our data indicate that the ascorbate based Halliwell-Asada-Cycle and the 2-Cys Prx-catalyzed alternative water-water cycle function partly independently, but are linked via the redox-poise of low molecular weight antioxidants.

Oxidized 2-Cys Prx needs to be regenerated. In yeast, the 2-Cys Prx is regenerated by thioredoxin and thioredoxin reductase. Accordingly, in plants the thioredoxin/ferredoxin-system might be the main electron source for 2-Cys Prx regeneration in the light (Baier and Dietz, 1997). This mechanism would link the regeneration reaction to the photosynthetic electron transport chain. But apart from some experimental evidence obtained with Chinese cabbage in which reduction of 2-Cys Prx by the thioredoxin/thioredoxin reductase-system has been shown *in vitro* (Cheong et al., 1999), the actual regeneration mechanism is still unknown. In this communication we address the questions (1) whether low molecular weight antioxidants could be involved in 2-CP regeneration and (2) how specific the enzymatic regeneration system is?

Materials and Methods

Barley (*Hordeum vulgare* var. Gerbel) was grown on soil for 8 - 10 days either at a light/dark cycle of 14 h at a PAR of 100 µmol quanta m⁻² s⁻¹ and 25 °C/10 h darkness at 15 °C. For the experiment shown in Fig. 2 half of the plants were illuminated for 1 h at 350 µmol quanta m⁻² s⁻¹ after 18 h in darkness, while the rest of the plants were kept in the dark.

Barley leaves were extracted in 100 mM sodium phosphate buffer (pH 7.0) supplemented with 10 mM ascorbate, dehydroascorbate (DHA), reduced (GSH) or oxidized glutathione (GSSG), dithiothreitol (DTT), NADH, NADPH or 12.5 or 50 mM DTNB (5,5-Dithiobis-[2-nitrobenzoic acid]), respectively. As indicated in Fig. 4 in one of the experiments 10 mM NADPH, 5 µg recombinant *E. coli* thioredoxin or 10 µg recombinant *E. coli* thioredoxin

reductase were added to the extracts. After addition of 2-times concentrated loading buffer (without reducing agent), the samples were analyzed by polyacrylamide gel electrophoresis and Western blot analysis with 2-Cys Prx peptide antibody as described before (Baier and Dietz, 1996).

Results and Discussion

Both, in the light and in the dark, 2-Cys Prx is present in 3 redox states in the cell: (1) The fully oxidized form in which the two homomeric subunits are linked by two disulfide bonds, (2) the partially oxidized form with only one disulfide bond and (3) the fully reduced form which separates into monomers following SDS treatment and heating. The different redox states can easily be analyzed by polyacrylamide gel electrophoresis (Fig. 2).



Fig. 2. The redox state of native 2-Cys Prx: The redox state was arrested by denaturing the protein or reacting the reduced cysteine residues with DTNB during extraction. The samples were analyzed by gel electrophoresis and Western Blot analysis. The leaves were harvested either after 19 h dark treatment or after 1 h light treatment following an 18 h dark period.

In an oxygenic environment the protein is rapidly oxidized (control in Fig. 2). For redox monitoring, the redox state can be arrested by immediate denaturing of the protein and by blocking the sulfhydryl groups with saturating amounts of DTNB. Interestingly, no major differences could be observed in plant samples maintained in darkness for 19 h or illuminated for 1 h at 350 μ mol quanta m⁻² s⁻¹ after 18 h in darkness. Most of the protein was in the partially or fully reduced form. From this observation two conclusions can be drawn: (1) Increased photooxidative ROS formation and peroxidation during a 1h light treatment did not overburden the 2-CP capacity. (2) There exists an electron source for 2-Cys Prx reduction that is independent of light and keeps the 2-Cys Prx partly reduced even in darkness. Apparently, the photosynthetic electron transport chain is not the exclusive mechanism to reduce oxidized 2-Cys Prx.

To test the effects of low molecular weight antioxidants and the reducing equivalents NADH and NADPH on the redox state of 2-Cys Prx, equimolar amounts of ascorbate, glutathione, DTT, NADH and NADPH were added to the extraction buffer. For control,

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extractions were performed in the presence of dehydroascorbate and oxidized glutathione (Fig. 3). In this experiment only DTT fully reduced 2-CP. Under denaturing conditions the DTT-reduced protein dimer monomerized, and reconstitution of the dimer, for instance by incubation in an oxygenic environment or by mild treatment with H_2O_2 , was not possible (data not shown).



Fig. 3. Redox state of barley 2-Cys Prx in dependence of the extraction conditions. Crude extracts of barley primary leaves from light grown plants were extracted in the presence of 10 mM ascorbate, dehydroascorbate, reduced or oxidized glutathione, DTT, NADH and NADPH, respectively. The redox state of 2-Cys Prx was analyzed by Western blotting following separation in non-reducing SDS-PAGE.

The reduced form of 2-CP was partially maintained by application of ascorbate, while NADH and NADPH supplementation further increased the amount of reduced 2-CP (Fig. 3). From this data it is concluded that the two major low molecular weight antioxidants, ascorbate and glutathione, cannot reduce 2-CP directly.

The ascorbate-dependent stabilization could be a direct or indirect effect. The added ascorbate might reduce the oxidative pressure on 2-Cys Prx by reacting directly with reactive oxygen species. It is more likely that ascorbate indirectly protected the 2-Cys Prx from oxidation by stabilizing the H_2O_2 reducing capacity of the ascorbate peroxidase system, because addition of equimolar amounts of reduced glutathione did not stabilize the redox state of 2-Cys Prx to the same extent as ascorbate did.

The different effects of ascorbate, glutathione and NADH or NADPH suggest that the 2-Cys Prx is regenerated enzymatically. It may be postulated that electrons are transferred on 2-Cys Prx via various NADH- and NADPH-dependent oxidoreductases or via an oxidoreductases with little preference for NADH or NADPH.

Thioredoxins and thioredoxin-like proteins are important redox-carriers in plants. That they could also be involved in the regeneration of oxidized 2-Cys Prx has been shown for yeast and Chinese cabbage 2-Cys Prx by Chae et al. (1994) and Cheong et al. (1999), respectively. Here, *E. coli* thioredoxin and thioredoxin reductase and NADPH were added to crude desalted barley extracts. They catalyzed partial reduction of the 2-Cys Prx as visualized on denaturing SDS-polyacrylamide gels (Fig. 4).



Fig. 4. Effect of thioredoxin and thioredoxin reductase on 2-Cys Prx. In 3 parallel experiments extracts incubated for 5 min at room temperature were supplemented consecutively with NADPH, recombinant *E. coli* thioredoxin (Trx) and recombinant *E. coli* thioredoxin reductase (TR) in the order as indicated. Each component was added after 5 min of incubation with the previous components. The redox states of 2-Cys Prx were visualized by Western blot analysis.

Control reactions with thioredoxin reductase and NADPH only and, especially, thioredoxin and NADPH only, demonstrate that endogenous redox-components contained in the extract can bridge the electron transfer from NADPH to 2-Cys Prx. Even in the absence of NADPH as electron source, addition of thioredoxin or thioredoxin reductase/thioredoxin caused partial reduction of the 2-Cys Prx. Since the extract had been desalted and pre-incubated under oxidizing condition for 5 min, it is unlikely that it contained any reducing equivalents or reduced low molecular thiols as alternative electron sources. 2-Cys Prx is almost fully oxidized under these conditions (Fig. 4). The occurrence of the reduced form of 2-Cys Prx may indicate electron transfer between proteins under these conditions.

Conclusions:

The results of this paper characterize the redox state of the chloroplast 2-Cys Prx *in situ* and potential electron donors *in vitro*. The observations were:

- (1) Both in darkness and low light, the 2-Cys Prx is partly reduced. Apparently, there exists a regeneration system for oxidized 2-Cys Prx, which is independent of the photosynthetic electron transport chain.
- (2) 2-Cys Prx is efficiently oxidized under oxygenic conditions in vitro, and possibly also in vivo.
- (3) Oxidized 2-Cys Prx can partly be reduced by adding NADPH or NADH to the extraction medium or extracts. This may indicate the involvement of NAD(P)H-dependent reductases in the alternative reduction reaction of 2-Cys Prx.
- (4) *E. coli*-Trx/TR can also reduce oxidized 2-Cys Prx in crude desalted extracts confirming results by Cheong et al. (1999). However, the functional capacity of chloroplast Trx-m or Trx-f will have to be investigated in the regeneration reaction of 2-Cys Prx.

The partial reduction of 2-Cys Prx in the dark deserves attention. The redox potential of 2-Cys Prx is below –300 mV (König, Baier, Dietz unpublished). Consequently, reduction of 2-Cys Prx would only occur at stromal redox conditions leading to full activation of redox-regulated Calvin cycles enzymes. In the dark, the thioredoxin system is oxidized and the

regulated Calvin cycle enzymes are inactive; nevertheless the 2-Cys Prx was partly reduced. This observation indicates alternative reduction pathways at least in the dark and a strong electron accepting capacity of oxidized 2-Cys Prx.

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References

Baier M, Dietz K-J (1996) Plant Molecular Biology 31, 553 - 564

Baier M, Dietz K-J (1997) Plant Journal 12, 179-190

Baier M, Dietz K-J (1999) Plant Physiology 119, 1407 - 1414

Baier M, Noctor G, Foyer CH, Dietz K-J (2000) Plant Physiology 124, 823 - 832

Chae HZ, Chung SJ, Rhee SG (1994) Journal of Biological Chemistry 269, 27670 – 27678

Cheong NE, Choi YO, Lee KO, Kim WY, Jung BG, Chi YH, Jeong JS, Kim K, Cho MJ, Lee SY (1999) *Plant Molecular Biology* **40**, 825 – 834

Dietz KJ, Horling F, König J, Baier M (2001) *Journal of Experimental Botany* in press Horling F, Baier M, Dietz KJ (2001) *Planta* in press.