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Isolation and characterization of a thioredoxin-dependent peroxidase from *Chlamydomonas reinhardtii*

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Introduction

Peroxiredoxins (Prx) form a ubiquitous group of peroxidases that were found in almost all living organisms. Prx featuring 2 conserved cysteine residues (2Cys-Prx) are reduced by the AhpF protein in bacteria, and by the thioredoxin/thioredoxin reductase system in yeast, animals and plants. 2Cys-Prxs catalyze *in vitro* the reduction of alkyl hydroperoxides and hydrogen peroxide. They exist as homodimers, both subunits are linked by an intermolecular disulfide (Chae *et al*., 1994). In animals, yeast, and plants, the disulfide is reduced *via* a thiol/disulfide redox interchange with reduced thioredoxin (Trx), thus regenerating an active peroxidase.

The present study was aimed at setting up a purification system for trapping proteins that react with thioredoxin, based on their ability to form mixed disulfide-linked adducts with a single cysteine mutant thioredoxin. The system allowed the purification and identification of a 2Cys-Prx protein (Ch-Prx1) from the green alga *Chlamydomonas reinhardtii*. The purified protein was characterized by its peroxidase activity, and its ability to use different thioredoxin isoforms as hydrogen donors. The regulation of the *Ch-Prx1* gene expression suggests that Ch-Prx1 is involved in detoxification of ROS in the *Chlamydomonas* chloroplast.

Materials and methods

Algal strains and culture conditions-Chlamydomonas reinhardtii cell-wall-less strain CW15 and strain CC 125 were grown in a photoautotrophic minimal medium (HSM) at 25°C or 32°C under continuous stirring and bubbling with 2 or 5 % CO_2 enriched air. Light intensity was 150 or 300 µmol/m^2sec. Cultures were kept in a continuous light regime or in a 12 h light/dark regime.
Purification of the Ch-Prx1 native protein from Chlamydomonas cells-Chlamydomonas CW15 cells were pelleted, resuspended in 30 mM Tris-HCl pH 7.9 (Tris buffer), and broken by 2 cycles of freeze-thawing. Broken cells were centrifuged, the supernatant was adjusted to 2 % (w/v) streptomycin sulfate and precipitated nucleic acids were pelleted. The supernatant was adjusted to 95 % (w/v) ammonium sulfate. Precipitated proteins were pelleted, resuspended in Tris buffer, and dialyzed against the same buffer. The protein solution was loaded onto a column made of a mutated *Chlamydomonas* h-type thioredoxin (C39S mutant) grafted on a CNBr activated sepharose support according to recommandations of the supplier (Pharmacia-Amersham) and equilibrated with Tris buffer. After loading on the column, the

proteins were eluted with DTT. The eluted proteins were dialyzed against Tris buffer and re-applied on the affinity column. Purification of the recombinant Ch-Prx1 expressed in *E. coli*-BL21 (DE3) was essentially done using the same procedure.
RNA isolations and northern blot analysis-RNA were isolated by two different methods, either TRIzol reagent method or magnetic oligo d(T) beads (Dynal) protocol (Salvador *et al*., 1993). Northern blot analysis were performed as described in (Salvador *et al*., 1993; Sambrook *et al*., 1989).
Thioredoxin-dependent peroxidase activity of Ch-Prx1-An assay, avoiding the need of a thioredoxin reductase, was used, based on colorimetric determination of hydrogen peroxide or alkyl hydroperoxide with the PeroXOquant kit (Pierce) following the supplier's recommandations.

Results-Discussion

In an attempt to isolate new Trx targets in *Chlamydomonas*, a strategy was used based on the formation of stable mixed disulfides between a Trx, mutated in its less reactive active-site cysteine (C39S), and its potential targets. The approach has previously been used successfully to characterize *in vivo* complexes of thioredoxin with some of its target proteins (Verdoucq *et al*., 1999; Kishigami *et al*., 1995) and to identify *in vitro* the most reactive internal cysteine (Cys 207) of *Sorghum* NADP-MDH (Goyer *et al*., 1999). Among the *Chlamydomonas* proteins that were retained on the column was a major protein of about 21 kDa (Fig.1). Computer database searches based on the amino-acid sequences of sequenced peptides of this protein revealed 75 % homology with a thioredoxin-dependent peroxidase, also named peroxiredoxin (PRX), of barley (Baier and Dietz, 1996) and *Arabidopsis* (Baier and Dietz, 1997) (BAS1 proteins).

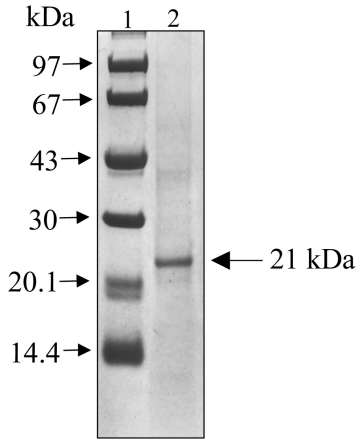

Fig.1. Analysis of elution products from the thioredoxin affinity column by reducing SDS-PAGE. Protein extracts of *Chlamydomonas* cultures were applied on a Trx h C39S affinity column. The elution was performed with DTT.
Lane 1, molcular weight markers;
lane 2, elution products.

These proteins belong to the 2Cys-Prx group (Baier and Dietz, 1997). *Arabidopsis* BAS1 was shown to be a chloroplastic protein. The homology of our peptides with BAS1 and the presence of two cysteines in alignment with the conserved cysteines of BAS1 proteins suggested that our 21 kDa protein (Ch-Prx1) also belongs to this protein family, and could be chloroplastic.

We isolated the cDNA (Lambda gt11 library) and the gene encoding Ch-Prx1 (*Chlamydomonas* genomic BAC library, GenomeSystemsInc., St. Louis, USA) (accession numbers: AJ304856; AJ304857). The gene contains 2 introns and 3 exons. A 12 bp exon separates both introns. The amino acid sequence deduced from the gene sequence revealed a putative chloroplast transit peptide.

Thioredoxin-dependent peroxidase activity of Ch-Prx1 toward t-butylhydroxyperoxide (t-BOOH) was examined by following the degradation of t-BOOH, using the peroXOquant kit, in the presence of DTT as electron donor to Trx. The rate of disappearance of t-BOOH was identical with Trx m and Trx h from *Chlamydomonas* used at the same concentration (Fig.2). When Trx was omitted, the rate of disappearance of t-BOOH was negligible, proving that DTT alone, used at low concentration, cannot significantly reduce Ch-Prx1. In order to determine whether different chloroplastic Trx differ in their abilities to function with Ch-Prx1, we compared the efficiencies of two Trx isoforms f and m from spinach, because no Trx f from *Chlamydomonas* has been isolated until now. Both were active with Ch-Prx1, but while Trx f was as efficient as Trx h and m from *Chlamydomonas*, Trx m from spinach showed a lower efficiency, suggesting that Trx f is the preferred electron donor to Prx *in vivo*.

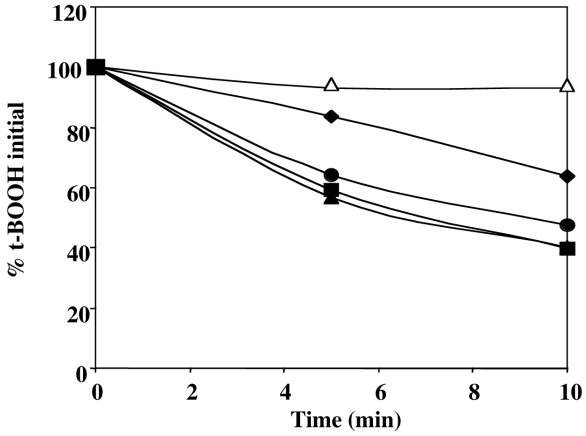

Fig. 2. Peroxidase activity of recombinant Ch-Prx1 towards t-butyl hydroperoxide with various thioredoxins. The concentration of t-BOOH was measured colorimetrically and expressed as a percentage of the initial concentration. Chlamydomonas Trx h (closed triangles) or Trx m (closed squares), spinach Trx f (closed circles) or Trx m (closed diamonds). Control without Trx (open triangles).Ch-Prx1 (43.8 µM) was incubated with 400 µM DTT, 16.6 µM Trx and 500 µM t-butyl hydroperoxide in 50 µL of 30 mM Tris-HCl, pH 7,9, buffer. The quantity of t-BOOH was measured on 5 µL aliquots added to a spectrophotometer cuvette containing 500 µL of PeroXOquant medium. The activity was estimated from the decrease in absorbance at 595 nm. Spinach Trx were a kind gift of Prof. P. Schürmann.

To find possible roles for Ch-Prx1 *in vivo*, the expression of the *Ch-Prx1* gene was monitored under different culture conditions. In the dark, levels of *Ch-Prx1* gene transcripts are relatively low (Fig. 3, lane 1). They were markedly increased in illuminated cells (lane 2). Moreover, the highest levels of *Ch-Prx1* transcripts were found in cells bubbled with oxygen for 90 min in the dark (lane 3). These results show that light and oxidative stress affect *Ch-Prx1* gene expression.

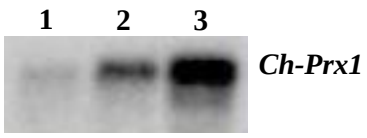

Fig.3. *Ch-Prx1* gene expression. Induction of *Ch-Prx1* gene expression in *Chlamydomonas* by light and by bubbling with oxygen. Lane 1, cells taken after 11 hrs in the dark; lane 2, cells taken 3 hrs after the start of the light period; lane 3, cells taken after 11 hrs in the dark after 90 min bubbling with O_2.

Because *Ch-Prx1* gene expression occurs in conditions known to stimulate the production of ROS, and because Ch-Prx1 is able to remove peroxides, it is likely that Ch-Prx1 is involved in detoxification of ROS in the *Chlamydomonas* chloroplast. It should be of interest

to over- and under-express Ch-Prx1 in *Chlamydomonas* to better understand its importance in protection against oxidative stress.

References

Baier M and Dietz KJ (1996) *Plant Mol. Biol*. **31**, 553-564.

Baier M and Dietz KJ (1997) *Plant J*. **12,** 179-190.

Chae HZ, Chung SJ and Rhee SG (1994) *J. Biol. Chem*. **269**, 27670-27678.

Goyer A, Decottignies P, Lemaire S, Ruelland E, Issakidis-Bourguet E, Jacquot JP and Miginiac-Maslow M (1999) *FEBS Lett*. **444**, 165-169.

Kishigami S, Kanaya E, Kikuchi M and Ito K (1995) *J. Biol. Chem.* **270**, 17072-17074.

Salvador ML, Klein U and Bogorad L (1993) *Plant J.* **3,** 213-219.

Sambrook J, Fritsch EF, Maniatis T (1989) in *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Verdoucq L, Vignols F, Jacquot JP, Chartier Y and Meyer Y (1999) *J. Biol. Chem.* **274**, 19714-19722.