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ORIGINAL PAPER

Functional characterisation of the peroxiredoxin gene family members of *Synechococcus elongatus* PCC 7942

Tina Stork · Miriam Laxa · Marina S. Dietz · Karl-Josef Dietz

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Abstract The genome of *Synechococcus elongatus* PCC 7942 encodes six peroxiredoxins (Prx). Single genes are present each for a 1-Cys Prx and a 2-Cys Prx, while four genes code for PrxQ-like proteins (*prxQ-A1*, *-A2*, *-A3* and *B*). Their transcript accumulation varies with growth conditions in a gene-specific manner (Stork et al. in J Exp Bot 56:3193–3206, 2005). To address their functional properties, members of the *prx* gene family were produced as recombinant proteins and analysed for their peroxide detoxification capacity and quaternary structure by size exclusion chromatography. Independent of the reduction state, the 2-Cys Prx separated as oligomer, the 1-Cys Prx as dimer and the PrxQ-A1 as monomer. PrxQ-A2 was inactive in our assays, 1-Cys Prx activity was unaffected by addition of TrxA, while all others were stimulated to a variable extent by addition of *E. coli* thioredoxin. Sensitivity towards cumene hydroperoxide treatment of *E. coli* BL21 cells expressing the cyanobacterial PrxQ-A1 to A3 proteins was greatly reduced, while expression of the other Prx had no effect. The study shows differentiation of Prx functions in *S. elongatus* PCC 7942 which is discussed in relation to potential roles in site- and stress-specific defence.

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T. Stork · M. Laxa · M. S. Dietz · K.-J. Dietz (✉)
Biochemistry and Physiology of Plants,
Faculty of Biology, W5-134, Bielefeld University,
33501 Bielefeld, Germany
e-mail: karl-josef.dietz@uni-bielefeld.de

Present Address:
M. Laxa
Department of Plant Sciences,
University of Oxford, South Parks Road,
Oxford OX1 3RB, UK

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Abbreviations

COOH	Cumene hydroperoxide
DTT	Dithiothreitol
Grx	Glutaredoxin
GSH	Glutathione
IPTG	Isopropyl-β-D-thiogalactopyranoside
Prx	Peroxiredoxin
tBOOH	Tertiary butylhydroperoxide
TrxA	Thioredoxin

Introduction

Following the evolution of the first cyanobacteria about 3.5 billion years ago the earth atmosphere enriched in oxygen and slowly reached the present day concentration of 21% (v/v) (Schopf 1993). From early times on cyanobacteria had to cope with three types of oxidative threats, (1) O_2 released in the vicinity of the thylakoid membranes by their own oxygenic metabolism of photosynthesis, (2) the local O_2 enrichment in photosynthesising cyanobacterial populations, and (3) the rising atmospheric O_2 concentration. Since in addition a cyanobacterium served as endocytobiont that turned into the chloroplast of plants, exploring the antioxidant system of cyanobacteria promises important insight into the requirements for antioxidant defence of organisms with oxygenic photosynthesis (Koksharova et al. 2006; Olson and Blankenship 2004).

Cyanobacteria constitute a taxonomic group distinct from other eubacteria, namely the gram-negative and -positive

bacteria. This separate classification is justified not only due to their photosynthetic capacity but also based on other characteristics such as the multilayer cell wall (Xiong et al. 2000; Hansmann and Martin 2000). *Synechococcus elongatus* PCC 7942 (synonymous to *Anacystis nidulans R2*) which was used in this study is an obligate photoautotrophic cyanobacterium that as early as in 1970 was transformed with foreign DNA (Shestakov and Khyen 1970). Its full genome sequence is available at NCBI (NC_007604).

Imbalances in energy metabolism result in increased production rates of reactive oxygen species (ROS) such as the superoxide anion radical, hydrogen peroxide and hydroxyl radical. ROS in turn damage macromolecules and elicit lipid peroxidation or peroxinitrite generation. In respiratory and photosynthetic electron transport particularly under conditions of overreduction ROS are produced at various sites such as photosystem II, the plastoquinon pool, photosystem I in photosynthesis, and complex I and III in respiration (Elstner 1990). To counteract oxidative stress, cells maintain an elaborate antioxidant defence system. *Synechocystis* PCC 6803 is the cyanobacterium studied best with respect to its antioxidant system. It expresses a superoxide dismutase (*slr1516*), a catalase peroxidase (*sll1987*) and five peroxiredoxins (Prx) (Kaneko et al. 1996; Kobayashi et al. 2004; Hosoya-Matsuda et al. 2005; Dietz et al. 2005; Stork et al. 2005). Perelman et al. (2003) analysed a double mutant of *S. elongatus* PCC 7942 deficient in both the catalase and the 2-Cys Prx homologue. The cells survived in the presence of toxic H_2O_2. The authors concluded that additional H_2O_2 detoxification pathways must coexist with the catalase and 2-Cys Prx in *S. elongatus* PCC 7942 cells. In the light of the presence of a complex Prx protein family with six members, such a functional redundancy may not be surprising.

Prx constitute a protein family of evolutionary old peroxidases with high affinity to their peroxide substrates (Hofmann et al. 2002). Prx are present in all organisms. Their enzymatic mechanism of peroxide reduction depends on a catalytic cysteinyl residue in conserved amino acid environment that reacts with the peroxide substrate under formation of a sulphenic acid intermediate (Dietz 2003). Hydrogen peroxide, alkyl hydroperoxides and peroxinitrite are reported Prx substrates. The catalytic efficiency with diverse substrates varies among the four different Prx forms which are distinguished based on the intramolecular location of their catalytic Cys residues and the subsequent mechanism of active site regeneration: (1) The first identified Prx group was later on denominated 2-Cys Prx. 2-Cys Prx function as homodimers in a head-to-tail arrangement. Each subunit has a catalytic and a resolving cysteinyl residue (Kim et al. 1988; Chae et al. 1993; Baier and Dietz 1996). Upon reaction with the peroxide substrate, an intermolecular disulphide bridge is formed between both subunits following the intermittent formation of the sulphenic acid derivative of the catalytic Cys. Reductive regeneration of 2-Cys Prx is achieved by thioredoxins and cyclophilins (Collin et al. 2003; Dietz 2003; Laxa et al. 2007). Plant 2-Cys Prx are posttranslationally imported into chloroplasts (Baier and Dietz 1997); (2) 1-Cys Prx lack a resolving Cys. The sulphenic acid intermediate is stabilised within the molecule prior to the direct regeneration by a yet unknown thiol regenerant (Stacy et al. 1996, 1999); (3) The PrxQ protein carries the two interacting Cys in close vicinity spaced by only four amino acids. Higher plant PrxQ is a chloroplast enzyme (Rouhier et al. 2004) attached to the thylakoid membrane (Lamkemeyer et al. 2006) and possibly imported into the thylakoid lumen (Petersson et al. 2006). PrxQ are absent from animals. In prokaryotic systems, the PrxQ is often named bacterioferritin comigratory protein (Bcp). Some bacterial PrxQ isoforms lack the resolving Cys. Plant PrxQ functions in context of photosynthesis (Lamkemeyer et al. 2006); (4) Type II Prx, together with PrxQ, also called atypical 2-Cys Prx form the disulphide bridge within one polypeptide. In this case the catalytic Cys is in 24 aa distance from the resolving Cys. Type II Prx are regenerated by glutaredoxins, thioredoxins or glutathione (Rouhier et al. 2001; Bréhélin et al. 2003; Finkemeier et al. 2005).

With its five members in *Synechocystis* PCC 6803, six in *S. elongatus* PCC 7942 (Stork et al. 2005) and seven in *Anabaena* sp. 7120 (Latifi et al. 2007), respectively, the peroxiredoxin gene families in cyanobacteria are larger than in other prokaryotic organisms. All organisms appear to possess at least one Prx, which most frequently is a 2-Cys Prx. In addition and with a high degree of variability most organisms contain an atypical 2-Cys Prx, i.e. either a type II Prx or a PrxQ-like protein, and a 1-Cys Prx, respectively. For example, in gram-negative and -positive bacteria, the Prx count gives figures between one (*Lactobacillus plantarum*) and four (*Mycobacterium bovis* AF2111/97, Engemann 2006) with most often two or three members. As representative example, *Helicobacter pylori* expresses one 2-Cys Prx (AhpC thiol-specific antioxidant), a Bcp and a thiol peroxidase (Tpx) (Wang et al. 2006). It is tempting to assume that the large Prx number in cyanobacteria is linked to various metabolic requirements in context of oxygenic photosynthesis.

This work aimed at characterising the *prx* gene family previously identified in *S. elongatus* PCC 7942 (named Se-Prx; Stork et al. 2005). To this end *prx* genes were cloned as cDNAs, heterologously expressed in *E. coli*, purified and analysed for their catalytic properties, as well as characterised for their functional aggregation state. In addition a peroxide tolerance test was performed with *E. coli* BL21 that expressed cyanobacterial *prx* genes. The study shows pronounced differences for these Prx proteins and suggests distinct functions in *S. elongatus* PCC 7942.

Table 1 Primer sequences employed for heterologous expression of Prx proteins

Primer	Gene number	Product-size (bp)	Characteristics	Sequence 5′→3′
Synechococcus elongatus PCC 7942				
1-cys prx F	*Gene 2449*	639	21mer, position 1	ATGTCTCTTCGTCTCGGCGAT
1-cys prx R			18mer, position 636	(CTA)-CTAGCGGTTGGGTTG
2-cys prx F	*Gene 2309*	603	18mer, position 1	(GGT)-ATGACCGAAGGAGCC
2-cys prx R			20mer, position 597	(CTA)-CTAGACTGCAGCGAAGA
prxQ-A1 F	*Gene 2180*	444	20mer, position 1	(GGT)-ATGGCTTTGACTGTAGG
prxQ-A1 R			21mer, position 438	(CTA)-CTAGAGTCCTAGGTCAGC
prxQ-A2 F	*Gene 1806*	480	19mer, position 1	(GGT)-ATGCCGTTACAAGTGG
prxQ-A2 R			18mer, position 474	(CTA)-TCACTCGGCAACGGC
prxQ-A3 F	*Gene 1942*	456	19mer, position 1	ATGGCGATCGCAGTTGGTG
prxQ-A3 R			22mer, position 453	(CTA)-CTAGAGCGATCGCAGAATG
prxQ-B F	*Gene 0642*	552	18mer, position 1	(GGT)-ATGCCGGTCAGTCGT
prxQ-B R			19mer, position 546	(CTA)-CTACCCCGCTTGCAAC
T7 F	–	Variable	20mer, position 20	TAATACGACTCACTATAGGG

Materials and methods

Strains and cDNA cloning

S. elongatus PCC 7942 was originally obtained from the Pasteur Culture Collection (Paris, France) and maintained in the collection of the institute. Cells were cultivated in 250 ml gas wash-bottles containing BG11 medium and aerated with air enriched in 2% CO_2 at 30°C (Stephan et al. 2000). The medium was inoculated with cells at an optical density of OD_{750} nm = 0.35. The RNA was isolated using a hot acidic phenol extraction procedure (Reddy et al. 1990) and the RNeasy Kit (Qiagen, Hilden, Germany). Using the gene-specific primers compiled in Table 1, the coding regions were reverse-transcribed from the RNA with MMLV reverse transcriptase (Promega) and the obtained cDNA amplified with Phusion™-polymerase (Finnzymes Oy, Espoo, Finland). PCR products were purified with 'Wizard SV Gel and PCR Clean-Up System' (Promega, Mannheim) and cloned into pCR T7/NT-TOPO-TA vector (Invitrogen). TOP10 F′-*E. coli* cells were transformed and tested for the presence of insertions by colony PCR. Following confirmation of the sequence fidelity by DNA sequencing, heterologous expression of the proteins was conducted in BL21(DE3)pLysS *E. coli* cells (Horling et al. 2002).

Production of recombinant proteins

Each *E. coli* BL21 strain harbouring the specified Prx/pCR T7/NT-TOPO-TA vector was grown over night in two Erlenmeyer flasks with 100 ml LB medium each supplemented with 100 µg/ml ampicillin. The precultures were transferred to 1 L medium and grown further to an OD 0.6–0.8. To induce expression IPTG (isopropyl-β-D-thiogalactopyranoside) was added to 0.36 mmol/L final concentration. Protein accumulation was monitored in harvested aliquots by SDS-PAGE analysis. After 4 h, the cells were sedimented by centrifugation and stored at −20°C. The thawed cell pellets were lysed in 0 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 10 mmol/L imidazole, 10 mmol/L ascorbate, pH 8.0 with repeated ultrasonication. Recombinant proteins were purified by Ni-NTA affinity chromatography as described before (Horling et al. 2003). Purified and quantified proteins were dialysed against 40 mmol/L potassium phosphate buffer (pH 7.0) three times for 4 h, each. Protein concentrations were determined using the BioRad protein reagent with bovine serum albumin as standard. Analysis by SDS polyacrylamide gel electrophoresis was conducted according to standard protocols. *E. coli* TrxA and thioredoxin reductase were overexpressed and purified as described by Yamamoto et al. (1999). The anti his-tag antibody was obtained from SIGMA Chemicals.

Peroxiredoxin activity

The enzyme assay mixture contained 40 mmol/L potassium phosphate buffer (pH 7.0), 10 mmol/L dithiothreitol and 2.5 to 5 µmol/L recombinant peroxiredoxin. Hydrogen peroxide, butylhydroperoxide and cumene hydroperoxide at the concentrations as indicated were employed as substrates for the Prx reaction. For activity determination, 10 µl aliquots were taken after 0, 30, 60, 90, 120, 180, 240 and 300 s of incubation and injected to 160 µl 12.5% trichloroacetic acid. After addition of 40 µl of 10 mmol/L $(NH_4)_2Fe(SO_4)_2$ and 20 µl of 2.5 mol/L KSCN, the colour development was assayed at 480 nm by use of a microtiterplate reader. Appropriate blanks and controls were subtracted. Quantification was achieved by comparing the absorption obtained in the

enzyme assay with absorption values of defined peroxide standards. The NADPH-coupled peroxidase activity was conducted in 40 mmol/L K-phosphate buffer, pH 7.0 supplemented with 1 mmol/L ethylenediamine tetraacetic acid (EDTA), 220 µmol/L NADPH + H^+, 0.5 µmol/L *E. coli* thioredoxin reductase, 3 µmol/L *E. coli* thioredoxin TrxA, 2 µmol/L 2-Cys Prx or 5 µmol/L PrxQ-A1/3. The reaction was started by addition of H_2O_2 with concentrations varying between 10 and 100 µmol/L. The change in absorption was monitored at 340 nm and rates were calculated with the molar extinction coefficient ε_{NADPH} = 6,220 L/mol cm.

Size exclusion chromatography

Prx proteins (100 µl at 1 µg/µL) were loaded at the flow rate of 0.5 ml/min on the Superose 12 (10/300) column equilibrated with 40 mmol/L K-phosphate buffer (pH 7.0). The proteins were reduced in the presence of 10 mmol/L dithiothreitol (20 min, 4°C) or oxidised with 500 µmol/L H_2O_2 (15 min) with subsequent addition of 2.5 mmol/L ascorbate (final concentration, 5 min) to quench the H_2O_2 in the test solution. The well established separation behaviour of the system (König et al. 2003) was routinely confirmed by recalibration of the column with ribonuclease A and albumin.

Peroxide tolerance test of transgenic *E.coli*

Single colonies of *E. coli* BL21 carrying the pCR T7/NT-TOPO-TA vectors with inserted *prx* genes were transferred to 20 ml LB medium supplemented with 35 µg/ml chloramphenicol and 100 µg/ml ampicillin for over night culture.

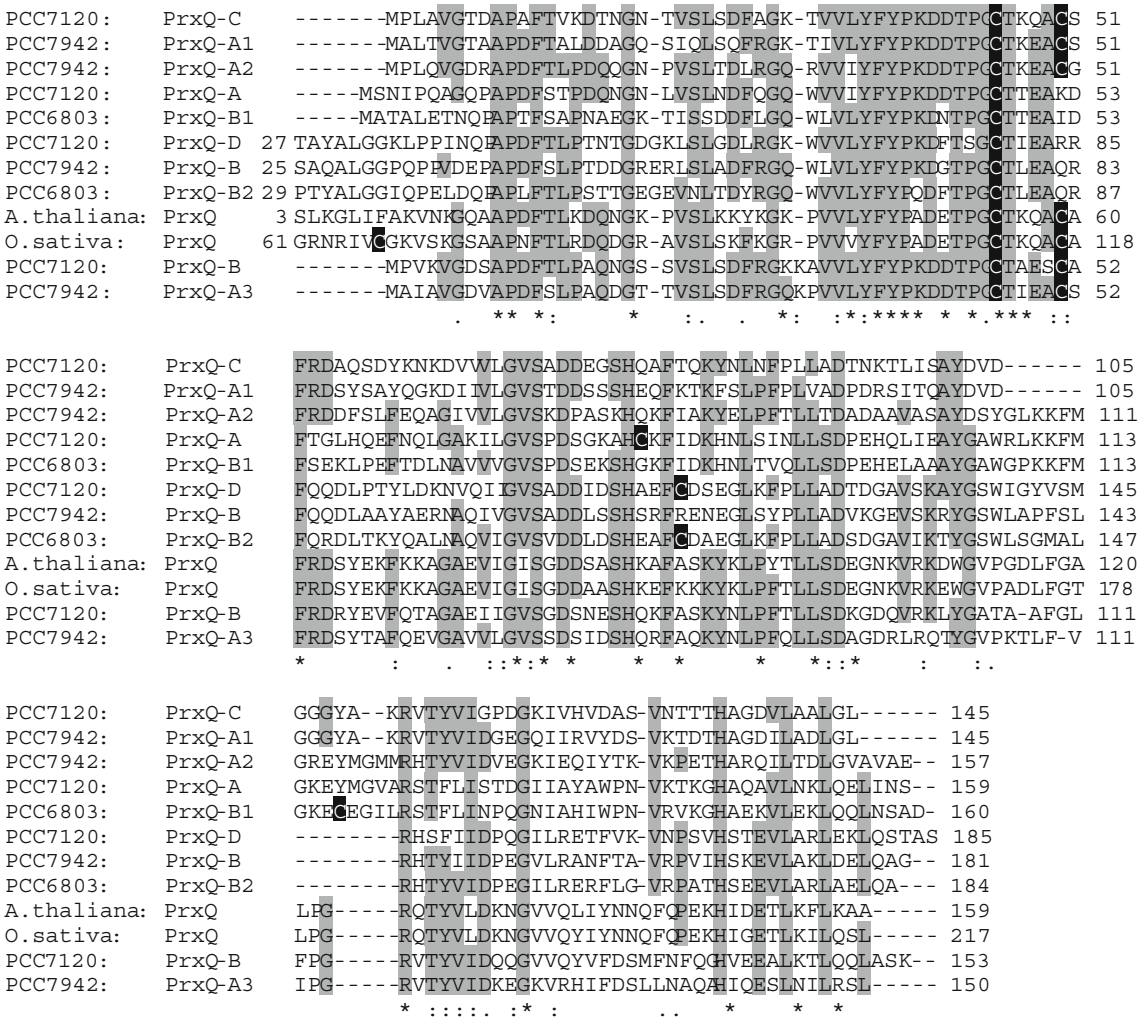

Fig. 1 PrxQ amino acid sequence alignment. The alignment was constructed with the PrxQ sequences from *S. elongatus* PCC 7942 (PrxQ-A1, -A2, -A3 and B), *Synechocystis* sp. PCC 6803 (PrxQ-B1 and -B2) (Stork et al. 2005), *Anabaena* sp. PCC 7120 (PrxQ-A, -B, -C and -D) (Latifi et al. 2007), *Arabidopsis thaliana* (PrxQ) and *Oryza sativa* (PrxQ"217"). Cys residues are in *black*, amino acid residues conserved in at least six sequences are in *grey*. *Asterisks* denote fully conserved, *colon* denotes conserved and *dot* denotes partly conserved amino acid positions. The chloroplast PrxQ-sequence of *A. thaliana* was included in the comparison without the transit sequence

100 µl suspension was transferred to new 20 ml LB with 100 µg/ml ampicillin and grown for 4.5 h. The expression of *prx* genes was induced by adding isopropyl-β-D-thiogalactopyranoside at 10 µmol/L final concentration for 30 min. This regime had been optimised in series of test experiments with variable IPTG concentrations and induction times in order to get significant expression without loss of bacterial cell viability. The control *E. coli* cells contained the pCR T7/NT-TOPO-TA vector with a short synthetic 27 bp insertion, namely GGAATTGCTCTGCAAGGATTATTCATC. The control cells were treated like the *prx*-expressing strains. Cells were collected by centrifugation (12,000×*g* for 5 min), washed once with 10 mM $MgCl_2$, and resuspended in 10 mM $MgCl_2$ at a titre of 2×10^7 colony-forming units (cfu) mL^{-1}. Bacteria (10^6 cfu/mL) were subsequently incubated at 37°C for 30 min in 10 mL potassium phosphate buffer (50 mmol/L, pH 7.4) in the presence or absence of 0.1 mmol/L cumene hydroperoxide and morpholinosydnonimine (SIN-1) as organic peroxide and peroxinitrite donor, respectively. After 30 min incubation, the cell suspension was diluted 1:10- and 1:100-fold, respectively, in 10 mmol/L $MgCl_2$. In triplicates, 10 µl were spread on 1.5% (w/v) LB agar plates, grown over night at 37°C, and number of surviving colonies was counted.

Statistical tests

Results from activity determinations were evaluated by Fisher LSD-test with one-way ANOVA (Statistica 6.0) with if not indicated otherwise $p < 0.2$. Significance of difference was calculated for the resistance test using Student's *t* test.

Results and discussion

From the six Prx proteins encoded in the *S. elongatus* PCC 7942 genome, the 1- and 2-Cys Prx are highly similar to their *Arabidopsis thaliana* orthologues with 68% and 83% (Stork et al. 2005). The four PrxQ isoforms, denominated A1-3 and B, have similarities of 66, 58, 71 and 54% to At-PrxQ, respectively. Figure 1 depicts the aa sequence alignment of the four Se-PrxQ, the four PrxQ identified in the *Anabaena* sp. PCC 7120 genome, the two PrxQ sequences from *Synechocystis* PCC 6803 and the single Q-type Prx At-PrxQ and Os-PrxQ present in *A. thaliana* and rice. SePrxQ-B lacks the resolving Cys and is the protein most dissimilar to the cognate At-PrxQ. Like Se-PrxQ, also PrxQ-A and PrxQ-D from *Anabaena* PCC7120 and PrxQ-B1 and PrxQ-B2 from *Synechococcus* PCC6803 each of which lack the resolving Cys residue.

Since none of the *S. elongatus* PCC 7942 Prx proteins has been characterised up to now, a comprehensive biochemical analysis was sought with recombinant proteins. The coding regions of the *1-cys prx*, *2-cys prx* and the four *prxQ-A1*, *-A2*, *-A3* and *-B1* from *S. elongatus* PCC 7942 were fused in frame to the 6xHis-extension of the pCR T7/NT-TOPO-vector. Recombinant proteins of the Se-Prxs and Ec-TrxA were purified by affinity chromatography on Ni-NTA material, and highly enriched fractions were obtained (Fig. 2).

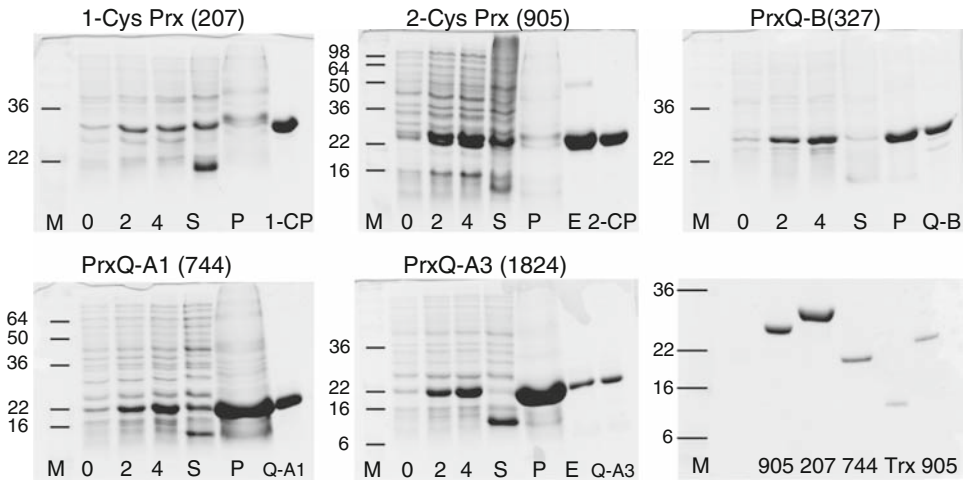

Fig. 2 Heterologous expression of the six *S. elongatus* PCC 7942 Prx proteins and the *E. coli* TrxA protein. Protein expression was induced by addition of IPTG to *E. coli* lines harbouring the Prx-constructs. Cells were lysed and the Prx proteins and the TrxA protein enriched by Ni-NTA affinity chromatography. The obtained proteins corresponded to the expected size. The complete purification is represented. M 4 µl Seablue protein marker, 0, 2, 4 denote the time in hours elapsed after IPTG induction, *S* supernatant, which was passed through the Ni-NTA column, *P* pellet-containing particulate cell components like membrane fragments after centrifugation and separation of the protein-containing supernatant for the column passage, *E* washing eluate from Ni-NTA-column, last lane purified protein after dialysis. The gels were stained with Coomassie brilliant blue

Table 2 Activity measurements of *S. elongatus* PCC 7942 Prx proteins with and without *E. coli* TrxA. Hydrogen peroxide was added at 100 µmol/L concentrations, DTT at a concentration of 10 mmol/L and TrxA at 5 µmol/L. The data are means of $n = 28–45$ determinations, ±SE

Substrate reductant activity	H_2O_2 DTT (µmol H_2O_2/min × µmol Prx)	H_2O_2 DTT + TrxA (µmol H_2O_2/min × µmol Prx)
1-Cys Prx	4.0 ± 0.4 ($n = 39$)	3.8 ± 0.6 ($n = 24$)
2-Cys Prx	3.1 ± 0.7 ($n = 27$)	26.9 ± 1.5 ($n = 45$)
PrxQ-A1	1.6 ± 0.5 ($n = 18$)	11.3 ± 0.4 ($n = 39$)
PrxQ-A2	Activity was not detected	Activity was not detected
PrxQ-A3	1.0 ± 0.5 ($n = 15$)	5.5 ± 0.4 ($n = 38$)
PrxQ-B	7.8 ± 1.1 ($n = 21$)	10.2 ± 0.8 ($n = 43$)

Peroxide reduction assays were conducted to characterise the enzymatic properties of the six Prx (Table 2). To this end two tests were performed with hydrogen peroxide at 100 µmol/L concentration as substrate. In the first test, 10 mmol/L dithiothreitol as artificial electron donor was added as immediate regenerant. Peroxidase activities of five Se-Prx ranged between 1 and 7.8 µmol $H_2O_{2decomposed}$/(µmol Prx min), while PrxQ-A2 revealed no detectable activity (not shown). To check for thioredoxin-dependent regeneration, the DTT-containing assay was supplemented with *E. coli* thioredoxin TrxA. Prx-A2 showed no activity with or without TrxA, as with any other tested potential regenerant such as plant glutaredoxins (At-Grx as in Finkemeier et al. 2005, not shown). The peroxidase activity of the 1-Cys Prx was unchanged with TrxA. All other Prx

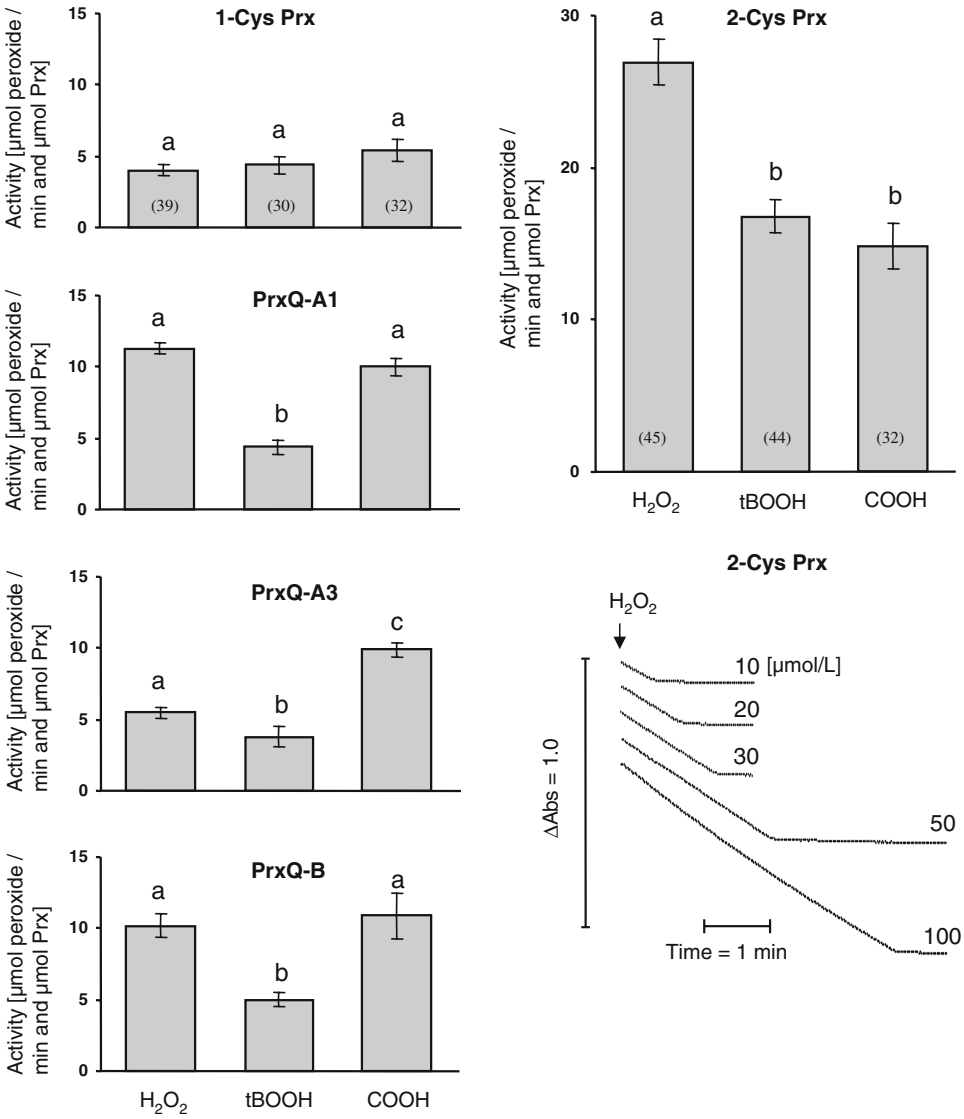

Fig. 3 Peroxidase activity of *S. elongatus* PCC 7942 Prx proteins. Activity measurements were performed in a colorimetric test with 1-Cys Prx, 2-Cys Prx, PrxQ-A1, -A3, -B and 2-Cys Prx, while PrxQ-A2 had no activity. Peroxide substrates were added at 100 µmol/L concentrations, DTT at a concentration of 10 mmol/L and TrxA at 5 µmol/L. The data are means of $n = 28–45$ determinations, ±SE. Different letters (*a*, *b*) indicate significance of difference at $p < 0.2$. In addition, the NADPH-linked peroxidase assay was performed with thioredoxin reductase, TrxA and 2-Cys Prx. H_2O_2 was added at concentrations between 10 and 100 µmol/L. Time dependent absorption changes were monitored at 340 nm

Table 3 Kinetic parameters of 2-Cys Prx, PrxQ-A1 and PrxQ-A3 as derived from the NADPH-dependent thioredoxin-linked peroxidase assay. H_2O_2 was taken as substrate. PrxQ-A1 and PrxQ-A3 were added at 5 µmol/L, 2-Cys Prx at 2 µmol/L concentration. TrxA concentration was 3 µmol/l. Other conditions are given in M&M section. The data are means of $n = 4$ (±SD, $n - 1$)

Prx type	K_M (H_2O_2) [µmol/L]	k_{cat} [s^{-1}]	k_{cat}/K_M [L mol^{-1} s^{-1}]
2-Cys Prx	2.0 ± 0.3	14.9 ± 1.5	1.2×10^5
PrxQ-A1	3.5 ± 0.5	13.8 ± 1.9	0.7×10^5
Prx-Q-A3	4.1 ± 0.8	4.2 ± 0.3	0.2×10^5

showed significant activity stimulation after addition of the TrxA protein despite the rather high concentration of 10 mmol DTT/L (Table 2). The least increase was seen with PrxQ-B with 1.3-fold stimulation, while a 5.5-fold stimulation was measured for PrxQ-A3, a 7.1-fold one for PrxQ-A1 and an 8.7-fold one for 2-Cys Prx, showing efficient regeneration by the heterologous *E. coli* thioredoxin.

Three different peroxide substrates, i.e. hydrogen peroxide, butylhydroperoxide (tBOOH) and cumene hydroperoxide (COOH), were compared in the TrxA-dependent assay, except for the case of 1-Cys Prx where TrxA was omitted since TrxA had failed to stimulate the activity (Fig. 3). The 1-Cys Prx detoxified H_2O_2, tBOOH and COOH with similar efficiencies. All three PrxQ-A revealed the lowest activity with tBOOH, but high efficiencies with COOH and equal or slightly lower activity with H_2O_2. *S. elongatus* 2-Cys Prx revealed a significant substrate preference for H_2O_2 relative to tBOOH and COOH, which exceeded that of plant 2-Cys Prx (König et al. 2003) but resembled that, e.g. of the malarial parasite *Plasmodium falciparum* (Rahlfs and Becker 2001).

In addition an NADPH-linked peroxidase activity test was performed, where thioredoxin reductase, TrxA were coupled to H_2O_2 detoxification. Reliable results were obtained with 2-Cys Prx, PrxQ-A1 and PrxQ-A3. Figure 3 shows the data for 2-Cys Prx. The initial slope of the NADPH oxidation curve was highly similar irrespective of the H_2O_2 concentration. The reaction proceeded with same speed until the substrate was almost completely exhausted. The experiments allowed us to derive the kinetic constants for these three Prx (Table 3). Concentration dependency revealed K_M values between 2 and 4.1 µmol/L, turnover numbers between 4.2 and 14.9 and values for the catalytic efficiency that decreased from 2-Cys Prx to PrxQ-A1 and PrxQ-A3. The turnover numbers of PrxQ-A1 and PrxQ3 were similar for the colorimetric and NADPH-dependent test. The lower activity of 2-Cys Prx in the NADPH-test might be caused by the lower TrxA concentration of 3 µmol/L as compared to 5 µmol/L in the colorimetric test and may indicate a regeneration limitation. The very low stimulation by TrxA of the monocysteinic Se-PrxQ-B had to be expected since TrxA most efficiently catalyses two electron transfer reactions. Glutaredoxins, glutathione-S-transferases in conjunction with GSH may serve as electron donors as in the case of 1-Cys Prx (Manevich et al. 2004; Noguera-Mazon et al. 2006).

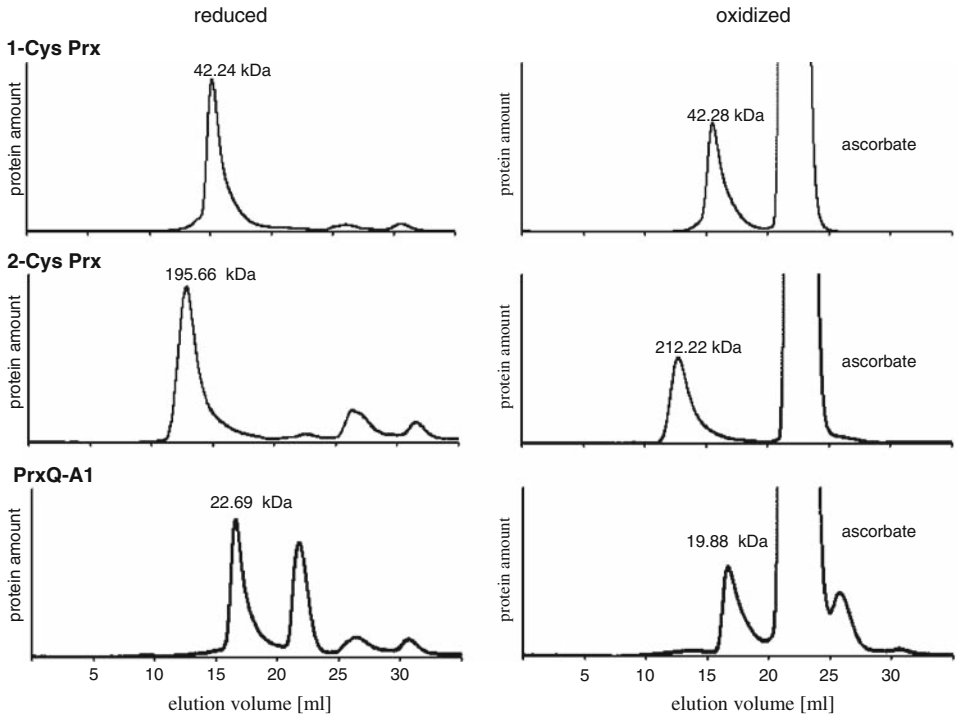

Fig. 4 Aggregation state of *S. elongatus* PCC 7942 1-Cys Prx, 2-Cys Prx and PrxQ-A1. Recombinant Prx proteins in reduced (10 mM DTT for 20 min) or oxidised state (0.5 mM H_2O_2 for 15 min, subsequent treatment with 2.5 mM ascorbate for 5 min) were separated by size exclusion chromatography on Superose 12 column. Elution of protein was monitored at 280 nm. Ascorbate was detected as a peak between 20–25 ml elution volume. Peaks representing Prx protein are labelled with the apparent molecular masses in kDa. The given curves are representative of 2–5 different independent fractionations

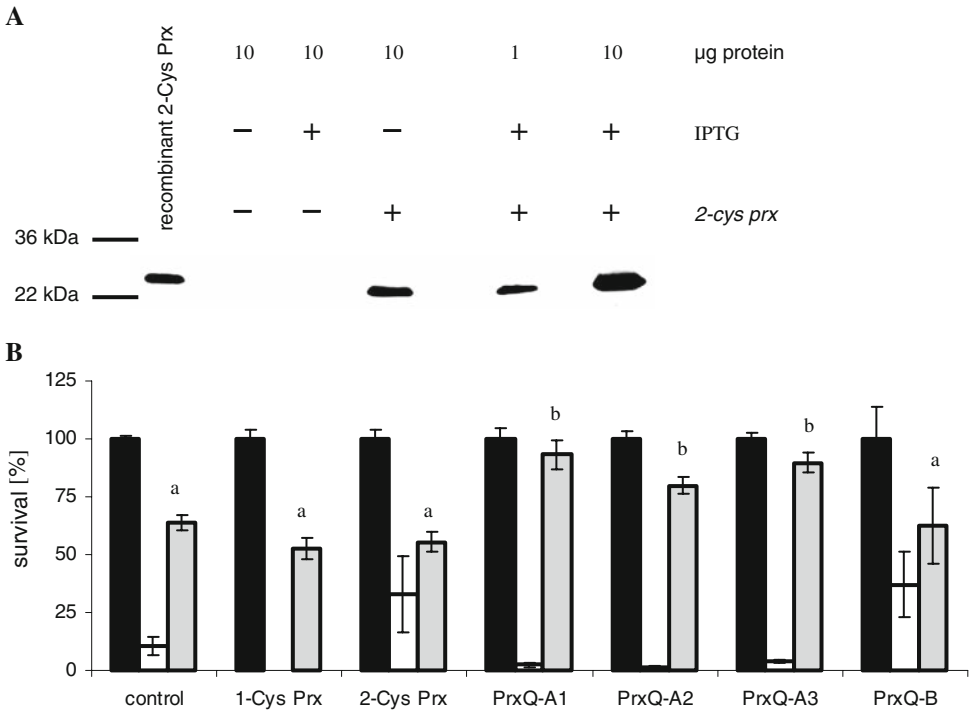

Fig. 5 Western Blot detection of 2-Cys Prx in *E. coli* lysates with and without IPTG (A) and in vivo protection of *E. coli* expressing Se-Prx against peroxynitrite ($ONOO^-$) and cumene hydrogen peroxide (COOH). **a** Transgenic *E. coli* BL21 cells with and without *2-cys prx* gene-carrying expression vector were grown for 4 h 30 min and then incubated with or without IPTG for 30 min. After washing and centrifugation lysates were obtained, which were separated after protein amount determination on SDS-PAGE. Detection of 2-Cys Prx was performed after blotting on nitrocellulose membrane with anti His antibody. As control 100 ng purified recombinant 2-Cys Prx protein was separated on the gel. **b** *E. coli* cells with T7/NT-TOPO-TA vector-containing one of each *prx* genes of *S. elongatus* PCC 7942 or vector with a small insertion (control) were grown for 4 h 30 min, incubated for 30 min with 10 µM IPTG, washed and diluted in 40 mM KPi buffer pH 7.4 up to 1×10^6 cells. After 30 min treatment with 100 µM COOH (*grey bars*), 1 mM SIN1 (*open bars*) or water (control, *black bars*) dilutions of the cell cultures were spread on agar plates, incubated at 37°C over night, colonies counted and survival relative to control calculated. Mean values ± SE were as follows: 1-Cys Prx, PrxQ-A2, PrxQ-A3 and PrxQ-B: $n = 6$ from two independent experiments; 2-Cys Prx and PrxQ-A1: $n = 9$ from three independent experiments; control: $n = 42$. Significant differences ($p < 0.01$) were calculated in the case of COOH in comparison to the control and indicated by different letters (*a*, *b*)

Prx function as monomers, dimers or oligomers that partly undergo dynamic transitions depending on the redox state. To characterise the protein family of *S. elongatus* PCC 7942, heterologously expressed proteins in reduced and oxidised state from each group, respectively, were separated by size exclusion chromatography (Fig. 4). 1-Cys Prx separated as dimer of 42 kDa, 2-Cys Prx as oligomer of ≥200 kDa and PrxQ-A1 as monomer of 20 kDa independent on the redox state. It should be noted that chaperones eventually present in *Synechococcus* but absent in *E. coli* during heterologous expression might affect the correct folding and aggregation. The unexpected stability of the cyanobacterial oxidised 2-Cys Prx is partly in line with results from a recent isothermal titration microcalorimetric study showing a critical assembly concentration for reduced human and plant 2-Cys Prx, that was not seen for the 2-Cys Prx from *S. elongatus* 2-Cys Prx (Barranco-Medina et al. 2008).

E. coli expresses two cytoplasmic peroxidoxins, the 2-Cys Prx AhpC (P0AE08) (Michan et al. 1999) and the PrxQ-like Bcp (P0AE52) (Jeong et al. 2000). In addition, a thioredoxin-dependent peroxidase is targeted to the periplasmic space (Cha et al. 1995). In the background of these endogenous Prx, the protective function of the Se-Prx isoforms was tested in vivo by assessing the viability of *E. coli* cells expressing the various cyanobacterial Prx in the presence of cumene hydroperoxide and peroxinitrite (Fig. 5). These peroxides were chosen based on the established toxicity in *E. coli* and the reported protective effect, e.g. of overexpressed plant 2-Cys Prx, e.g. against cumene hydroperoxide (Baier and Dietz 1997). Peroxinitrite has been investigated by Sakamoto et al. (2003) and Bryk et al. (2000). In initial experiments, the IPTG concentration routinely used to induce rapid and strong accumulation of recombinant protein was shown to drastically impair cell viability. To optimise the test system, the minimum IPTG concentration was searched and subsequently applied to induce significant synthesis of the respective Prx protein but to avoid detrimental overproduction. Figure 5 exemplarily shows a basal expression of 2-Cys Prx protein in

vector-containing cells even in the absence of IPTG. IPTG at 10 µmol/L concentration increased the amount of recombinant protein but did not affect cell viability (not shown). Application for 30 min of 100 µmol/L COOH resulted in 50–60% cell survival. Expression of 1-Cys Prx, 2-Cys Prx and PrxQ-B did not affect cell sensitivity towards COOH. In a converse manner expression of PrxQ-A1, −2 and −3 strongly increased cell survival almost to the level of the untreated control. 2-Cys Prx and PrxQ-B showed a trend towards protection of the cells against peroxinitrite; however, the difference to the treated control was statistically not significant.

Conclusions

Reverse genetic approaches using insertional inactivation of individual *prx* genes have implicated Prx in antioxidant defence of bacteria (Seaver and Imlay 2001; Wang et al. 2006), animals (Rhee et al. 2005) and plants (Baier and Dietz 1999; Finkemeier et al. 2005; Lamkemeyer et al. 2006). In *Synechocystis* PCC 6803, both deletion of the *1-cys prx* (*slr1198*) and the *type II prx* (*sll1621*) impaired cell survival (Hosoya-Matsuda et al. 2005). *S. elongatus* PCC 7942 lacks a type II Prx. This is interesting, since plant chloroplasts generally appear to contain a PrxII E (Horling et al. 2003). PrxII E is soluble in the stroma and was suggested to detoxify peroxides escaping from the thylakoids, generated in the stroma or diffusing from the cytosol into the chloroplast (Dietz et al. 2006). It is tempting to hypothesise that the lack of type II Prx is compensated by PrxQ in *S. elongatus.* The three Se-PrxQ-A proteins contain the reactive centre typical for PrxQ with two Cys residues spaced by four intervening amino acids. Se-PrxQ-A1 and -A3 showed significant activity stimulation in the presence of TrxA. Se-PrxQ-A2 revealed no activity under the various assay conditions tested, i.e. with DTT, DTT/TrxA, NADPH/thioredoxin reductase/TrxA, GSH and GSH/Grx. The reason for this inactivity is unknown. Inefficient regeneration is the most likely explanation. Surprisingly heterologous expression of all Se-PrxQ-A increased the tolerance of *E. coli* towards cumene hydroperoxide, showing that the required regeneration systems are present in *E. coli* cells. Comparing the gene family composition of *Synechocystis* PCC 6803 and *S. elongatus* PCC 7942 and Prx activities a functional substitution of PrxQ-A for the type II Prx present in some cyanobacteria may be assumed. This is in line with a strong transcript accumulation of the *Synechocystis type II prx* upon exposure to various stresses, e.g. in response to high light and H_2O_2 treatment. A similar pattern is seen for *prxQ-A*, particularly the *A1*-form in *S. elongatus* (Stork et al. 2005). The results show the structural and functional differentiation within the Prx family in *S. elongatus*. Future work should address the subcellular localisation and the processes affected by disturbed Prx function in single and multiple mutants in order to arrive at the next level of mechanistic understanding of the large Prx family in Cyanobaceteria.

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