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CHAPTER FOURTEEN

C0070

Comparative Analysis of Cyanobacterial and Plant Peroxiredoxins and Their Electron Donors: Peroxidase Activity and Susceptibility to Overoxidation

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Contents

Abstract

Peroxiredoxins (Prxs) are peroxidases that use thiol-based catalytic mechanisms implying redox-active cysteines. The different Prx families have homologs in all photosynthetic organisms, including plants, algae, and cyanobacteria. However, recent studies show that the physiological reduction systems that provide Prxs with reducing equivalents to sustain their activities differ considerably between cyanobacterial strains.

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Thus, for example, the filamentous cyanobacterium *Anabaena* sp. PCC 7120 is similar to the chloroplast in that it possesses an abundant 2-Cys Prx, which receives electrons from the NADPH-dependent thioredoxin reductase C (NTRC). In contrast, the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, which lacks NTRC, has little 2-Cys Prx but high amounts of PrxII and 1-Cys Prx. The characterization of cyanobacterial Prxs and their electron donors relies on straightforward enzymatic assays and tools to study the physiological relevance of these systems. Here, we present methods to measure peroxidase activities *in vitro* and peroxide decomposition *in vivo*. Several approaches to detect overoxidation of the active site cysteine in cyanobacterial 2-Cys Prxs are also described.

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1. INTRODUCTION

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Cyanobacteria are photosynthetic prokaryotes that share a common ancestor with plant and algal chloroplasts. Whereas reactive oxygen species (ROS) are generated during normal aerobic metabolism in most organisms, the oxygenic photosynthesis of cyanobacteria and chloroplasts leads to additional production of ROS, such as superoxide anion radicals and hydrogen peroxide. The ROS accumulated may cause oxidative damage to macromolecules and are therefore potentially harmful to the cell. However, some ROS, particularly hydrogen peroxide, have important functions as signaling molecules. Therefore, the intracellular levels of ROS are controlled by various enzymatic systems, some of which are common to prokaryotic and eukaryotic photosynthetic organisms. Peroxiredoxins (Prxs) represent a class of thiol-dependent peroxidases that are divided into four principal groups based on phylogeny, 2-cysteine peroxiredoxin (2-Cys Prx), 1-cysteine peroxiredoxin (1-Cys Prx), PrxII, and PrxQ. Cyanobacteria encode Prxs from all four groups (Bernroitner, Zamocky, Furtmüller, Peschek, & Obinger, 2009), whereas chloroplasts contain all but the 1-Cys Prx (Dietz, 2011; Dietz et al., 2006), which in plants is localized to the nucleus (Pulido, Cazalis, & Cejudo, 2009; Stacy, Nordeng, Culiáñez-Macià, & Aalen, 1999). Since the catalytic mechanism of Prxs implies oxidation of cysteines to cystine bridges, these enzymes must be regenerated through disulfide reduction. Chloroplast Prxs have been found to use a variety of endogenous electron donors, such as thioredoxins, glutaredoxins, or cyclophilin, to sustain their activities (Dietz, 2011). The ubiquitous chloroplast 2-Cys Prx together with its reductant, the NADPH thioredoxin reductase C (NTRC), constitutes one of the most efficient plant systems for peroxide detoxification reported to date (Pérez-Ruiz et al., 2006; Pulido et al., 2010). While all cyanobacterial species examined contain 2-Cys Prx, NTRC is

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present in some but not all cyanobacteria. For instance, NTRC is present in the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120, but not in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Pascual, Mata-Cabana, Florencio, Lindahl, & Cejudo, 2010). However, the five different *Synechocystis* Prxs may receive reducing equivalents from three of the *Synechocystis* thioredoxins (Pérez-Pérez, Mata-Cabana, Sánchez-Riego, Lindahl, & Florencio, 2009). This is just one example of the heterogeneity among the cyanobacterial Prx systems. This chapter describes some of the most useful tools for characterization of the cyanobacterial Prxs and their reductants, aimed at comparative studies with the plant chloroplast enzymes.

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2. EXPRESSION AND PURIFICATION OF RECOMBINANT Prxs AND THIOREDOXINS

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2.1. Cloning and expression of Prxs and thioredoxins

p0010 Cyanobacterial gene and protein sequences are obtained from the CyanoBase (http://genome.kazusa.or.jp/cyanobase) and plant gene and protein sequences may be retrieved from PlantGDB (http://www.plantgdb.org). DNA fragments corresponding to the entire coding region of each cyanobacterial gene of interest are amplified by PCR and cloned into the expression vectors, whereas for chloroplast proteins only the sequence encoding the mature protein, without the transit peptide, is amplified. If there are doubts regarding the length of the chloroplast transit peptides and the position of the cleavage sites, it might be useful to consult the ChloroP server (http://www.cbs.dtu.dk/services/ChloroP) or PSORT (http://www.psort.org) for a prediction of mature protein sequences. We recommend cloning of the PCR products into vectors that add a histidine tag to the protein in order to enable purification by nickel affinity chromatography. The histidine tag should be added to the N-terminus of the Prx proteins (Pérez-Pérez et al., 2009) in order to avoid interference with the C-terminal region that is closer to the catalytic site (Pascual, Mata-Cabana, Florencio, Lindahl, & Cejudo, 2011; Wood, Poole, & Karplus, 2003). Suitable vectors are, for example, pQE-30 (Qiagen) and pET28a (Novagen®, EMD Millipore). For protein production, *Escherichia coli* (e.g., strain BL21) cells are transformed with the resulting plasmids, grown at 37 °C and expression is induced by adding 1 m*M* isopropyl-L-D-thiogalactose at an optical density at 600 nm of about 0.4. Prxs and thioredoxins are usually well tolerated by the *E. coli* cells and expressed as soluble proteins at high levels.

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s0020 2.2. Purification of Prxs and thioredoxins

p0015 Ni-NTA (nickel-nitrilotriacetic acid) agarose resin (Qiagen), His bind® Resin (Novagen®, EMD Millipore), or HisTrap columns (GE Healthcare) may be used for purification of the expressed proteins. If additional purification would be necessary, we recommend exclusion chromatography (i.e., gel filtration) using, for example, a HiLoad 16/600 Superdex 75 column (GE Healthcare). Before carrying out this step, the pooled fractions containing the protein of interest eluted from the nickel affinity chromatography should be incubated with 20 m*M* DTT on ice for 1 h. This ensures that all disulfide bonds are broken prior to the second chromatography and that the protein shows a more consistent migration behavior in the gel filtration column. Exclusion chromatography also has the advantage that the high amounts of imidazole and NaCl, which are present in concentrations of up to 0.5 *M* in the eluates from nickel affinity chromatography, are eliminated along with the DTT. Furthermore, it offers the opportunity to change the buffer in accordance with requirements for subsequent analyses. In general terms, Prxs and thioredoxins from a wide variety of sources are straightforward to purify and give yields of about 10 mg of pure protein per liter of *E. coli* culture.

s0025 3. Prx ACTIVITY ASSAYS *IN VITRO*

s0030 3.1. Peroxide decomposition as measured by a colorimetric assay

p0020 Reduction of peroxides catalyzed by Prxs may be conveniently measured using the ferrous ion oxidation (FOX) assay in the presence of xylenol orange, as described by Wolff (1994). In dilute acid hydroperoxides oxidize selectively ferrous to ferric ions, which can be determined using ferric-sensitive dyes, such as xylenol orange. The so-called FOX1 reagent is composed of 100 μ*M* xylenol orange, 250 μ*M* ammonium ferrous sulfate $(NH_4^+)_2Fe(SO_4)_2$, 100 m*M* sorbitol and 25 m*M* H_2SO_4. Xylenol orange binds the ferric ion to produce a colored complex with an extinction coefficient of $1.5 \times 10^4\ M^{-1}\ cm^{-1}$ at 560 nm, the absorbance maximum. Hence, the rate of H_2O_2 decomposition catalyzed by Prx can be monitored through changes in the H_2O_2 concentration as determined by the colorimetric FOX assay.

p0025 For measurement of Prx activity the reaction mixture usually contains a buffer at pH 7.0. This buffer may be 50 m*M* Hepes–NaOH (pH 7.0) as in

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Pérez-Pérez et al. (2009) or 100 m*M* sodium phosphate buffer (pH 7.0) as in Pérez-Ruiz et al. (2006). The initial concentration of H_2O_2 or alkyl hydroperoxides used in this kind of assays reported in the literature ranges from 100 to 500 μ*M*. However, taking into account the susceptibility of some classes of Prx to hyperoxidation and concomitant inactivation at 500 μ*M* H_2O_2 (Pascual et al., 2010), we advice the use of the lower peroxide concentration, 100 μ*M*, in the reaction mixture. In order to facilitate the calculation of initial reaction rates and kinetic constants, the concentration of the enzyme, Prx, should be adjusted to decompose about half of the peroxide content in the first 2 min of reaction when saturated with reducing agent. This concentration is usually found between 5 and 15 μ*M* Prx, depending on the efficiency of the enzyme (Pérez-Pérez et al., 2009; Pérez-Ruiz et al., 2006).

p0030 Various kinds of electron donors may be tested as reducing agents for each Prx. When using Trxs, these need to be kept reduced throughout the assay by including a low concentration of DTT (0.2–0.5 m*M*) (Pérez-Pérez et al., 2009; Pérez-Ruiz et al., 2006). The concentration of DTT chosen should be sufficiently low as not to allow for direct reduction of the Prx, since this *per se* would sustain its peroxidase activity. It should also be noted that the ability to receive electrons from DTT varies considerably between different Prxs. For instance, the 2-Cys Prx from the cyanobacterium *Anabaena* sp. PCC 7120, much like the plant enzyme, is reduced to 15% by a 10-min incubation with 0.5 m*M* DTT, whereas the *Synechocystis* sp. PCC 6803 2-Cys Prx is not reduced under these conditions (Pascual et al., 2010). A series of reactions with increasing Trx concentrations will be needed for determination of kinetic parameters. A range of concentrations between 0.1 and 25 μ*M* Trx is suitable, since the apparent K_m values of the five Prxs from *Synechocystis* sp. PCC 6803 for three different Trxs as substrates were found in this range (Pérez-Pérez et al., 2009). Reaction volumes of 250–500 μL are recommended because larger volumes would consume unnecessarily high amounts of purified enzymes. The final volume of the reaction in the protocol detailed below is 400 μL. Prior to the assays, the enzymes should be diluted to stock solutions of 2 mg mL^{-1} for Prx and 1 mg mL^{-1} for Trx. The reactions are performed at 25 °C.

o0005 **1.** Pipette 200 μL 100 m*M* Hepes–NaOH (pH 7.0) into a 1.5-mL microfuge tube.

o0010 **2.** Add 40 μL 2 mg mL^{-1} Prx, which yields 0.2 mg mL^{-1} final concentration. This would correspond to 8 μ*M* for a Prx with a molecular mass of 25 kDa.

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o0015 **3.** Add 10 μL of 8 m*M* DTT, which gives a final concentration of 0.2 m*M*.

o0020 **4.** Add 125 μL 1 mg mL^{-1} Trx. For an average Trx of 12 kDa, this corresponds to approximately 25 μ*M* final concentration.

o0025 **5.** The reaction is started with the addition of 25 μL 1.6 m*M* H_2O_2, yielding 100 μ*M* initial concentration, followed by vigorous mixing.

o0030 **6.** At time points 0, 1, 2, 4,. . ., 10 min, 50 μL-aliquots of the reaction mixture are withdrawn and mixed with 950 μL of the FOX1 reagent. Color development takes about 30 min at room temperature and is thereafter stable for some hours.

p0065 The absorbance is read at 560 nm against a blank consisting of 950 μL FOX1 reagent mixed with 50 μL H_2O. Obviously, the maximal H_2O_2 concentration in the mixture between 950 μL FOX1 reagent and a 50 μL-aliquot of reaction withdrawn at time point 0 never exceeds 5 μ*M*, when the initial H_2O_2 concentration in the reaction is 100 μ*M*. This value is within the linear response range of the FOX assay (Wolff, 1994), which we have also confirmed independently. Therefore, the absorbance is directly proportional to the H_2O_2 content. For example, if the initial absorbance at time point 0 is 1.382 AU, then an absorbance of 0.846 AU after 1 min of reaction means that there remains 61% of 100 μ*M* H_2O_2, that is, 61 μ*M*, in the reaction mixture. The concentrations of Prx and Trx suggested here should be used as a guide to try out the best conditions for each couple of enzymes to be examined. If the aim of the assay is to determine the K_m value of a given Prx for a particular Trx as substrate, serial dilutions of the Trx stock solution should be performed in order to obtain progressively lower Trx concentrations. A graph representing the result of a typical assay of this kind is displayed in Fig. 14.1. As may be appreciated, longer times of reaction are needed to accurately determine the reaction rates at the lowest Trx concentrations, whereas 1 or 2 min are sufficient to determine the rates at the highest Trx concentrations.

s0035 3.2. The coupled NTRC/2-Cys Prx assay

p0070 The plant NTRC comprises a NADPH thioredoxin reductase (NTR)/thioredoxin system in a single polypeptide chain (Pérez-Ruiz et al., 2006; Serrato, Pérez-Ruiz, Spínola, & Cejudo, 2004). The functional quaternary structure of NTRC is a homodimer (Pérez-Ruiz, González, Spínola, Sandalio, & Cejudo, 2009), and there is evidence that electrons are transferred from the NTR domain of one subunit to the thioredoxin domain

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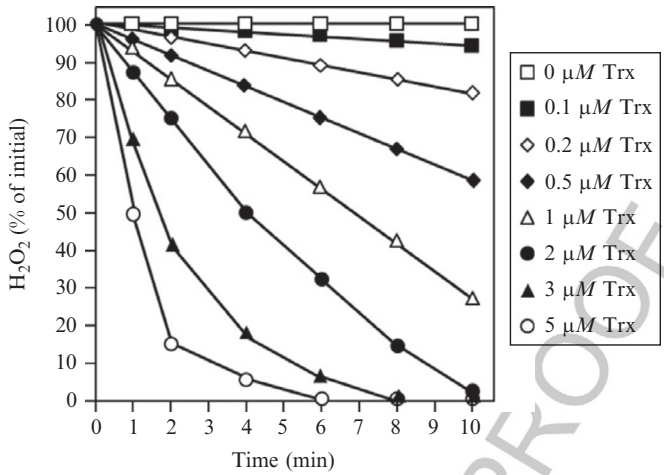

f0005 **Figure 14.1** Prx activity as a function of thioredoxin (Trx) concentration for determination of kinetic constants. The graph represents an example of H_2O_2 reduction catalyzed by a Prx *in vitro* using a Trx as electron donor. Each sample contains a low concentration of DTT, which does not *per se* sustain the Prx activity (0 μ*M* Trx, open squares). A series of Trx concentrations is required to accurately determine the kinetic parameters.

of the other subunit (Bernal-Bayard, Hervás, Cejudo, & Navarro, 2012; Cejudo, Ferrández, Cano, Puerto-Galán, & Guinea, 2012; Pérez-Ruiz & Cejudo, 2009). The chloroplast 2-Cys Prx receives reducing equivalents from NTRC *in vivo* (Kirchsteiger, Pulido, González, & Cejudo, 2009) and *in vitro* (Pérez-Ruiz et al., 2006). Thus, the activity of the NTRC/2-Cys Prx system that catalyzes the transfer of electrons from NADPH to H_2O_2 can be measured as consumption of NADPH, which absorbs light at 340 nm with an extinction coefficient of 6220 M^{-1} cm^{-1}. The following protocol has been used for the measurement of activity of NTRC from rice (Pérez-Ruiz et al., 2006) and from the cyanobacterium *Anabaena* sp. PCC 7120 (Pascual et al., 2011). The assay may be performed directly in a 500 μL quartz cuvette at room temperature. In order not to waste the purified enzymes, the reaction volume in this protocol is 200 μL. However, it might be necessary to adjust the reaction volume depending on the height of the light beam in the spectrophotometer. The purified NTRC and 2-Cys Prx should be diluted to stock solutions of 2 mg mL^{-1} and kept on ice.

o0035 **1.** Pipette 100 μL 200 m*M* sodium phosphate buffer into the cuvette.

o0040 **2.** Add 31 μL of water. (This volume should be adjusted to give a 200 μL final reaction volume.)

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o0045 **3.** Add 21 μL 2 mg mL^{-1} NTRC. This corresponds to about 4 μ*M* of the 53 kDa NTRC protein.

o0050 **4.** Add 18 μL 2 mg mL^{-1} 2-Cys Prx. This corresponds to about 8 μ*M* of the 23 kDa 2-Cys Prx protein.

o0055 **5.** Add 10 μL 5 m*M* NADPH to yield a 0.25 m*M* final concentration within the cuvette and mix well. This maximal concentration of NADPH gives an absorbance at 340 nm that is in the upper part of the linear range.

o0060 **6.** The reaction is started by adding 20 μL 1 m*M* H_2O_2, which results in a 100 μ*M* initial concentration.

p0105 The absorbance should be read continuously at 340 nm during 5 min against a blank containing all components except NADPH. Note that 4 μ*M* is a suitable concentration for the *Anabaena* sp. PCC 7120 NTRC (Pascual et al., 2011) but may be too high when measuring the activity of the plant chloroplast NTRC. Previous measurements of the rice NTRC activity have been performed using 2 μ*M* concentration (Pascual et al., 2011; Pérez-Ruiz et al., 2006). Since NTRC presents a low diaphorase activity (Pérez-Ruiz et al., 2006), a sample containing all components except Prx should also be prepared and the measured activity should be subtracted from each activity measured in the presence of Prxs. If the K_m value of NTRC for a particular Prx as substrate is to be determined, serial dilutions of the Prx stock solution should be performed in order to obtain a range of Prx concentrations between 0.1 and 20 μ*M*.

s0040 4. PEROXIDE DECOMPOSITION IN CYANOBACTERIA *IN VIVO*

p0110 Levels of endogenously produced peroxides in cyanobacteria are usually too low to be detected by colorimetric assays. However, the decomposition of peroxides exogenously added to cyanobacterial cultures may be measured using the FOX reagent. H_2O_2 readily diffuses through the cell wall and lipid membranes and is degraded within the cell. It must be remembered that the observed activity is the sum of all peroxidase and/or catalase activities of the cyanobacterium.

s0045 4.1. Growth of cyanobacterial cultures under standard conditions

p0115 *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120, as well as other strains, are grown in BG-11 medium (Rippka, Deruelles, Waterbury,

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Herdman, & Stanier, 1979) supplemented with 12 m*M* $NaHCO_3$ as carbon source. Cultures are bubbled with a stream of 1% (v/v) CO_2 in air under continuous illumination at a light intensity of 50 μmol photons m^{-2} s^{-1} and a temperature of 30 °C.

s0050 4.2. Chlorophyll measurement of intact cyanobacterial cells

p0120 The growth is monitored by measuring the chlorophyll concentration of the culture. To this end, 1 mL of cyanobacterial culture is centrifuged for 2 min at 12,000 × *g* at room temperature. Nine hundred microliter of the supernatant is discarded and the pellet containing the cells is resuspended in the remaining volume of medium by vortexing. Nine hundred microliter of methanol is added and mixed with the cell suspension by vigorous vortexing for 30 s. The sample is centrifuged for 2 min at 12,000 × *g*, and the absorbance of the supernatant is read at 665 nm. According to Mackinney (1941), the extinction coefficient of chlorophyll *a* at 665 nm is 74.46 mM^{-1} cm^{-1} when using this method of extraction. For all practical purposes, the absorbance at 665 nm multiplied by 13.43 equals the amount of chlorophyll (μg) in the sample. For example, an absorbance of 0.361 AU × 13.43 yields an amount of 4.8 μg of chlorophyll, that is, the chlorophyll concentration in the culture is 4.8 μg mL^{-1}. An exponentially growing culture has a chlorophyll concentration ranging from 3 to 5 μg mL^{-1}.

s0055 4.3. Quantification of peroxides in cyanobacterial cultures

p0125 When comparing peroxide reduction rates between cyanobacterial strains and mutants, exponentially growing cultures should first be diluted to equal chlorophyll concentrations. The following protocol was used to compare peroxide decomposition in *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 (Pascual et al., 2010). Since the cyanobacterial catalase-peroxidase KatG has been reported to be specifically inhibited by hydroxylamine (NH_2OH) (Miller, Hunter, O'Leary, & Hart, 2000), peroxide decomposition may be analyzed with and without 100 μ*M* NH_2OH to determine the proportion of activity due to KatG (Pascual et al., 2010).

o0065 **1.** Aliquots of 20 mL cyanobacterial cultures at a concentration of 3.5 μg chlorophyll per mL are pipetted into 100-mL E-flasks and placed on an orbital shaker at 100 rpm in order to avoid sedimentation of cells.

o0070 **2.** The temperature should be kept at 30 °C and the light intensity at 50 μmol photons m^{-2} s^{-1}.

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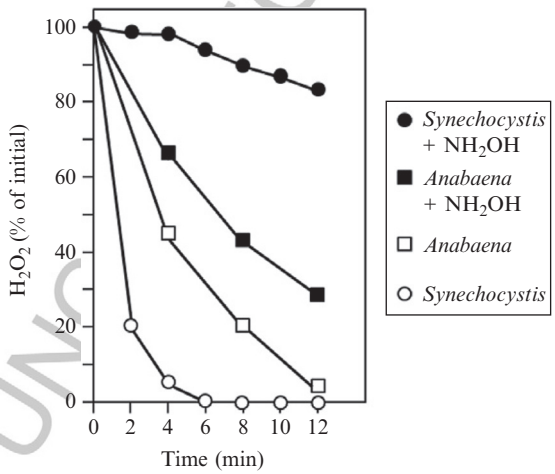
o0075 **3.** Add 4 μL of 0.5 *M* NH_2OH to the aliquots, which are to contain 100 μ*M* NH_2OH for inhibition of KatG.

o0080 **4.** Each reaction is started with the addition of 100 μL 100 m*M* H_2O_2, yielding a 0.5 m*M* initial concentration. It is important to mix rapidly the peroxide with the culture through a circular movement of the E-flask and to withdraw instantaneously the time 0 aliquot, since some strains display very high rates of peroxide decomposition.

o0085 **5.** At regular time intervals up to 15 min, 20 μL-aliquots of the reaction mixture are withdrawn and mixed with 1.98 mL of the FOX1 reagent in 2-mL microfuge tubes, to obtain a maximal H_2O_2 concentration of 5 μ*M*.

o0090 **6.** After at least 30 min color development at room temperature, the absorbance is read at 560 nm against a blank consisting of 1.98 mL FOX1 reagent mixed with 20 μL H_2O.

p0160 The outcome of a typical experiment comparing peroxide decomposition rates between *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 in the presence and in the absence of NH_2OH is shown in Fig. 14.2. The *Synechocystis* cells decompose 80% of the H_2O_2 within the first 2 min of reaction in the absence of NH_2OH, but less than 5% in the presence of NH_2OH (Fig. 14.2). This is in agreement with a highly active and/or abundant KatG



f0010 **Figure 14.2** Decomposition of H_2O_2 *in vivo* in cyanobacterial cultures. Exponentially, growing *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 decompose 0.5 m*M* exogenously added H_2O_2 within minutes. The strong inhibition of peroxide elimination in *Synechocystis* by 100 μ*M* NH_2OH shows that its high peroxide detoxifying activity is largely due to the catalase-peroxidase KatG.

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enzyme that is responsible for most of the peroxide detoxification in this organism, as has previously been reported in a study of a *Synechocystis* KatG deletion mutant (Tichy & Vermaas, 1999). In contrast, the *Anabaena* cells display considerably lower rates of peroxide decomposition but are much less affected by NH_2OH (Fig. 14.2).

s0060

5. OVEROXIDATION OF PLANT AND CYANOBACTERIAL 2-Cys Prx

p0165 Eukaryotic and some prokaryotic 2-Cys Prxs, for example, the cyanobacterial 2-Cys Prxs, are sensitive to overoxidation, also referred to as "hyperoxidation," at elevated peroxide concentrations (Pascual et al., 2010). This means that one of the catalytic cysteines is oxidized by the peroxide substrate to a sulfinic acid, which renders the enzyme inactive (Wood et al., 2003). However, the active form of 2-Cys Prx may be restored through the action of sulfiredoxin (Biteau, Labarre, & Toledano, 2003), which has also been identified in plant chloroplasts (Iglesias-Baena et al., 2010; Rey et al., 2007) and in the cyanobacterium Anabaena sp. PCC 7120 (Boileau et al., 2011). The physiological importance of Prx hyperoxidation in eukaryotes and cyanobacteria has been debated (Jeong, Bae, Toledano, & Rhee, 2012; Pascual et al., 2010). There are several methods to detect the overoxidized form of a 2-Cys Prx.

s0065

5.1. Immunological detection of overoxidized 2-Cys Prx *in vitro*

p0170 Some years ago, polyclonal antibodies were raised in rabbit against a peptide comprising 10 amino acids from the active site of a human 2-Cys Prx carrying the catalytic cysteine in the sulfonic acid form (Woo et al., 2003). These antibodies, which are commercially available (LabFrontier, Seoul, South Korea), recognize the sulfinic and sulfonic acid forms of the enzyme, but not the thiol-, disulfide-, or sulfenic acid forms (Woo et al., 2003). Since the antigenic peptide is completely conserved in plant 2-Cys Prx, a protocol for Western blot analysis was developed for detection of overoxidized *Arabidopsis thaliana* 2-Cys Prx *in vivo* (Iglesias-Baena et al., 2010). To this end, 10 μg of total soluble leaf protein is loaded on the SDS-PAGE gels and the antibodies against overoxidized 2-Cys Prx are used at a dilution of 1:2000 (Iglesias-Baena et al., 2010). Western blot has also been used to detect overoxidation *in vitro* of plant and cyanobacterial 2-Cys Prx (Pascual et al., 2010).

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p0175 In this protocol 25 μg each of purified 2-Cys Prx from rice, *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 are first incubated for at least 15 min with 10 m*M* DTT and 5 m*M* H_2O_2 at room temperature. The presence of DTT is absolutely necessary, since the disulfide-linked dimer of 2-Cys Prx is inert to peroxide treatment, and the enzyme must be in the reduced form to be susceptible to overoxidation. Thereafter, the proteins are subjected to normal SDS-PAGE gels under reducing conditions, electroblotted onto nitrocellulose membranes and immunodetected using the aforementioned antibodies at a dilution of 1:2000 (Pascual et al., 2010). It should be noted that, although the antigenic decapeptide is conserved also in cyanobacteria, the detection of the overoxidized cyanobacterial 2-Cys Prx is less efficient than the detection of the overoxidized rice 2-Cys Prx using these antibodies. Therefore, 5 μg of the cyanobacterial proteins are loaded per lane, whereas 0.3 μg of the rice protein is loaded per lane, in order to obtain strong signals (Pascual et al., 2010). Moreover, this is one of the reasons why the immunological method has limited utility for the analysis of 2-Cys Prx overoxidation in cyanobacteria *in vivo*. The other reason is that the abundance of 2-Cys Prx varies substantially between cyanobacterial species. The *Anabaena* 2-Cys Prx is an abundant protein that accounts for about 1% of the total cytosolic protein (Pascual et al., 2010), which is similar to the plant 2-Cys Prx that constitutes about 0.6% of the total chloroplast protein (Dietz et al., 2006). In contrast, the *Synechocystis* 2-Cys Prx is at least 20 times less abundant (Pascual et al., 2010). It is possible to load 50 μg of total protein per lane in a small (9×8 cm^2) SDS-PAGE gel without loss of resolution and this would correspond to 0.5 μg of *Anabaena* 2-Cys Prx and 0.3 μg of plant chloroplast 2-Cys Prx. However, since 50 μg of *Synechocystis* cytosolic protein corresponds to just 25 ng 2-Cys Prx, this would be below the detection level, even when the protein is fully overoxidized.

s0070 5.2. Overoxidation of cyanobacterial 2-Cys Prx *in vivo* as detected by nonreducing SDS-PAGE and Western blot

p0180 A simple way to assess the degree of overoxidation *in vivo* is to resolve cytosolic proteins from cyanobacteria using one-dimensional SDS-PAGE under nonreducing conditions combined with Western blot analysis. The overoxidized sulfinic acid form of 2-Cys Prx is unable to form disulfide-linked dimers and, therefore, migrates as a monomer also under nonreducing conditions. In contrast, the pools of 2-Cys Prx that at the moment of isolation were found in the thiol- or sulfenic acid forms will rapidly form disulfides

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and migrate as dimers. Thus, the monomeric pool of the enzyme detected by Western blot corresponds exclusively to overoxidized 2-Cys Prx.

p0185 The susceptibility to overoxidation *in vivo* differs between 2-Cys Prxs from different cyanobacterial species. The *Anabaena* sp. PCC 7120 2-Cys Prx is highly sensitive to overoxidation induced by illumination at high light intensity or by addition of H_2O_2 at high concentrations, whereas the *Synechocystis* sp. PCC 6803 2-Cys Prx is largely inert to these treatments (Pascual et al., 2010). The following protocol describes the detection of *Anabaena* 2-Cys Prx overoxidation by nonreducing SDS-PAGE and Western blot following exposure of cultures to high light intensities. Since maximal overoxidation of the *Anabaena* 2-Cys Prx is observed after 15–30 min under high light conditions (Pascual et al., 2010), the duration of the light exposures in a trial experiment should be 0 min (control), 15, 30, and 60 min.

s0075 5.2.1 High light treatment of cyanobacterial cultures

o0095 **1.** *Anabaena* sp. PCC 7120 cultures are grown under standard conditions to mid-exponential phase (3–5 μg chlorophyll per mL).

o0100 **2.** Aliquots of 40 mL culture diluted to 2.5 μg chlorophyll per mL are poured into 50-mL glass tubes and placed in a transparent water bath kept at 30 °C. Cultures are continuously bubbled with a stream of 1% (v/v) CO_2 in air, which also avoids sedimentation of cells during the light treatment.

o0105 **3.** A light source emitting strong white light is placed directly in front of the water bath and the intensity should be measured inside a glass beaker placed in the bath at the same distance from the light as the tubes containing the cultures. The intensity for high light treatment should be at least 500 μmol photons $m^{-2}\,s^{-1}$ and, hence, the temperature of the water bath must be controlled through a cooling system to maintain 30 °C.

s0080 5.2.2 Isolation of cytosolic extract from cyanobacterial cells

o0110 **1.** At each time point after the onset of high light exposure, cells are harvested by centrifugation at 14,000 × *g* for 30 min at 4 °C.

o0115 **2.** The pellets are resuspended in 350 μL of Buffer A consisting of 25 m*M* Hepes–NaOH (pH 7.6), 15% (v/v) glycerol, and supplemented with 1 m*M* phenylmethylsulfonyl fluoride. These suspensions are transferred to 1.5-mL microfuge tubes and kept on ice.

o0120 **3.** Add to each tube 0.5 mL glass beads with a diameter of 212–300 μm.

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o0125 **4.** Cells are broken through vigorous shaking on a vortex at maximum speed. Thirty seconds of vortexing should be followed by 30 s of cooling on ice to avoid heating of the material. Repeat this procedure six times in order to achieve the best possible lysis.

o0130 **5.** Pipette the lysate to a fresh tube, rinse the glass beads with 150 μL Buffer A, and pool this volume with the rest of the lysate.

o0135 **6.** Centrifuge at $2300 \times g$ for 5 min at 4 °C, to remove unbroken cells. Thereafter, centrifuge the supernatant at $16{,}000 \times g$ for 30 min at 4 °C to pellet the total cellular membranes, that is, thylakoid and plasma membranes. The remaining supernatant corresponds to the cytosolic extract.

s0085 *5.2.3 One-dimensional nonreducing SDS-PAGE and Western blot analysis of 2-Cys Prx*

p0245 Five microgram of *Anabaena* cytosolic proteins are loaded per lane on 15% acrylamide gels. The sample buffer must be devoid of reductants, such as DTT and β-mercaptoethanol, in order to leave disulfides intact. Gels are electroblotted onto nitrocellulose membranes and the antibodies raised against rice 2-Cys Prx (Pérez-Ruiz et al., 2006) are used at a dilution of 1:1500. If *Synechocystis* sp. PCC 6803 2-Cys Prx is to be analyzed, 25 μg of *Synechocystis* cytosolic proteins should be loaded per lane and the antibodies raised against *Synechocystis* 2-Cys Prx (Pascual et al., 2010) are used at a dilution of 1:1000. It should be remarked that overoxidation of *Synechocystis* 2-Cys Prx has not been previously observed after illumination at high light intensities, though addition of H_2O_2 at m*M* concentrations to *Synechocystis* cultures does lead to some overoxidation of 2-Cys Prx (Pascual et al., 2010). Figure 14.3A illustrates a typical experiment demonstrating *Anabaena* 2-Cys Prx overoxidation following high light treatment.

s0090 5.3. Overoxidation of cyanobacterial 2-Cys Prx as detected by two-dimensional isoelectric focusing/SDS-PAGE and Western blot

p0250 In this protocol, both isoelectric focusing and SDS-PAGE are performed under denaturing conditions in the presence of high concentrations of DTT. Therefore, the entire pool of 2-Cys Prx remains monomeric throughout the procedure. The sulfinic acid-containing 2-Cys Prx is distinguished by a slightly lower pI value and, thus, the overoxidized form of the enzyme is found in a spot shifted toward the acidic side of the gel (Fig. 14.3B). The pI values of unmodified 2-Cys Prxs from most cyanobacteria and plants are close to 5.

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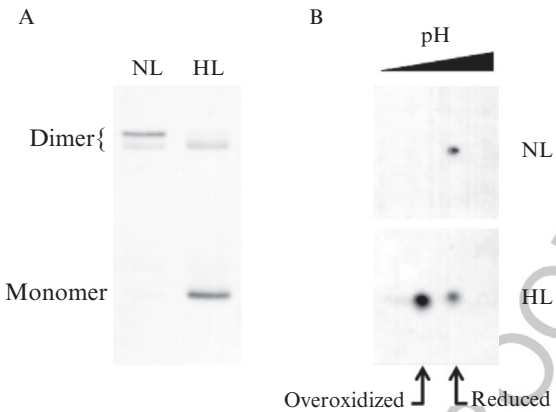

f0015 **Figure 14.3** Overoxidation of the *Anabaena* sp. PCC 7120 2-Cys Prx. *Anabaena* cultures are kept at normal light intensity (NL; 50 μmol photons m^{-2} s^{-1}) or exposed to high light intensity (HL; 800 μmol photons m^{-2} s^{-1}) for 15 min. The cytosolic extracts are thereafter analyzed by one-dimensional SDS-PAGE under nonreducing conditions (A) and two-dimensional isoelectric focusing/SDS-PAGE (B) combined with Western blot. The overoxidised 2-Cys Prx is characterized by its migration as a monomer in (A) and by the pI shift toward the acidic side in (B).

o0140 **1.** *Anabaena* cytosolic extracts containing a minimum of 15 μg of protein are precipitated with 5% trichloroacetic acid for 30 min on ice and centrifuged at 16,000 × *g* for 20 min at 4 °C.

o0145 **2.** The supernatant is discarded and the pellet is washed twice with 200 μL acetone and centrifuged as above. The acetone is removed and the pellet should be well dried in air.

o0150 **3.** Add 100 μL rehydration buffer (e.g., ReadyPrep™, Bio-Rad) containing 8 *M* urea, 2% CHAPS, 50 m*M* DTT, and 0.2% ampholytes. Do not attempt to resuspend the pellet immediately, but leave it overnight at −20 °C to absorb slowly the buffer.

o0155 **4.** Mix the proteins solubilized in dehydration buffer using the pipette tip and centrifuge the sample at 16,000 × *g* for 5 min to remove nonsolubilized material.

o0160 **5.** Resolve the proteins by isoelectric focusing with an immobilized pH gradient (pH 4–7) of 11 cm, using for instance ReadyStrip™ IPG strips (Bio-Rad).

o0165 **6.** The second dimension SDS-PAGE should be performed on 16-cm long 15% acrylamide gels. After electrophoresis, the proteins are transferred onto nitrocellulose membranes and probed with the antibody against rice 2-Cys Prx (Pérez-Ruiz et al., 2006) at a dilution of 1:1500.

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6. CONCLUDING REMARKS

p0285

The precise roles of the different families of Prxs in peroxide detoxification and signaling of plants and cyanobacteria still remain to be established (Dietz, 2011). This chapter should serve as a practical guide in the effort to explore the Prx systems of cyanobacteria and chloroplasts. The methods describing measurement of activity may be used to test the substrate specificities of various Prxs with respect to electron donors as well as peroxides and to examine the importance of particular residues or domains for Prx activity in site-directed mutant versions. The protocols for *in vivo* measurements of peroxide decomposition should be useful to assess the detoxifying capacity of knockout mutants lacking one or more Prxs. Finally, the degree of overoxidation of 2-Cys Prx *in vivo* under different physiological conditions might be informative for future studies on peroxide-mediated signaling in photosynthetic organisms.

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