NADPH-dependent thioredoxin reductase and 2-Cys peroxiredoxins are needed for the protection of Mg–protoporphyrin monomethyl ester cyclase

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Abstract The chloroplast-localized NADPH-dependent thioredoxin reductase (NTRC) has been found to be able to reduce hydrogen peroxide scavenging 2-Cys peroxiredoxins. We show that the Arabidopsis ntrc mutant is perturbed in chlorophyll biosynthesis and accumulate intermediates preceding protochlorophyllide. A specific involvement of NTRC during biosynthesis of protochlorophyllide is indicated from in vitro aerobic cyclase assays in which the conversion of Mg–protoporphyrin monomethyl ester into protochlorophyllide is stimulated by addition of the NTRC/2-Cys peroxiredoxin system. These findings support the hypothesis that this NADPH-dependent hydrogen peroxide scavenging system is particularly important during periods with limited reducing power from photosynthesis, e.g. under chloroplast biogenesis. © 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Reactive oxygen species (ROS) affect an immense variety of cellular mechanisms [1,2]. Recent evidence indicates that chlorophyll biosynthesis is also perturbed by ROS. Aarti et al. [3] have observed that chlorophyll intermediates, in particular protoporphyrin IX (PPIX) and Mg–protoporphyrin monomethyl ester (MgP-MME) accumulate in cucumber seedlings when exposed to either high light or methylviologen. Both treatments have observed that chlorophyll intermediates, in particular protochlorophyllide formation. A specific involvement of NTRC during biosynthesis of protochlorophyllide is indicated from in vitro aerobic cyclase assays in which the conversion of Mg–protoporphyrin monomethyl ester into protochlorophyllide is stimulated by addition of the NTRC/2-Cys peroxiredoxin system. These findings support the hypothesis that this NADPH-dependent hydrogen peroxide scavenging system is particularly important during periods with limited reducing power from photosynthesis, e.g. under chloroplast biogenesis.

Chlorophyll precursors themselves are highly phototoxic molecules and, therefore, their production is tightly regulated [5]. Moreover, some intermediates, in particular Mg–protoporphyrin IX (MgP), have been proposed to be involved in plastid to nucleus signaling [6] underlining the importance of having a well-functioning and carefully regulated chlorophyll biosynthetic pathway. Therefore, it is highly interesting to find enzymes involved in protecting and regulating the chlorophyll biosynthetic steps.

To search for antioxidative enzymes involved in protection of chlorophyll biosynthesis we performed a bioinformatics screen for Arabidopsis genes co-regulated with genes involved in chlorophyll biosynthesis. One of the candidates identified in this screen, the NADPH-dependent thioredoxin reductase (NTRC), has previously been biochemically characterized as an electron-donor to 2-Cys peroxiredoxins [7,8]. Peroxiredoxins (Prx) are antioxidant enzymes of 17–20 kDa which employ a Cys-thiol-based mechanism to reduce peroxides including H$_2$O$_2$. The Arabidopsis chloroplast contains three different Prx types; two 2-Cys Prx’s (A and B), Prx Q and PrxII E [9,10]. Besides NTRC, Trx X, CDSP32 and CYP20-3 have also been found to reduce 2-Cys Prx in vitro [11–13]. The NADPH-dependent NTRC/2-Cys Prx system constitutes a chloroplast-localized H$_2$O$_2$-scavenging system alternative to the ascorbate–glutathione cycle [14] and is proposed to be especially important under short day conditions [15]. This is supported by the fact that ntrc mutants perform much better under long day conditions than under short day conditions [8] (E. Rintamäki, personal communication).

In this study, we propose that NTRC and 2-Cys Prx are also involved in the protection of at least the later part of the chlorophyll biosynthetic pathway and show that NTRC and 2-Cys Prx in vitro stimulate the aerobic cyclase step of this pathway.

2. Materials and methods

2.1. Bioinformatics screen

Genes that are co-regulated with known genes of the chlorophyll biosynthetic pathway were identified using the Arabidopsis thaliana Co-Response Data base, hosted at Max-Planck-Institute of Molecular Plant Physiology (http://csbdb.mpimp-golm.mpg.de/csbdb/dbcor/ath.html) [16].
2.2. Plant material

Seeds from 4 T-DNA insertion lines affected in an exon of the NTRC gene (At2g41680) (SALK_012208, SALK_114293, SALK_096776) [17] and one line where the T-DNA was placed in the 3′-UTR of the chlorophyll biosynthetic pathway (GAI) were obtained. Likewise seeds from one line affected in the promoter of the aerobic cyclase diiron component CHL27 (At1g56940) was obtained (SALK_009052). Wild-type and T-DNA tagged Arabidopsis thaliana (L.) Heyn cv. Columbia were grown on peat in a controlled environment chamber (Percival AR-60L, Percival, Perry, IA) at a photosynthetic flux of 100–120 μmol photons m⁻² s⁻¹, 20 °C, 70% relative humidity and a daily photoperiod of 8 h. Grains of barley (Hordeum vulgare L. cultivar Bonus) were planted in moist vermiculite and grown in darkness at 20 °C for 6 or 7 days.

2.3.ALA feeding and HPLC analysis of chlorophyll precursors

Feeding with 5-aminolevulinic acid (ALA) (Ala) and subsequent HPLC analysis were performed as described previously [19] except that an Agilent Zorbax Extend-C18 column was used. Excised leaves were fed with ALA in darkness for 16 h. Extraction volumes were normalized to the fresh weight of the leaf material. Chlorophyll intermediates were identified based on their spectroscopic characteristics as well as comparison to known standards (as described [19]).

2.4. Cloning and purification of NTRC and Prx’s

2-73, 0.3 M glycerol and 0.02 M Tris–HCl, pH 7.9, according to the manufacturer’s instructions (GE Healthcare, Buckinghamshire, UK). The barley NTRC gene (GenBank accession number EU360810 bases 5–1426) was cloned into a pET-15b vector with an N-terminal 6XHis-tag (GE Healthcare) according to the manufacturer’s instructions (Ala). The barley NTRC gene was expressed in Escherichia coli BL21. The recombinant protein was purified using a HiTrap chelating HP column (GE Healthcare) according to the manufacturer’s instructions. The protein solution was desalted with a NAP-10 column (GE Healthcare) into 30 mM Tris–HCl, 1 mM DTT, 1 mM PMSF, pH 8.

2.5. Thioredoxin reductase assay

The ability of NTRC to reduce 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) by NADPH to two molecules of 5-thionitrobenzoic acid (TNB) was performed essentially as described [21].

2.6. Preparation of barley etioplasts

Intact barley etioplasts were isolated from 6 to 7 day old dark grown seedlings, as previously described [22], except that the Percoll step was modified with 45% Percoll (GE Healthcare) and centrifuged at 13000 × g at 4 °C for 2 min. Through all steps, the material was kept in darkness. Intact barley etioplasts were subsequently lysed in a minimal volume of lysis buffer (20 mM Tricine, pH 8.1, 10 mM HEPES, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 1 mM PMSF). For long term storage at -80 °C, glycerol was added to a final concentration of 15%.

2.7. Aerobic cyclase in vitro activity assays

To deplete the endogenous pool of Pchlide found in the etioplast extract from dark grown barley, the preparations were exposed to light, 10 μmol photons m⁻² s⁻¹ (Schott, KL 1500) in the presence of 1 μM NADPH at room temperature for 5 min. The aerobic cyclase activity assays were performed in assay buffer (lysis buffer supplemented with 25 mM MgCl₂) containing 10 mM D-glucose-6-phosphate, 0.025 U/ml glucose-6-phosphate dehydrogenase, 0.6 mM NADPH (added to the etioplast extract during light treatment) and 12.5 μg MgP-MME. Etioplast extract was added to a final concentration between 1/5 and 1/4 of the total assay dependent on concentration of the etioplast preparation. Thirteen units per microliter of catalase (C-30) were included in the assay, where indicated. All chemicals and enzymes were obtained from Sigma, except MgP-MME which was obtained from mutant bacteria [23]. Incubations were performed at 30 °C in an Eppendorf Thermomixer with shaking at 1000 rpm. Precautions were taken through out the experiment to avoid exposure to any other light sources than dim green light. At indicated time points, 10 μl aliquots of the assay mixture were withdrawn, quenched by adding 300 μl alkaline acetone (acetone/water/25% ammonia (80:20:1, vol/vol/vol)) and centrifuged at 18000 × g at 4 °C for 2 min. Fluorescence emission spectra of the supernatants were recorded at room temperature from 550 nm to 700 nm with excitation wavelengths set to 440 nm using a spectrophotometer (Photon Technology International, Lawrenceville, NJ). Routinely the slit widths were set to 2 nm. When desired, assay mixtures were exposed to light, 10 μmol photons m⁻² s⁻¹ (Schott, KL 1500) in room temperature for 5 min to further metabolize the in vitro synthesized Pchlide.

3. Results

3.1. Restricted flow through the chlorophyll biosynthetic pathway in ntrc plants

The T-DNA insertions lines affected in the NTRC gene were clearly chlorotic and failed to assemble wild-type amounts of chlorophyll containing complexes when grown under short day conditions with 8 h light (Fig. 1). Four independent T-DNA lines showed identical phenotypes indicating that it is indeed the At2g41680 gene that is affected by the T-DNA. The lack of NTRC protein in one of these lines (SALK_012208) has previously been confirmed by Western blotting [24] and all subsequent work was done with this line. Compared to the chl27 mutant which has a reduced expression of the diiron component of the aerobic cyclase catalyzing the conversion of MgP-MME into Pchlide due to a T-DNA insertion immediately upstream the CHL27 start codon (Andreas Hansson, unpublished results) the ntrc mutant showed a more spotted chlorotic pattern (Fig. 1).

Due to tightly regulated chlorophyll biosynthesis, plants do not normally accumulate chlorophyll precursors [25]. This regulation can be overcome by feeding ALA in darkness resulting in an unrestricted flow through the pathway up to the light-dependent Pchlide oxidoreductase with large amounts of Pchlide accumulating (Fig. 2). Therefore, ALA-feeding is a valuable tool to investigate putative mutants directly affected in or otherwise restricted in chlorophyll biosynthesis [25]. To investigate if the chlorotic phenotype of ntrc was due to restric-
tions in chlorophyll biosynthesis, ntrc, chl27 and wild-type plants were analyzed for accumulating chlorophyll intermediates using ALA-feeding followed by HPLC analysis.

The HPLC analysis showed as expected that ALA-fed wild-type plants accumulate high amounts of Pchlide and minor amounts of PPIX (Fig. 3). Plants leaky for CHL27 expression were clearly restricted in accumulation of Pchlide and instead accumulated MgP and MgP-MME and to a minor extent PPIX as previously demonstrated for antisense CHL27 plants [19]. The ALA-fed ntrc mutant accumulated MgP and MgP-MME as the chl27 plants; however, the ratio was changed with more MgP accumulating (Fig. 3). From these results we conclude that the chlorophyll biosynthetic pathway is restricted in ntrc mutant plants with lower activities of especially the methyltransferase and the aerobic cyclase, synthesizing MgP-MME and Pchlide, respectively (Fig. 2). From this experiment it appears that NTRC is required for optimal activity of one or more enzymes involved in chlorophyll biosynthesis.

3.2. Stimulatory involvement of NTRC and 2-Cys Prx on in vitro aerobic cyclase activity

Chlorophyll biosynthesis is perturbed by conditions leading to ROS-formation [3] and NTRC and 2-Cys Prx has been characterized as a H$_2$O$_2$-scavenging system [7,8]. This combined with the fact that accumulation of NTRC transcripts co-regulate with known chlorophyll biosynthesis genes prompted us to investigate if a specific connection exists between the NTRC/2-Cys Prx-system and enzymatic steps in chlorophyll biosynthesis.

Recombinant His-tagged NTRC from barley and 2-Cys Prx (A and B) from *Arabidopsis* were expressed in *E. coli*. A thiol-redoxin reductase assay showed that NTRC is active and capable of reducing DNTB to TNB using NADPH with an average activity of 6.5 ± 0.9 pmol formed TNB per minute and microgram added NTRC (results not shown).

The aerobic cyclase catalyzing the conversion of MgP-MME into Pchlide consists of at least one diiron enzyme which requires molecular oxygen for oxygenation of MgP-MME [19,26,27]. Bollivar and Beale [28] have previously shown that in vitro aerobic cyclase activity can be stimulated by addition of catalase and ascorbate probably due to scavenging of ROS produced in the oxygenase reaction. It was therefore obvious to test if the chloroplast-localized H$_2$O$_2$-scavenging NTRC/2-Cys Prx system at least in vitro could stimulate the aerobic cyclase reaction. An in vitro aerobic cyclase assay using plastid extract from etiolated barley seedlings was established, where activity is measured by the buildup of the product Pchlide. Indeed, by adding NTRC and 2-Cys Prx A to the aerobic cyclase assay, a stimulatory effect similar to that seen by adding catalase was obtained (Fig. 4A). Similar results were obtained...
with NTRC in combination with 2-Cys Prx A, the second isoform present in the \textit{A. thaliana} chloroplast (results not shown). Adding either NTRC or 2-Cys Prx A alone did not result in any activity stimulation (results not shown) showing that both components are required. Likewise, adding NTRC in combination with PrxII E which is a stromal type II peroxiredoxin [20] failed to stimulate cyclase activity to almost the same extent as catalase. Adding higher amounts of PrxII E (6 or 9 µM) or 9 µM together with catalase diminished this stimulation. An NADPH regenerating system consisting of 10 mM D-glucose-6-phosphate, 0.025 U/µl glucose-6-phosphate dehydrogenase and 0.6 mM NADPH was present in all assays.

and the NTRC/2-Cys Prx-system, respectively (Fig. 4B). The mutually exchangeable stimulatory effect on aerobic cyclase activity by the three different H$_2$O$_2$-scavenging systems (catalase, NTRC + 2-Cys Prx and PrxII E + 10 mM DTT) indicates that H$_2$O$_2$ produced in the assay needs to be removed continuously in order to avoid inhibition of cyclase activity.

As shown in Figs. 4 and 5, addition of either catalase or NTRC combined with 2-Cys Prx had a profound effect on the aerobic cyclase activity with only very small amounts of Pchlide formed without the presence of a H$_2$O$_2$-scavenging system. It has previously been suggested that this is due to the breakdown of tetrapyrroles [28], however it is shown that in the absence of catalase, at least Chlide was stable and not degraded through out the assay incubation (Fig. 5). The observation that the in vitro formed Pchlide can be further metabolized to Chlide, by exposing the assay mixtures to light, shows that the catalytic steps associated with the aerobic cyclase have been completed (Fig. 5).

4. Discussion and conclusion

The pale phenotype and the accumulation of significant amounts of MgP and MgP-MME as evidenced by the HPLC analysis in the \textit{ntrc} mutant clearly showed that chlorophyll biosynthesis is perturbed in this mutant. Moreover we show a direct stimulation of aerobic cyclase activity in vitro by addition of NTRC and 2-Cys Prx. It has previously been shown that catalase can stimulate aerobic cyclase activity in vitro [28]. However, catalase is localized in peroxisomes [30] and a chloroplast-localized scavenging system would be required in planta. The chloroplast has an extensive and finely balanced ROS-scavenging system [14,30] of which NTRC and 2-Cys Prx has been proposed to play a role especially during darkness [15]. NTRC is a dual domain protein combining the thioredoxin reductase function with a thioredoxin activity in one polypeptide. It receives its electrons for reduction of target
proteins from the NADPH-pool. In darkened chloroplasts the NADPH system is maintained reduced by import of substrates and activation of the oxidative pentose phosphate cycle. Thus, the NTRC/2-Cys Prx-system is an efficient and short electron transport system to detoxify peroxides at the expense of NADPH. In the light, reduction of 2-Cys Prx might be linked to the photosynthetic electron transport chain via ferredoxin and thioredoxin [13].

As the in vitro assay is performed in darkness, the generation of the H₂O₂ is not light-dependent. Rather, one could speculate that the H₂O₂ is a product of the oxygenase reaction itself or other metabolic reactions occurring in the plastids. As the catalytic aerobic cyclase enzyme is a diiron enzyme protein this H₂O₂ would together with the Fe in the diiron center produce extremely damaging hydroxyl radicals [31].

Not only aerobic cyclase activity is decreased without a H₂O₂-scapenging system in vivo. In the ntrc mutant also other chlorophyll biosynthetic intermediates accumulate, in particular the methyltransferase substrate MgP, which could be due to a negative feedback inhibition by the restrained aerobic cyclase complex or damage to the methyltransferase enzyme itself. The accumulation of several different chlorophyll intermediates in the ntrc mutant is in agreement with the findings by Aartii et al. [3], who also found accumulation of PPIX, MgP and MgP-MME after methylviologen treatment.

At present it cannot be ruled out that NTRC plays a specific role in the aerobic cyclase reaction for example by interacting with one of the peroxo-intermediates proposed to be part of the catalytic cycle of diiron enzymes [26]. However, the deletion of NTRC does not prevent chlorophyll biosynthesis and therefore NTRC is not essential for biosynthesis of chlorophyll. This is further supported by the fact that ntrc mutants grown under long-day conditions, i.e. 16 h light perform much better than mutants grown under 8 h light suggesting that under long day conditions other ROS-scapenging pathway can complement the lack of NTRC. As discussed above, other electron donors are likely to substitute for NTRC in the light [13].

In conclusion, the NTRC and 2-Cys Prx system is suggested to be important for scavenging H₂O₂ independent of light-driven generation of reducing equivalents. This is a situation which is prevalent in darkness or before photosynthesis is established, i.e. during chlorophyll biosynthesis and biogenesis of the photosynthetic apparatus. In this context it is interesting to note that the 2-Cys Prx expression precedes expression of other antioxidants during early seedling establishment [32] and that suppression of 2-Cys Prx expression evokes an inhibition of photosynthesis during plant development [33]. Both, the early requirement for 2-Cys Prx during leaf development and the effect of insufficient 2-Cys Prx amounts on photosynthesis might be also linked to the protection of chlorophyll biosynthesis.

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References


