Thiol-based Control of Tetrapyrrole Metabolism: Posttranslational Control of NADPH-Dependent Thioredoxin Reductase C on Enzymes of Tetrapyrrole Biosynthesis

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Summary

The NADPH-dependent thioredoxin reductase C (NTRC), thioredoxin and related proteins are involved in thiol/disulfide exchange reactions affecting many key enzymes in central metabolic pathways of photosynthetic organisms. This posttranslational control applies also to tetrapyrrole biosynthesis to fine-tune enzyme activities in response to environmental and endogenous cues and to prevent accumulation of photoreactive tetrapyrrole metabolites. In previous studies several proteins of the tetrapyrrole biosynthetic pathway have been identified to interact with these thiol-reductants. In my group, studies on posttranslational control of tetrapyrrole biosynthesis have been already performed and confirm the regulatory impact of thiol-disulfide exchanges between thioredoxin and NTRC and two proteins, the CHLI subunit of the Mg chelatase (MgCh) complex and the MgProto methyltransferase (CHLM), respectively (Luo et al. 2012, Richter et al. 2013). Reduced content of glutamyl-tRNA reductase (GluTR), CHLM and protochlorophyllide oxidoreductase (POR) has been found in a pale-green *Arabidopsis ntrc* mutant (Richter et al., 2013) indicating that NTRC is essential for stability and/or activity of enzymes in tetrapyrrole biosynthesis and likely a central redox regulator of tetrapyrrole biosynthesis. The main objective of the proposed project is to identify and validate the thiol-based redox switches of the three enzymes of the tetrapyrrole biosynthetic pathway, CHLM, GluTR and POR, which are catalysed by NTRC, and to elucidate the physiological effects of the posttranslational modifications of these NTRC target enzymes as well as, in consequence, the balanced supply of the end-products, chlorophyll and heme, in the balanced quantities. After confirmation of NTRC interactions with tetrapyrrole biosynthesis enzymes by alternative biochemical methods, it is intended (i) to examine the redox-active thiol switches that control the activity and stability of these enzymes and (ii) to analyse *in planta* the modulated enzyme activity of Cys>Ser substitution mutants of these enzymes in comparison to wild type always in an *Arabidopsis* knock-out mutant background for the respective analysed enzyme. It is expected that elucidation of details of the thiol-disulfide exchange reactions on enzymes of tetrapyrrole biosynthesis will improve understanding on redox-controlled tetrapyrrole biosynthesis, which includes changes of enzyme activities, protein structure and stability as well as interaction of proteins to others components of the pathway and their localization within the chloroplast.

State of the Art and preliminary work

Photosynthetic electron transfer is a major source of redox-related processes performed by redox-active compounds of the photosynthetic complex which enables the production of reducing equivalents and ATP. During acclimation to the continuous environmental changes, for example light intensity and temperature, these redox-active components/intermediates of the photosynthetic electron transfer chain are dynamically modulated. In turn, this regulatory mechanism is embedded in a redox network and part of a complex chloroplast redox poise that adjusts not only photosynthesis but also the organellar redox-controlled metabolic and genetic activities.

Modification of the redox status by formation and reduction of disulfide bonds is a crucial posttranslational regulation of protein activity, folding and stability. Cysteine residues are the main

redox-active component for redox regulation of proteins. Thereby, the thiol group of cysteine residues can be reversibly oxidized to stabile disulfides to form either intra- or intermolecular disulfide bonds. Thiol-based control of plastid processes could be driven either by the light and ferredoxin-thioredoxin-dependent or the NADPH-thioredoxin-dependent pathway.

It is generally accepted that normal growth conditions keep intracellular compartments (cytoplasm, plastidal stroma or mitochondrial matrix) reductive, while the extracellular area (including apoplastic space, vacuole, ER) is in an oxidative state. However, regulatory disulfide bonds can be typically formed or released in spite of the prevailing reducing or oxidizing conditions to modulate biochemical activity or protein stability. Modifications of thiols of cysteines are also crucial, as they are particular susceptible to oxidation by reaction with reactive oxygen species (ROS, sulfenation) and nitric oxide (NO, S-nitrosylation). Then, as a consequence of sulfenation, stable intra- and intermolecular disulfide bonds or bonds to glutathione (GSH) and less stable sulfenic, or sulfonic acid side chains can be formed dependent on the mid-point redox potential of a given thiol group, its accessibility to the oxidant and the locally restricted oxidative conditions.

Thiol-based control of tetrapyrrole biosynthesis

Tetrapyrrole biosynthesis in plants forms multiple end products including chlorophyll, heme, siroheme and phytochromobiline. These essential molecules belong to the most abundant macromolecules in the plant kingdom. During the last decades, almost all enzymes of tetrapyrrole biosynthesis and their complex network of transcriptional regulation have been comprehensively studied (Tanaka et al., 2011; Brzezowski and Grimm, 2013). These studies revealed a complex control of the expression of genes encoding enzymes in the light-regulated chlorophyll (Chl)-synthesizing branch of tetrapyrrole metabolism and paved the way for current and future exploitation of posttranslational regulation of tetrapyrrole biosynthesis (Figure 1) But in contrast to the physiological significance of the pathway, very little is still known about its posttranslational control (Czarnecki and Grimm, 2012).

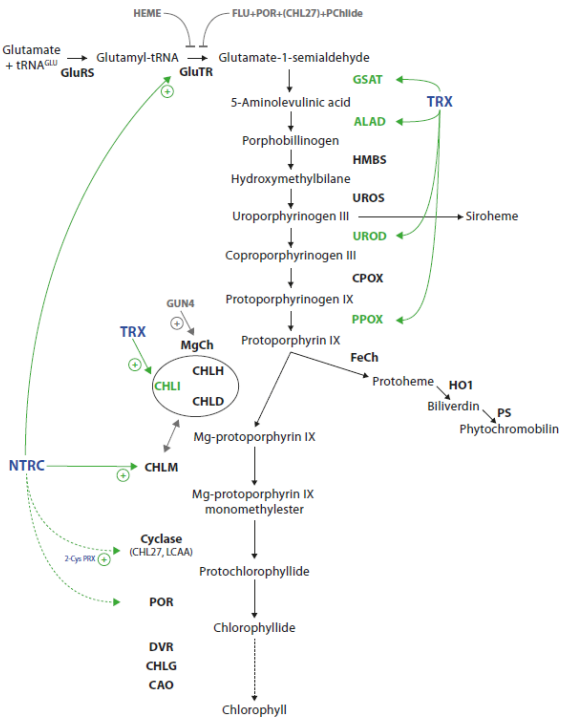

Figure 1: Scheme of the plant tetrapyrrole biosynthetic pathway. The coloring highlights the redox regulation and targets of redox regulation in green and the involved enzymes in blue. Further post-translational regulations are indicated in grey, including the proteins involved. Solid lines refer to verified interactions and dotted lines to potential targets of thiol-based regulation. Further details and the abbreviations of the enzymes are given in the text.

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Among the multiple posttranslational control mechanisms, redox regulation is an important mechanism to translate light signals into structural and enzymatic changes of enzymes involved in photosynthetic reactions (Buchanan and Balmer, 2005). The tetrapyrrole biosynthetic pathway

contains several target proteins susceptible to redox regulation. Using thioredoxin-bound affinity chromatography, thiol-based proteome studies revealed several target enzymes of tetrapyrrole biosynthesis. Interaction between thioredoxin and the following proteins have been reported: CHLI, a Mg chelatase subunit, glutamate-1-semialdehyde aminotransferase (GSAT), uroporphyrinogen III decarboxylase (UROD), protoporphyrinogen oxidase (PPOX) (Balmer et al., 2003) and ALA dehydratase (ALAD) (Lindahl and Florencio, 2003, Figure 1).

Initial evidence for a redox-dependent activity in tetrapyrrole biosynthesis was obtained in studies on the sensitivity of Mg chelatase (MgCh) activity from purified plastids to thiol-reacting reagents (Fuesler et al., 1984). The ATPase activity of CHLI, one of the three MgCh subunits, was abolished under oxidizing conditions and when conserved cysteines were blocked by the sulfhydryl reagent N-ethylmaleimide (NEM) (Walker and Weinstein, 1991 Jensen et al., 2000). Addition of *Arabidopsis* thioredoxin f to an *in vitro* assay stimulated ATPase activity of heterologously expressed CHLI (Ikegami et al., 2007). In conclusion, these results suggest that the redox-dependent mechanism stimulates the MgCh reaction. In addition, chlorophyll biosynthesis is also likely regulated at other enzymatic steps by the redox-state of the chloroplasts at other enzymatic steps (See also own contributions 1.2.1).

Apart from the ferredoxin-thioredoxin-dependent thiol-based redox regulation, electrons are also transferred from NADPH to target proteins via NADPH-dependent thioredoxin reductase (NTR). This enzyme contains a flavin-adenine-diphosphate-binding domain and a double cysteine peptide motif (CXXC) in the catalytic center. Three isoforms of NTR (NTRA, NTRB and NTRC) are reported in *Arabidopsis thaliana* that link TRXs to multiple target proteins of metabolic processes. While NTRA and NTRB are dually targeted to cytosol or mitochondria, NTRC is plastid-localized. NTRC is a bifunctional protein composed of the typical NTR module at the N-terminus and a thioredoxin module at the C-terminus (Serrato et al., 2004). Electrons are transferred from NADPH to FAD which reduces an internal disulfide, followed by the reduction of the thioredoxin domain by the reduced cysteines. It was initially demonstrated that NTRC reduces 2-Cys peroxiredoxins (2-CysPrx), which are small hydrogen peroxide (H_2O_2) peroxidases (Pulido et al., 2010). NTRC contributes to the reduction of H_2O_2 via donation of two electrons to 2-CysPrx indicating a role of NTRC in the detoxification of reactive oxygen species (ROS) during oxidative stress (Perez-Ruiz et al., 2006). Thus, it was assumed that NTRC constitutes an alternative reduction system for protection of chloroplasts against oxidative damage which could be of particular importance in the dark when photoreduced ferredoxin is not available for reduction of thioredoxin. Subsequently, NTRC has been shown to reduce heterotetrameric ADP-Glc pyrophosphorylase (AGP), a key enzyme in starch synthesis (Michalska et al., 2009). However, it is also reported that the activity of AGP is not influenced by the light-induced and redox-dependent switch between monomeric and dimeric forms of AGP (Li et al., 2012).

Interestingly, an *Arabidopsis ntrc* mutant shows a chlorotic phenotype under normal growth conditions, which is due to accumulation of ROS, but also attributed to reduced chlorophyll biosynthesis. In comparison to wild-type seedlings, the chlorophyll deficiency of *ntrc* increases during shorter photoperiods and upon increasing light intensities (Lepisto et al., 2009). It was recently shown that NTRC stimulates the reactions of the Mg protoporphyrin monomethylester (MgPME) cyclase in *in vitro* assays by scavenging peroxides (Stenbaek et al., 2008). As the *ntrc* mutant accumulates MgPME upon feeding with 5-aminolevulinic acid (ALA), the authors proposed that the inhibited activity of the MgPME cyclase results from a compromised NTRC-2-CysPrx action which leads to impaired ROS scavenging.

In summary, NTRC is apparently one of the main redox regulators in photosynthetically active and non-active plastids and contributes likely to the functional separation of day and night activities of enzymes in photosynthetic tissue (above-ground tissue) (Serrato et al., 2004; Perez-Ruiz et al.,

2006; Spinola et al., 2008; Kirchsteiger et al., 2009; Lepisto et al., 2009; Perez-Ruiz and Cejudo, 2009; Pulido et al., 2010; Kirchsteiger et al., 2012).

Thioredoxin-controlled activation of Mg chelatase

Initial biochemical investigations indicated posttranslational redox regulation of thioredoxin and NTRC at different steps of chlorophyll biosynthesis (Stenbaek and Jensen, 2010; Wulff et al., 2011, Richter and Grimm, 2013, submitted). The need of the thiol-based regulation of the tetrapyrrole biosynthetic pathway was proposed to result from regulatory interdependency of a tightly regulated metabolic flow in higher plants, which is due to the functional separation of day and night activities, as well as essential response to rapid environmental changes and avoidance of the accumulation of photosensitizing tetrapyrrole metabolites.

Among the thiol-reducing systems that control the redox state of proteins in plastids is the ferredoxin-thioredoxin-reductase, which catalyses the transfer of electrons from the photosynthetic electron transport chain to target proteins to reduce their disulfide bridges. In the course of our investigation of posttranslational regulation in tetrapyrrole biosynthesis, we contributed to the thiol-based control of the CHLI subunit of Mg chelatase *in vitro* and *in vivo* studies. Supply of thioredoxin stimulates ATPase activity of CHLI and Mg chelation of MgCh (Luo et al., 2012). The ferredoxin-dependent thioredoxin-mediated redox control of CHLI and MgCh argues for the photosynthesis-dependent allocation of tetrapyrrole metabolites into the Mg branch (Luo et al., 2012). Light-dependent activation of MgCh ensures a high activity in day time and a rapid inactivation of MgCh during darkness (Papenbrock et al., 1999). Using a virus-inducible gene silencing approach in pea plants, the simultaneous inactivation of TRX f and TRX m gene expression caused a reduced MgCh activity, a feedback controlled reduced ALA synthesis and a retrograde signaling-dependent reduction of nuclear gene expression of photosynthesis associated nuclear genes (PhANGs) (Luo et al., 2012). The observed functional redundancy of these two types of thioredoxins in terms of their capacity to reduce thiol groups of the same target protein most likely explains the observed lack of phenotypical effects when the thioredoxin-encoding genes were separately inactivated (Luo et al., 2012).

NTRC-controlled stability and activity of enzymes in tetrapyrrole biosynthesis

NTRC has been shown to be crucial for reduction of H_2O_2, but also for the redox control of enzymes in the primary metabolism, including tetrapyrrole biosynthesis (s. references in 1.1.2). In *in vitro* enzyme assays of MgPME cyclase, NTRC and 2-CysPrx caused an enhanced synthesis of Pchlide (Stenbaek et al., 2008).

In combination with initial characterization of the *ntrc* mutant (Lepisto et al., 2009) these findings encouraged us to examine the impact of NTRC on the entire metabolic pathway of tetrapyrrole biosynthesis. We reinvestigated the role of NTRC in tetrapyrrole synthesis by analysing the steady-state levels of tetrapyrrole intermediates, protein levels and enzyme activities in chlorophyll biosynthesis and found that the *ntrc* mutant shows reduced MgP methyltransferase (CHLM) and ALA-synthesizing activities. *ntrc* contained reduced levels of glutamyl-tRNA reductase (GluTR), CHLM and protochlorophyllide oxidoreductase (POR) (Figure 2A), although their encoding mRNA levels are wild-type-like. In addition, *ntrc* accumulates Mg protoporphyrin (MgP), the substrate of methyltransferase, rather than MgPME. A bimolecular fluorescence complementation (BiFC) assay revealed that all three enzymes, GluTR, CHLM and POR interact with NTRC in chloroplasts (Richter et al., 2013). GluTR reduces activated glutamate of glutamyl-tRNA to glutamate-1-semialdehyde, which is the first committed and highly regulated step in tetrapyrrole biosynthesis.

CHLM methylates MgP to MgPME in an S-adenosyl methionine-dependent step. POR reduces strictly light-dependent protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (Figure 1).

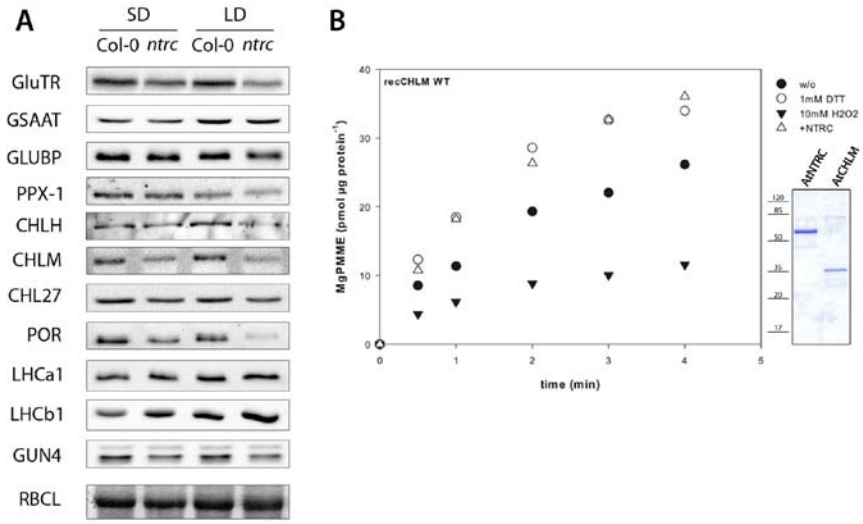


Figure 2: A) Contents of tetrapyrrole synthesis enzymes of 14-d-old wild-type (Col-0) and ntrc seedlings grown under short (SD) or long day (LD) conditions. GluTR, GSAAT, Glu-1-semialdehyde aminotransferase; GLUBP, GluTR-binding protein; PPX-1, protoporphyrinogen IX oxidase; CHLH, CHLM, CHL27, POR, LHCa1 and LHCb1, light-harvesting chlorophyll binding protein a1 and b1;GUN4, RBC, RuBisCo large subunit (Coomassie stain). B) CHLM activity assay with similar amounts of wild-type enzyme under reducing (1mM DTT) and oxidizing conditions, DTT is replaced by NTRC.

Recombinant CHLM activity requires substitution of DTT and is redox-dependently enhanced by NTRC, while oxidizing conditions lower the activity of CHLM (Figure 2B). The importance of the reduced status of CHLM and GluTR was shown in non-reducing SDS-PAGE. In the *ntrc* mutant, CHLM was found to be in a partial oxidized state and its CHLM activity and stability are compromised, while the *Arabidopsis* wild-type plants contain the fully reduced CHLM protein (Richter et al., 2013). In conclusion, it was proposed that reduced stability and enzyme activity of at least GluTR, CHLM and POR are associated with NTRC deficiency.

Objectives

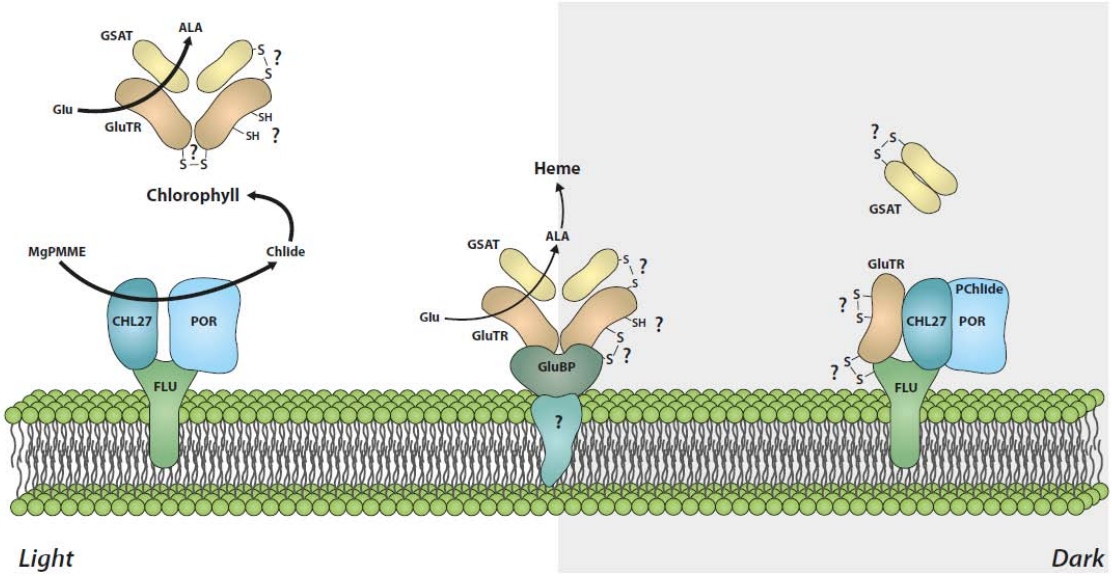
Almost all key enzymes in central metabolic pathways of photosynthetic organisms were proposed to be controlled by one of the thiol-based systems. NTRC, thioredoxin and related proteins perform these thiol/disulfide exchange reactions. This posttranslational control applies also to tetrapyrrole biosynthesis to fine-tune enzyme activities in response to environmental and endogenous cues.

The main objective of this project is to identify and validate the thiol-based redox switches of the enzymes that are controlled by NTRC and to elucidate the physiological consequence of the posttranslational modifications of these NTRC-target enzymes for tetrapyrrole biosynthesis and in consequence, the balanced supply of the end-products, chlorophyll and heme, in the balanced quantities.

The objectives are grouped into five subgoals.

1. Confirmation of NTRC target proteins in tetrapyrrole biosynthesis.
2. S-thiolation of CHLM and the physiological, biochemical and structural impact of NTRC-catalysed-thiol switch of CHLM.

3. S-thiolation of *in vitro* GluTR activity and analysis of modulated *in planta* activity of (Cys→Ser) mutants of GluTR.
4. NTRC-catalysed-thiol-based reactions of the GluTR: Studies on GluTR interaction with adjacent enzymes and regulators, and analysis of the light-dark-dependent alteration in GluTR activity and its accumulation in protein complexes.
5. Redox control of NTRC affecting other proteins of tetrapyrrole biosynthesis.



Model of the redox-dependent organization of the ALA synthesising enzyme complexes. (From Richter and Grimm, Front Plant Sci. 2013 Sep 20;4:371. eCollection 2013

In light, the tetrapyrrole biosynthetic pathway is actively channeling substrates from GluTR to GSAT, which ensures high activity of the ALA synthesising enzymes and allocation of sufficient amounts of the metabolites for the synthesis of different end products. In darkness, GluTR interacts with FLU, which is assembled with CHL27 and POR. This protein complex represses ALA synthesis. It is assumed that the reduction of target cysteine residues in intramolecular disulfide bonds of GluTR is a pre-condition for the formation of a heterotetrameric $GluTR_2$-$GSAT_2$ complex or for high activity of ALA synthesising enzymes in light. Oxidation of cysteine residues or formation of intra-/intermolecular disulfide bonds in darkness could additionally participate in the deactivation of GluTR (e.g. by stabilising the interaction with FLU), and thus mediating the dark-repression of ALA synthesis. Spatial separation of GluTR for heme synthesis by the GluTR-binding protein (GluBP) ensures ALA synthesis for the heme-synthesising branch, although the bulk of ALA

Researchers, who are involved in the proposed project

- Prof. Dr. Ulrich Brandt, U. Nijmegen: mass spectrometry on NTRC-interactomes in tetrapyrrole biosynthesis
- Prof. Dr. Karl-Josef Dietz, U. Bielefeld: 2CysPrx deficiency and chlorophyll biosynthesis
- Prof. Dr. Francisco Javier Cejudo, U. de Sevilla, Spain – CSIC, Sevilla, Spain
- Prof. Dr. Hongbin Wang, Sun Yat-Sen University, Guangzhou, China

Scientific equipment

At the Institute of Biology and in the Department of Plant Physiology all equipment and instrumentation for plant growth and molecular genetics, biochemical and physiological work is available and accessible: A greenhouse (S1), growth cabinets for *Arabidopsis*, radionuclide laboratory, HPLC equipped with an diode array and fluorescence detector, FPLC, microtiter plate reader (with fluorescence detection), uv/vis photometer, PAM-2000 fluorometer, Licor

photosynthesis device, fluorescence microscope, ultracentrifuges and high speed centrifuges including rotors, clean benches, incubators for growth of genetically modified bacteria and yeast, PCR machines, confocal laser scanning microscope.

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