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Review

Chloroplast-targeted ferredoxin-NADP$^+$ oxidoreductase (FNR): Structure, function and location ☆

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ABSTRACT

Ferredoxin-NADP$^+$ oxidoreductase (FNR) is a ubiquitous flavin adenine dinucleotide (FAD)-binding enzyme encoded by a small nuclear gene family in higher plants. The chloroplast targeted FNR isoforms are known to be responsible for the final step of linear electron flow transferring electrons from ferredoxin to NADP$^+$, while the putative role of FNR in cyclic electron transfer has been under discussion for decades. FNR has been found from three distinct chloroplast compartments (i) at the thylakoid membrane, (ii) in the soluble stroma, and (iii) at chloroplast inner envelope. Recent *in vivo* studies have indicated that besides the membrane-bound FNR, also the soluble FNR is photosynthetically active. Two chloroplast proteins, Tic62 and TROL, were recently identified and shown to form high molecular weight protein complexes with FNR at the thylakoid membrane, and thus seem to act as the long-sought molecular anchors of FNR to the thylakoid membrane. Tic62–FNR complexes are not directly involved in photosynthetic reactions, but Tic62 protects FNR from inactivation during the dark periods. TROL–FNR complexes, however, have an impact on the photosynthetic performance of the plants. This article is part of a Special Issue entitled: Regulation of Electron Transport in Chloroplasts.

1. Introduction

Ferredoxin-NADP$^+$ oxidoreductase (FNR; EC 1.18.1.2) is a ubiquitous enzyme, which has been identified in various organisms including heterotrophic and phototrophic bacteria, in mitochondria and plastids of higher plants and algae, as well as apicoplasts of some intracellular parasites (see [1] for a review). FNR harbors one molecule of noncovalently bound FAD as a prosthetic group [2–5], and it catalyzes reversible electron transfer between ferredoxin (Fd) (or flavodoxin) and NAD(P)H. The most extensively studied reaction catalyzed by FNR is the last step of the linear electron transfer chain in chloroplasts (Fig. 1A). There, FNR mediates the transfer of electrons from reduced Fd to NADP$^+$ for production of NADPH according to the reaction 2 $Fd_{red} + NADP^+ + H^+ \rightarrow 2\ Fd_{ox} + NADPH$. The FAD cofactor of FNR functions as an one-to-two electron switch by reduction of FAD to a semiquinone form FADH$^\cdot$, followed by another round of reduction to FADH$^-$, and hydride transfer from FADH$^-$ to NADP$^+$. Ordered formation of a ternary complex, e.g. binding of NADP$^+$ first and then Fd to FNR is considered to be central for the catalytic process [6–9]. NADPH, in turn, is utilized mainly for CO_2 fixation in the Calvin–Benson cycle. In addition to the chloroplast FNRs, FNR proteins can also be found in non-photosynthetic plastids of higher plants [10]. The non-photosynthetic FNR isoforms that function in nitrogen metabolism [11] are, however, beyond the scope of this review.

2. Structure of FNR

Chloroplast FNR proteins are hydrophilic proteins with a molecular weight of approximately 35 kDa. Sequence similarity of FNRs from various plant species varies from 40% to 97% (see [4]), and especially regions involved in FAD and NADP$^+$ binding share high degree of identity. The three dimensional structure of chloroplast FNR has been described in many species, at the best resolution of 1.7 Å [8,9,12–18]. The topology of all chloroplast FNRs is highly conserved, as the FNR protein in all studied species is composed of two distinct domains connected by a loop [8], which shows the biggest variance between the species. The N-terminal domain (ca. 150 amino acids) is involved in FAD binding, while the C-terminal domain (ca. 150 amino acids) is mainly responsible for binding of NADP$^+$ [12] (Fig. 2). FAD-binding domain is made up of a β-barrel structure built of six antiparallel β-strands and capped by an α-helix and a long loop, while the NADP$^+$-binding domain consists of a central five-stranded parallel β-sheet surrounded by six α-helices. The NADP$^+$-binding domain can be further divided into two subdomains [19]. The C-terminal subdomain shows dynamic conformation at stromal daytime pH, allowing increased binding of NADP$^+$ and thereby effective functional control for photosynthesis according to ambient illumination [19].

Abbreviations: Cyt, Cytochrome; Fd, ferredoxin; FQR, ferredoxin-plastoquinone oxidoreductase; FNR, ferredoxin-NADP$^+$ oxidoreductase; FAD, flavin adenine dinucleotide; LHC, light harvesting complex; NDH, NAD(P)H dehydrogenase; PS, Photosystem; PAGE, polyacryl amide gel electrophoresis; Tic, Translocon at the inner envelope of chloroplast

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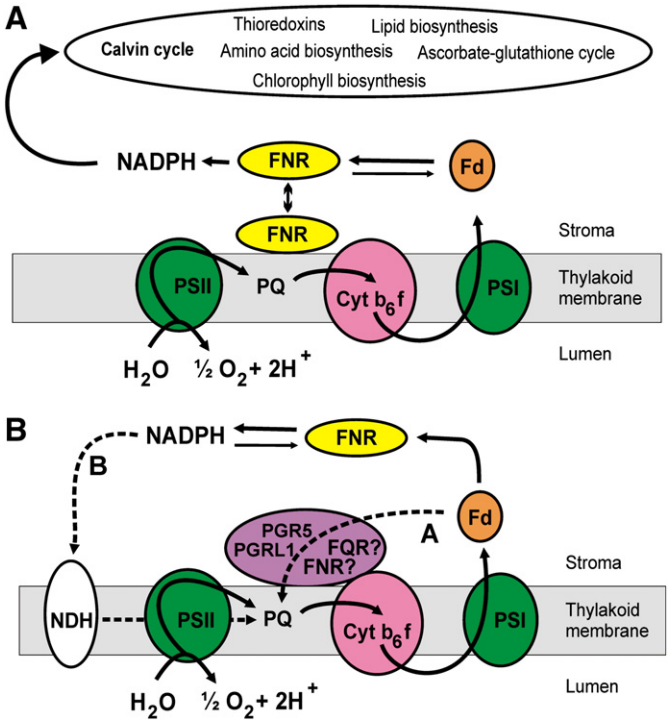

Fig. 1. FNR functions in the crossing of various electron transfer pathways. A. Linear electron transfer from water via PSII, plastoquinone (PQ) pool, Cyt b_6f complex, PSI, Fd and FNR to $NADP^+$ is presented as solid arrows. FNR is present both as soluble and thylakoid-bound forms. Reducing power may be used either for carbon assimilation (Calvin cycle), amino acid, lipid and chlorophyll biosynthesis or reduction of stromal redox-active components. B. Possible routes for cyclic electron transfer are shown as dotted arrows. In Fd-dependent pathway, electrons are transferred from Fd to Cyt b_6f complex via a partly uncharacterized route involving PGR5 and PGRL1 proteins and possibly FNR as well as hypothetical FQR. Alternatively, electrons may be transferred from Fd to PQ pool via the NDH-dependent pathway, which functions in two steps. Firstly, electrons are transferred from NADPH to NDH-1 complex, and secondly the PQ pool is reduced by the NDH-1 complex.

Fd is bound to the large, shallow cleft between the two domains [20] by interaction of basic residues of FNR and acidic residues of Fd, as well as by van der Waals forces, hydrophobic packing interactions and hydrogen bonding (see [21] for a review) (Fig. 2). The amino acids essential for the formation and activity of the Fd-FNR complex have been identified in detail, especially in cyanobacteria, using continuum electrostatic computations [22] and a wide set of site-directed mutants of both FNR and Fd [17,23–30]. Nuclear magnetic resonance and mutagenesis studies have further revealed that the flexible N-terminus of FNR is also involved in the interaction with Fd [31].

3. Expression of the *FNR* genes and import of the pre-protein to the chloroplast

3.1. FNR gene family

In higher plants, chloroplast-targeted FNR is encoded by a small nuclear gene family with one to three *FNR* genes sharing approximately 80% homology with each other [10,32–36]. All higher plants studied to date contain two different types of FNR, more acidic and more basic ones [33]. The chloroplast FNR proteins seem to be at least partly redundant, but they also possess unique properties, which are probably required for adjustment of chloroplast metabolism according to changes in the ambient environment. Properties of the well studied *FNR* gene families in the (i) dicot C_3 plant *Arabidopsis thaliana* (thale cress), (ii) monocot C3 plant *Triticum aestivum* (wheat), and (iii) monocot C_4 plant *Zea mays* (maize) are described below. The nomenclature and classification of the FNR isoforms in Arabidopsis, wheat and maize are presented in Table 1.

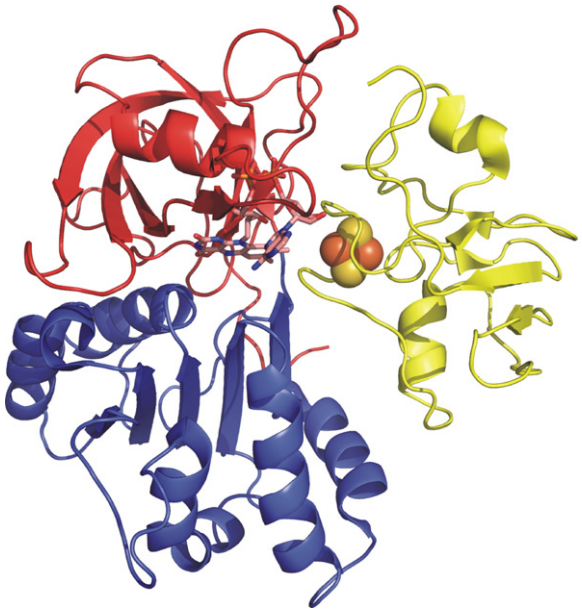

Fig. 2. Structure of FNR in complex with Fd [15]. The N-terminal domain, involved in FAD binding, is shown in red, and the C-terminal domain harboring $NADP^+$ is shown in blue. FAD-binding domain is made up of a β-barrel structure built of six antiparallel β-strands and capped by an α-helix and a long loop, while the $NADP^+$-binding domain consists of a central five-stranded parallel β-sheet surrounded by six α-helices. Fd (shown in yellow) is bound to the large, shallow cleft between the two domains. The FAD cofactor is shown as sticks and the ironsulfur cluster of Fd as spheres. The figure was prepared with Pymol (http://www.pymol.org). *(Courtesy of Dr. Tiina A. Salminen, Åbo Akademi University, Finland).*

3.1.1. A. thaliana

In Arabidopsis two genes, At5g66190 and At1g20020, encode the two distinct ~32 kDa leaf isoforms FNR1 and FNR2, respectively [33,37]. Sixty-eight percent of total chloroplast *FNR* transcripts is composed of *FNR1* mRNA, whereas *FNR2* transcripts represent only one third [38]. Both genes are predominantly expressed in the rosette leaves, whereas only minor amount of mRNA could be detected in the stems, flowers and siliques [33]. Chloroplast *FNR* transcripts or FNR proteins could not be detected in the root tissue [33]. Inactivation of one chloroplast *FNR* isoform did not result in upregulation of the expression of the other, neither at the level of transcription nor translation [37,38]. Mature FNR1 has a more acidic pI (5.54) than FNR2 (6.19) [33], which can be exploited to separate the isoforms in native polyacrylamide gel electrophoresis (PAGE) [38,39]. The presence of both chloroplast FNR proteins as two distinct spots in 2D-PAGE indicates that the FNR proteins are post-translationally modified [37], possibly by N-terminal processing and/or phosphorylation [40,41]. Inactivation of either *FNR* gene resulted in general

Table 1
Classification of the FNR isoforms in *A. thaliana*, *T. aestivum* and *Z. mays*. Gene name denotes the name of each isoform given in defined reference. Classification of the FNR isoforms in more basic (pFNRI) and more acidic (pFNRII) forms according to [36].

Organism	Gene ID	Gene name	References	pI	Classification
A. thaliana	TAIR ID: At5g66190	FNR1	[33]	5.54	pFNRII
	TAIR ID: At1g20020	FNR2		6.19	pFNRI
T. aestivum	GenBank ID: CAD30025	FNRI	[36]	5.9–6.2	pFNRI
		FNR-B	[35]		
	GenBank ID: CAD30024	FNRII	[36]	5.3–5.5	pFNRII
		FNR-A	[35]		
Z. mays	GenBank ID: AB035644	FNR1	[34]	6.76	pFNRI
	GenBabk ID: AB035645	FNR2		5.50	pFNRII
	GenBank ID: ACF85815	FNR3		6.1	pFNRI

down-regulation of the photosynthetic machinery, but neither of the isoforms showed any specific function in linear or cyclic electron flow of photosynthesis, or other alternative electron transfer reactions [37,38,42]. Growth of the *fnr* knock-out plants under adverse conditions (e.g. low temperature), however, revealed a unique role for FNR2 in redistribution of electrons to various redox reactions [38]. Moreover, the growth of Arabidopsis on different nitrogen regimes induces differential expression of the two chloroplast *FNR* genes (see Section 3.2; [33]).

3.1.2. T. aestivum

The proteome of wheat has revealed four distinct leaf FNR isoforms based on isoelectric focusing and SDS-PAGE. These isoforms can be divided into two groups, FNRI and FNRII. Both groups contain two proteins, which differ from each other by truncation of the N-terminus by two or three amino acids [35,36]. Moreover, recently four more FNR spots were detected after 2D-PAGE, and these spots match the isoelectric positions predicted to arise with protein phosphorylation [40]. The alternative processing and (putative) phosphorylation of the FNR does not markedly change the electrophoretic mobility of FNR isoforms, but has an effect on the pI of the FNR isoform. Differences in the N-terminus of the wheat FNR isoforms seem to result in changes of FNR activity, subchloroplast localization as well as affinity of FNR to different Fd isoforms [36,40].

3.1.3. Z. mays

Maize is unique among the higher plants, since the genome of maize codes for three distinct leaf FNR genes. These genes share 83–92% homology with each other, and are present in the leaves at approximately equivalent concentration [34]. FNR1 is found at the thylakoid membrane, while FNR3 is an exclusively soluble enzyme. FNR2, in turn, has a dual location [34]. The activity of FNR2 is similar to FNR3, and higher than that of the FNR1, and the mode of interaction between Fd(s) and the FNR isoforms is dependent on both pH and redox status of the chloroplast [34].

3.2. Regulation of gene expression

Although transcription of the *FNR* genes is not under circadian regulation, light controls the expression of the *FNR* genes via the calcium and cGMP-dependent phytochrome signal transduction pathway [43–47]. Light quality and inhibitors of photosynthetic electron transfer (DCMU and DBMIB), however, do not have any effects on the transcriptional activity of the spinach *FNR* gene [48], while high level of nitrate induces the gene(s) encoding the more basic FNR isoform [33,36]. Expression of the gene(s) encoding the more acidic FNR isoform(s), however, does not respond to changes in nitrogen supply [33,36].

FNR is synthesized on cytosolic polysomes as a precursor containing an amino-terminal transit peptide, which is responsible for targeting the protein to the chloroplasts [49]. The concerted action of chloroplast import machinery composed of Toc (Translocon at the outer envelope of chloroplast) and Tic (Translocon at the inner envelope of chloroplast) guides the vast majority of chloroplast targeted preproteins into the organelle. The Tic complex includes seven subunits, and intriguingly one of them, the Tic62 protein, has been found to interact with FNR [50–53]. The N-terminus of Tic62 binds pyridine nucleotides, while the stroma exposed C-terminus contains repetitive, highly conserved FNR-binding domains [51]. Database searches have verified the presence of the FNR-binding domains only in the Tic62 protein of vascular plants [52]. The function of FNR in the Tic complex has been suggested to link redox regulation to chloroplast protein import. Indeed, *in vitro* experiments with compounds interfering either with NAD binding or NAD(P)/NAD(P)H ratio (deamino-NAD and ruthenium hexamine trichloride, respectively) modulate the import characteristics of the leaf FNR isoforms: FNR1 is translocated preferentially at high NAD(P)/NAD(P)H ratio, while the translocation of FNR2 is not influenced by the redox status [51]. Moreover, distinct isoforms of maize Fd and FNR have been shown to possess unique chloroplast import properties, e.g. import of pre-FdI to chloroplast stroma is independent on illumination, while pre-FdIII and preFNR were efficiently targeted into stroma only in darkness [54]. These results imply that the diurnal changes in the chloroplast redox poise may control import characteristics of the organelle. It was recently shown that Tic62 shuttles between the soluble stroma and the chloroplast membranes, and that oxidation of stroma results in stronger association of Tic62 to the membrane fraction [50]. Importantly, FNR shows similar shuttling behavior, and the Tic62–FNR interaction is likewise dependent on chloroplast redox state [50]. Therefore, the control of chloroplast protein import seems to be an important step in the regulation of gene expression, and it is conceivable that FNR plays a role in this process as a chloroplast redox sensor [51].

The successful chloroplast import of FNR is followed by cleavage of the transit peptide by a stromal protease [55]. Thereafter FAD incorporation, required for maintenance of the native structure, takes place. Binding of FAD is also a prerequisite for membrane binding of FNR [56]. *In vitro* studies have shown that the newly imported FNR interacts transiently with stromal Hsp70 (heat shock protein of 70 kDa), and thereafter with Cpn60 (chaperonin of 60 kDa) [57], which probably assist in the proper folding of FNR.

Finally, regulation of the enzyme activity has been suggested to take place via binding of FNR to the thylakoid membrane. Although soluble and membrane-bound FNR form a complex with Fd with the same dissociation constant, the rate constant of $NADP^+$ photoreduction has been shown to be much higher in the membrane bound than in the soluble complex *in vitro* [58]. Further *in vitro* experiments have suggested that association of FNR via connectein (see Section 5) is indispensable for the $NADP^+$ photoreducing activity [59]. However, since the Arabidopsis *fnr1* knock out mutant does not contain any membrane-bound FNR and still the plants possess more or less normal photosynthetic performance, it may be concluded that *in planta* the soluble FNR is photosynthetically competent [37], and thus the solubility of FNR as such is not a crucial determinant of enzyme activity.

4. FNR exists in three distinct chloroplast pools

It has been long known that FNR exists as a soluble pool in chloroplast stroma, and either loosely or tightly bound to the thylakoid membrane [60–62]. Recently, FNR has been found to bind also to the inner envelope membrane of the chloroplast via Tic62, which is a subunit of chloroplast protein import machinery [50–52].

In Arabidopsis, chloroplast FNR1 has been shown to be more abundant in the membrane fraction [33], especially at the stroma thylakoids [53], whereas FNR2 accumulates in higher amounts in the soluble stroma [33]. Indeed, FNR1 serves as a membrane anchor to FNR2, since upon inactivation of *FNR1* all the chloroplast FNR (FNR2) exists as a soluble protein [37]. Therefore, it seems plausible that FNR *in vivo* exists as a dimer. This possibility has been supported by a number of studies, e.g. structural analyses [15,37,63,64] and various biochemical studies, which have shown appearance of a high molecular weight form of FNR [65–71]. Recently, also formation of large (~330 kDa) FNR oligomers, devoid of other proteins, has been documented upon low ionic strength and neutral pH [35].

5. Location of FNR in chloroplasts

Despite intense study focusing on the location and binding mechanisms of FNR at the thylakoid membrane, the field is still controversial. Early studies have suggested that FNR is bound to the thylakoid membrane via specific FNR binding proteins (base protein and/or connectein). Various studies have shown interaction of FNR

with the photosynthetic protein complexes Cytochrome (Cyt) b_6f, Photosystem (PS) I or NAD(P)H dehydrogenase (NDH) complex, but also interaction with glyceraldehyde-3-phosphate dehydrogenase, or direct membrane attachment have been suggested. Recently, two novel proteins Tic62 and TROL have been identified and shown to bind FNR. The studies introducing the potential thylakoid binding sites are described and discussed below.

5.1. Base protein and connectein

Early studies suggested that FNR was bound to the thylakoid membrane via a specific "binding" or "base" protein. The binding protein was implied to be 16.5–17.5 kDa protein, which apparently mediates membrane attachment of FNR [72,73] as a trimer [74]. The binding protein is located on the stromal side of the thylakoid membrane [72,75], which seems to be contradictory with the fact that the binding protein shares high similarity with the 16.5 kDa PsbQ(like) protein of oxygen evolving complex, which is localized on the lumenal side of the thylakoid membrane [75,76]. Nevertheless, it is an intriguing detail that occasionally FNR seems to co-purify with the oxygen evolving complex proteins [35]. Also a heat stable "connectein" protein of 10 kDa has been suggested to bind two molecules of FNR, and to be involved in membrane binding [77,78]. Although FNR may bind to the thylakoid membrane even in the absence of connectein, it has been suggested that connectein may be required for the guidance of FNR to the correct position at the thylakoid membrane in order to attain full $NADP^+$ photoreducing activity [59]. Since connectein can not be stained either with Coomassie brilliant blue or silver stain, its molecular identity has unfortunately remained obscure [71,77].

5.2. Photosynthetic protein complexes

FNR has been shown to co-purify with the Cyt b_6f complex [79,80]. Although the presence of bound FNR in the spinach Cyt b_6f complex does not affect the activity of the complex, NADPH is able to reduce Cyt b_6 only in the presence of Fd and FNR [80,81]. Measuring the activity of plastoquinone reduction *in vitro* has implied that FNR indeed may provide electrons for plastoquinone reduction, either via NDH complex (chlororespiration) or Cyt b_6f complex (FQR-dependent cyclic electron transfer; see Section 6.1) [82]. However, the ultimate significance of FNR-Cyt b_6f complex formation has remained contradictory [83]. Besides Cyt b_6f complex, FNR has been shown to interact with the PsaE subunit of PSI [70], but also opposing results about interaction of FNR and PsaE exist [84–86].

The chloroplast NDH complex is composed of several chloroplast and nuclear encoded subunits (see [87] for a review), and it has been shown to be involved in chlororespiration [88] and cyclic electron transfer around PSI [89,90]. In contrast to *Escherichia coli*, neither the catalytic subunits of the NDH complex nor the genes encoding them have yet been found in cyanobacteria or higher plants, which has raised conjectures about possible involvement of FNR in working as a diaphorase (i.e. oxidizing NADPH) in the vicinity of the NDH complex. Indeed, FNR has been shown to be associated with the NDH complex in potato and barley [91,92], although some studies have not evidenced such interaction [53,93–95].

5.3. Glyceraldehyde-3-phosphate dehydrogenase and direct attachment to membrane

Recently, FNR has been shown to co-localize with a Calvin cycle enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD), both in the stroma and at the thylakoid membrane [96]. This might provide high local concentration of NADPH for carbon fixation, with only spill-over of reducing power remaining for other stromal synthases, such as malate and glutamate dehydrogenases. Another mode of interaction has been shown to be binding of the FNR protein to the model membranes without mediation of any additional proteins [97].

5.4. Tic62

Proteomics studies have identified Tic62 in the chloroplast envelope, stroma and thylakoid fraction [53,98]. Intriguingly, Tic62 at the thylakoid membrane was found in several high molecular mass protein complexes (250–500 kDa), and it was shown to be tightly associated with both chloroplast FNR isoforms ([53]; Fig. 3). Tic62 binds on the FNR surface opposite to the catalytic site, and thus does not compete with the binding of Fd or $NADP^+$ [53]. The FNR–Tic62 complexes do not comigrate with PSI, Cyt b_6f complex or NDH-complex in the Blue Native gels [53]. When Tic62 was knocked out, formation of high molecular weight FNR protein complexes was hindered, while some free FNR still was detected at the thylakoids of *tic62* plants. The amount of FNR in the soluble pool, however, remained more or less constant. *Vice versa*, if either one of the chloroplast targeted FNR isoforms was missing, the membrane binding of the Tic62 protein was prevented. Since no changes in the *FNR* gene expression or in the FNR pre-protein import could be detected in the *tic62* plants, reduction of FNR level most probably resulted from differences in the turnover of FNR isoforms inside the chloroplasts [53].

What might be the function of the Tic62–FNR complexes? One obvious answer would be photosynthetic electron transfer, especially because membrane-bound FNR has been suggested to be the photosynthetically active form of the protein [58]. However, the lack of Tic62 and consequently the lack of Tic62–FNR complexes did not have any effects on the plant phenotype or photosynthetic properties, neither linear nor cyclic electron transfer [53], implying that the Tic62–FNR complexes serve for some other purpose(s) than photosynthesis. Although the Tic62–FNR complexes were not involved in photosynthesis, they still responded readily to light. The membrane-bound Tic62–FNR protein complexes were most abundant in the dark, while increase in light intensity resulted in the disassembly of the complex (Fig. 3). Similarly, *in vitro* alkalization of isolated thylakoids dissociated FNR and Tic62 [53]. It is also worth noting that the interaction of Tic62 with FNR stabilizes the

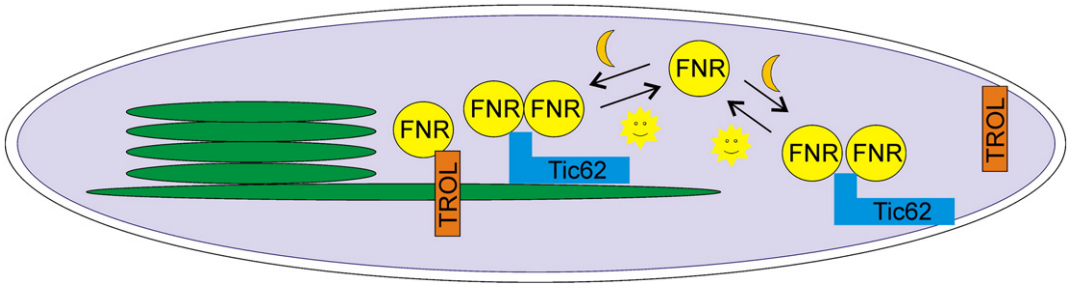

Fig. 3. FNR is located in three distinct chloroplast pools. In chloroplasts, FNR can be found in soluble stroma, attached to the stroma thylakoids as well as inner envelope membrane. In darkness, FNR forms large protein complexes at the thylakoids together with Tic62 and TROL. Similarly, Tic62 and presumably TROL bind FNR at the envelope. FNR is released from the membranes upon illumination.

activity of the FNR protein [53] and that FNR activity is lower in acidic than basic environment [19]. These results indicate that Tic62 acts as a chaperone for FNR, and protects the flavoenzyme from inactivation and degradation during the photosynthetically inactive periods, e.g. in darkness [53].

5.5. TROL (thylakoid rhodanase-like protein)

TROL is a nuclear encoded protein of 66 kDa, which contains two transmembrane helices and a centrally positioned (inactive) rhodanase domain [99]. As a transmembrane protein, TROL is firmly attached to the thylakoid membrane and can not be extracted from the membrane by high salt, urea or high pH treatments [99]. TROL, as FNR, is mainly located at the stroma thylakoids, and it can be also found embedded in the chloroplast inner envelope membrane in the non-processed form (70 kDa) (Fig. 3). Similarly as in Tic62, the C-terminus of TROL contains a conserved domain, which is capable of establishing a high-affinity interaction with FNR. Moreover, TROL forms a high molecular mass thylakoid protein complex together with FNR. The size of the complex is, however, smaller (190 kDa) than the Tic62–FNR complexes, which is in line with only one FNR binding motif found in the amino acid sequence of Arabidopsis TROL protein, as compared to four of such motifs present in the sequence of Arabidopsis Tic62. Although *in vitro* experiments indicate that the interaction between TROL and FNR is stronger than the interaction between FNR and Tic62 [99], the exposure of plants to high light intensity results in faster dissociation of FNR from the TROL–FNR complexes than from the Tic62–FNR complexes [53].

In contrast to *tic62* plants, *trol* knock out mutants have a clear photosynthetic phenotype [99]. The appearance of the *trol* plants is slightly smaller than the WT, but with no distinct differences in pigment composition. However, the mutant chloroplasts are small, irregular in morphology and show deteriorated thylakoid structure. The abnormalities in chloroplast structure are reflected on the function with marked differences in electron transfer rate under high light intensity (500 to 800 μmol photons m^{-2} s^{-1}). Since nonphotochemical quenching increases and variable chlorophyll fluorescence decreases in the *trol* leaves upon increasing illumination, it seems that the absence of TROL results in increased ability to dissipate excess absorbed energy.

6. Function of FNR

6.1. FNR and cyclic electron flow around PSI

Besides the apparent role of FNR in linear electron transfer reactions of photosynthesis, many studies have suggested the involvement of FNR also in cyclic electron flow around PSI (Fig. 1B). In cyclic electron flow, electrons are transferred from PSI to Cyt b_6f complex via Fd, with a concomitant formation of proton gradient (Fig. 1B). Thus, cyclic electron transfer produces ATP without accumulation of NADPH. It is generally accepted that cyclic electron transfer supplies the ATP needed for driving the CO_2 concentrating mechanism in the C_4 plants [100]. Description of the Arabidopsis *pgr5 crr2-2* mutant with impaired capacity for cyclic electron flow, leading to severe defects in photosynthesis and development of the plant, has recently evidenced the significance of cyclic electron flow also in C_3 plants [101]. At least two distinct cyclic electron transfer pathways have been described:

1) In the Fd- or ferredoxin-plastoquinone oxidoreductase (FQR)-dependent pathway electrons are funnelled from Fd to Cyt b_6f complex (possibly via the plastoquinone pool) and back to PSI using the so far uncharacterized FQR enzyme (see [102,103]). Antimycin sensitivity of the Fd-dependent pathway suggests that the stromal plastoquinone binding pocket Q_i of the Cyt b_6f complex [104,105] is involved in the Fd-dependent cyclic electron transfer route. To date the only characterized components of this route are the regulatory proteins PGR5 [101,106,107] and PGRL1 [86]. In Arabidopsis, PGRL1 has been shown to interact with FNR, Fd, PsaD and Cyt b_6 [86], which might imply that Cyt b_6f indeed accepts electrons from PSI via the concerted action of PGRL1, PGR5 and FNR. The interaction has been considered to be transient, since it has not been possible to detect any stable thylakoid super-complexes composed of these proteins in higher plants [83,86]. However, recent isolation of thylakoid supercomplexes composed of PSI, light harvesting complex (LHC) I, LHCII, Cyt b_6f, FNR and PGRL1 has provided evidence for the formation of the complex capable of cyclic electron transfer without any unidentified components (e.g. FQR), at least in *Chlamydomonas reinhardtii* [108]. Moreover, also earlier studies showing attachment of FNR to Cyt b_6f complex in higher plants [79,80] have allowed suggestions of FNR to oxidise Fd and feed the electrons to Cyt b_6f complex [109–111]. In line with the interaction studies, electron transfer properties of Arabidopsis *fnr1* and *fnr2* mutants have shown that the both FNR isoforms contribute equally to cyclic electron transfer, and that a specific isoform is not dedicated solely for cyclic, and the other for linear electron transfer [37,38].
2) In the NDH-dependent route, FNR oxidizes Fd for the production of NADPH, which may be subsequently reduced by the thylakoid-bound NDH complex. NDH complex, in turn, provides reducing power for reduction of the plastoquinone pool (Fig. 1B). Attachment of FNR to the NDH complex has implicated that FNR might serve also as the missing catalytic subunit of the NDH complex [91,92,112,113]. However, it is also possible that the NDH complex might be directly reduced by Fd, as PSI and NDH complex have been shown to form a supercomplex [114,115]. Since the concentration of NDH complex in the thylakoid membrane of the C_3 plants is very low, the significance of this antimycin insensitive route of cyclic electron transfer under physiological conditions has remained controversial [116,117]. Nevertheless, the copious accumulation of the NDH complex in the bundle sheath cells of C_4 plants with high ATP requirement suggests that the NDH complex energizes C_4 photosynthesis [102,118].

6.2. FNR and oxidative stress

PSII and PSI in the chloroplasts of higher plants are potential sources of harmful reactive oxygen species (ROS) in plant tissues. In *E. coli*, FNR is involved in quenching of ROS [119,120], and in methyl viologen resistant *Chlamydomonas reinhardtii* strains the steady state level of chloroplast FNR transcripts has been shown to be increased as compared to WT CC-125 [121]. Moreover, expression of plant FNR has been proven to restore the oxidative tolerance of a mutant *E. coli* [122,123]. These results have prompted research on the participation of FNR in oxidative stress responses of higher plants.

Propagation of superoxide with methyl viologen, and subsequent accumulation of H_2O_2 in wheat plants, has revealed that in contrast to bacterial cells, the content of *FNR* mRNA as well as protein in higher plants rather decreases than increases in response to induction of oxidative stress [124]. However, production of ROS results in marked release of FNR from the thylakoid membrane followed by reduction of $NADP^+$ photoreduction capacity, which might aim at maintaining the $NADP^+$/NADPH homeostasis of the stressed plants [124]. Recently, it has been shown that FNR releases from the thylakoids in the plants suffering from drought stress [125], and the FNR containing thylakoid protein complexes disassemble upon high light illumination [53].

If the amount of FNR is artificially reduced by antisense or silencing techniques [38,126], or by interruption of a *FNR* gene by T-DNA [37,127], the plants suffer from chlorosis and reduced photosynthetic activity, which finally results in reduced growth. Although the level of total NADP(H) was not affected in the mutants, the ratio of NADPH/

$NADP^+$ was strongly reduced [126,128]. These mutants are prone to photo-oxidative damage, and suffer from oxidative stress [38,129]. The redox poise of the NADP(H) pool is also likely to regulate photosynthetic electron transfer activity in order to balance production and consumption of reducing equivalents, and thereby to limit production of ROS in the chloroplasts [128]. Over-expression of FNR, however, did not markedly up-regulate the rate of $NADP^+$ photoreduction (20%) or CO_2 assimilation, but showed augmented tolerance to photodamage [130]. Although it seems conceivable that FNR is involved in the responses to oxidative stress, further studies are needed to clarify the ultimate role of FNR in these reactions.

7. Conclusions and future perspectives

Taken together, in the light of recent findings it seems that in darkness FNR is recruited to the thylakoid membranes via the Tic62 and TROL proteins, which contain a specific FNR binding domain. Tic62–FNR complexes are not involved in photosynthetic reactions, while TROL–FNR complexes may have a (indirect) link to photosynthesis. Under photosynthetically active period of the day, FNR-containing protein complexes are disassembled and FNR partly released from the membrane. The soluble enzyme produces NADPH, which is further utilized in carbon assimilation. At chloroplast envelope, FNR may have an additional role in regulating chloroplast protein import.

It will be intriguing to find out whether new interaction partners of FNR will be discovered either at the chloroplast membranes or in the soluble stroma. Moreover, it remains to be elucidated whether the previously described thylakoid binding proteins (connectein and/or base protein) might be, at least transiently, involved in FNR–Tic62 or FNR–TROL complex formation. Further interest, and complexity, to the system is likely to be provided by the (redox dependent) shuttling of different components between the membrane and soluble pools of the chloroplast. It is also possible that upon specific environmental cues FNR might be transiently attached to other thylakoid protein complexes (such as Cyt b_6f) to fulfil the metabolic and photoprotective needs of the chloroplasts. The dynamic nature of the structure and function of various FNR complexes will obviously be a focus of intense research also in the future.

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