Oxidative Stress Causes Ferredoxin-NADP\(^+\) Reductase Solubilization from the Thylakoid Membranes in Methyl Viologen-Treated Plants\(^1\)

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The flavoenzyme ferredoxin-NADP\(^+\) reductase (FNR) is a member of the cellular defense barrier against oxidative damage in *Escherichia coli*. We evaluated the responses of chloroplast FNR to methyl viologen, a superoxide radical propagator, in wheat (*Triticum aestivum* L.) plants and chloroplasts. Treatments with the herbicide showed little effect on the levels of FNR protein or transcripts, indicating that expression of this reductase is not up-regulated by oxidants in plants. Viologens and peroxides caused solubilization of active FNR from the thylakoids into the stroma, converting the enzyme from a membrane-bound NADPH producer to a soluble NADPH consumer. This response appeared specific for FNR, since other thylakoid proteins were unaffected by the treatments. The reductase-binding protein was released together with FNR, suggesting that it might be the target of oxidative modifications. Stromal accumulation of a functional NADPH reductase in response to oxidative stress is formally analogous to the induction of FNR synthesis observed in *E. coli* under similar conditions. FNR solubilization may be playing a crucial role in maintaining the NADPH/NADP\(^+\) homeostasis of the stressed plastid. The unchecked accumulation of NADPH might otherwise increase the risks of oxidative damage through a rise in the Mehler reaction rates and/or the production of hydroxyl radicals.

Chloroplasts are potentially the major source of toxic oxygen derivatives in plant tissues (Foyer et al., 1994; Allen, 1995). They generate singlet oxygen under high doses of illumination through the interaction of triplet-state chlorophyll with ground-state oxygen (Asada and Takahashi, 1987), and are also able to produce superoxide anion radicals (O\(_2^-\)) by univalent oxygen reduction at the level of PSI in the Mehler reaction (Foyer, 1996). H\(_2\)O\(_2\) is then synthesized by O\(_2^-\) disproportionation, either spontaneously or through the action of soluble and thylakoid-bound superoxide dismutases (Asada, 1994). Superoxide and peroxide molecules that escape detoxification may then participate in a metal-catalyzed Fenton-type pathway to generate highly toxic hydroxyl (OH\(^-\)) radicals (Cadenas, 1989).

Accumulation of active oxygen species is an unavoidable consequence of photosynthesis, even under the most favorable conditions. To cope with their toxicity, aerobic organisms have developed a highly efficient antioxidant defense system, composed of both enzymatic and nonenzymatic constituents. In plants a number of enzymes involved in free radical scavenging are normally induced in response to a variety of oxidative challenges. They include catalases, superoxide dismutases, peroxidases, and reductases (Foyer et al., 1994; Allen, 1995). Recent studies suggest that FNR serves a functional role in the oxidative tolerance to wild-type levels, indicating that a regulatory mechanism of oxidative stress responses analogous to that operating in bacterial systems may exist in these photosynthetic eukaryotes (Kitayama et al., 1995).

FNR isoforms are present in plant chloroplasts and in nonphotosynthetic plastids, participating in the NADP\(^+\) photoreduction and nitrate assimilation pathways (Shin and Arnon, 1965; Bowsher et al., 1993; Morigasaki et al., 1993; Arakaki et al., 1997). On the other hand, the FNR from *Escherichia coli* is specifically induced by superoxide radicals, and its inactivation leads to mutant bacteria that are abnormally sensitive to radical-propagating compounds such as MV (paraquat), a redox-cycling herbicide that mediates O\(_2^-\) generation in situ (Bianchi et al., 1995, and refs. therein). A comparable role for FNR in eukaryotes remains to be demonstrated, although expression of a cloned plant FNR gene in a mutant *E. coli* strain did restore the oxidative tolerance to wild-type levels, indicating that the eukaryotic flavoenzyme behaves as a toxic radical scavenger in the bacterial host (Krapp and Carrillo, 1995). Moreover, MV-resistant strains of *Chlamydomonas reinhardtii* show an increase in the steady-state levels of chloroplast FNR transcripts relative to wild-type cells, suggesting that a regulatory mechanism of oxidative stress responses analogous to that operating in bacterial systems may exist in these photosynthetic eukaryotes (Kitayama et al., 1995).

*Abbreviations: FNR, Fd-NADP\(^+\) reductase; LSU, large subunit of Rubisco; MV, methyl viologen.*

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In the present study we evaluated the response of FNR to MV-induced oxidative damage in whole wheat (Triticum aestivum L.) plants and in isolated chloroplasts. The steady-state levels of FNR transcripts and protein were hardly affected by treatment with the herbicide, except for a moderate degradation of the flavoprotein at high MV concentrations. The most striking feature of the FNR response to MV, however, was the progressive solubilization of the reductase and its putative binding protein from the thylakoid membranes, and the accumulation of active FNR in the stromal fraction. The possible relevance of this finding is discussed in the context of the stressed cell metabolism.

MATERIALS AND METHODS

Wheat (Triticum aestivum L. cv Oasis) plants were grown for 7 to 8 d in a growth chamber under a 16-h light/8-h dark regime, with a light intensity of 200 μmol m⁻² s⁻¹, 75% RH, and day/night temperatures of 24/19°C.

Oxidative Treatments

Chemical stress was imposed by spraying 40 seedlings with 20 mL of a 0.05% (v/v) Tween 20 solution containing different concentrations of MV. Plants were then illuminated (200 μmol m⁻² s⁻¹) for 8 h before sampling.

Chloroplast Isolation and Treatments

Intact wheat chloroplasts were isolated by Percoll gradient centrifugation and resuspended in buffer A (50 mm Hepes-NaOH [pH 8.0], 330 mm sorbitol, 5 mm MgCl₂, 0.5 mm MnCl₂, 1 mm EDTA, and 1 mm DTT) (Desimone et al., 1996). Chloroplast suspensions corresponding to 50 μg chlorophyll mL⁻¹ were incubated with MV for 1 h at 22°C under continuous illumination (600 μmol m⁻² s⁻¹). Plastids were then collected by centrifugation (3 min at 2,000g) and disrupted by osmotic shock in buffer A without sorbitol. The stromal fraction was separated from the membranes by centrifugation (5 min at 10,000g).

Isolation of Soluble and Membrane-Associated Proteins from Whole Plants

Wheat leaves were homogenized in a medium containing Tris-HCl (pH 7.5), 330 mm sorbitol, and 5 mm MgCl₂. Membrane and soluble fractions were separated by centrifugation (10 min at 10,000g). Membranes were resuspended in the homogenization buffer, and protein content was determined in both fractions according to Peterson (1977).

RNA Extraction and Analysis

Total RNA was isolated according to Nagy et al. (1988) and its concentration was determined spectrophotometrically. Ten micrograms of total RNA was subjected to denaturing electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde. Gels were stained with ethidium bromide, transferred to nylon membranes, and hybridized to the probes indicated below, which were 32P-labeled by random priming according to the manufacturer’s instructions (Boehringer Mannheim). After hybridization, filters were washed twice (30 min at 65°C) in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate [pH 7.0]), 0.1% SDS and exposed to XAR-5 film (Kodak). Stained gels and autoradiograms were scanned and analyzed using a CS9000 scanner (Shimadzu, Tokyo, Japan).

A 0.8-kb cDNA fragment encoding LSU from tobacco was obtained by digesting plasmid pTB29 with BamHI (Shinozaki and Sugiyama, 1982). A 1.2-kb cDNA probe encoding pea FNR was isolated by EcoRI/BamHI digestion of plasmid pCV105 (Ceccarelli et al., 1991).

Analytical Procedures

Proteins were fractionated by SDS-PAGE on 12% gels, and were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes. Immunodetection methods were carried out following the ECL protocols (Amersham). Antisera against various chloroplast proteins were raised in rabbits.

Immunoprecipitation Experiments

The FNR activity of chloroplast lysates was estimated by measuring the diaphorase reaction (Serra et al., 1993), before and after FNR immunoprecipitation. Anti-FNR IgG bound to protein A-Sepharose (Ausubel et al., 1987) was incubated for 2 h at 4°C with the stromal fractions, and suspensions were cleared by centrifugation before activity measurements in the supernatants. The amount of nonspecific binding was estimated by incubating the extracts with protein A-Sepharose under the same conditions described above, whereas the efficiency of the precipitation step was determined by immunodepletion of a titrated solution of recombinant pea FNR.

RESULTS

Effect of MV Treatment on Fd-NADP⁺ Reductase Expression and Stability

Experiments in Escherichia coli have shown that FNR expression is induced after a brief challenge of the bacterial cells with MV (Liochev et al., 1994). To investigate whether a similar activation occurs in plants, we sprayed 8-d-old wheat shoots with increasing concentrations of the herbicide. Visible effects were dependent on exposure time and MV concentration. After 8 h of continuous illumination, plants treated with 5 mm MV still looked healthy, but after that time the leaves began to tilt and necrotic spots became evident.

Figure 1A shows the total protein profiles obtained from control and treated plants after fractionation by SDS-PAGE. The main effect of oxidative injury at the protein level was the steady decline of a major band at 55 kD, most likely representing the LSU (Fig. 1A). Accordingly, this effect was also evident in immunoblots probed with Rubisco antisera (Fig. 1C). Both higher- and lower-molecular-mass immunoreactive peptides became appar-
Solubilization of Fd-NADP\(^{+}\) Reductase by Methyl Viologen

Figure 1. Effect of MV on leaf protein (A), FNR (B), and LSU (C) degradation. Wheat shoots were treated with the indicated concentrations of MV during 8 h in the light. Leaf extracts were fractionated by SDS-PAGE and either stained with Coomassie brilliant blue (A), or blotted onto nitrocellulose membranes for immunodetection of FNR (B) and LSU (C). Total leaf protein loaded onto each lane was 40 \(\mu\)g (A and B) or 0.4 \(\mu\)g (C).

Figure 2. Steady-state levels of FNR and LSU mRNAs in MV-treated plants. Blotted RNA (10 \(\mu\)g) isolated from control plants or from plants treated with the indicated concentration of MV was hybridized to \(^{32}\)P-labeled FNR (A) or LSU (B) DNA probes. The amount of rRNA loaded onto each lane was estimated by staining the gels with ethidium bromide (C). Values obtained from the densitometric scanning of each autoradiographic signal were divided by the intensities of the corresponding rRNA bands to correct for loading differences. Means and so values from three experiments are shown in D, expressed as percentages of the FNR or LSU values of untreated seedlings. Closed and open bars indicate FNR and LSU transcript levels, respectively.

Solubilization of Fd-NADP\(^{+}\) Reductase by MV-Induced Oxidative Stress

Soluble and membrane-associated proteins were isolated from MV-treated and control plants, subjected to SDS-PAGE, and analyzed by immunoblotting with FNR antisera. Application of the herbicide led to a substantial release of the flavoprotein into the soluble fraction (Fig. 3A). Solubilization was dependent on MV concentration (Fig. 3A) and exposure time (not shown). Similar results were obtained when the treatment was applied to isolated chloroplasts (Fig. 3B). Rates of NADP\(^{+}\) photoreduction by thylakoids from MV-treated and untreated plastids were 0.41 ± 0.11 and 1.06 ± 0.21 \(\mu\)mol NADPH min\(^{-1}\) mg\(^{-1}\)
Figure 3. Treatment of wheat shoots (A) or chloroplasts (B) with MV solubilizes FNR from thylakoid membranes. A, After spraying the plants with MV as described in “Materials and Methods,” soluble and membrane-associated proteins (10 µg) were fractionated on SDS-PAGE and blotted onto nitrocellulose. FNR was detected by immunoreaction with specific antisera. B, Wheat chloroplasts were isolated from 8-d-old untreated seedlings and incubated at a chlorophyll concentration of 50 µg mL⁻¹ with 0 or 100 µM MV in the light for 1 h at 22°C. Stroma (S) and thylakoid (T) membranes were fractionated by SDS-PAGE, and the presence of FNR in each fraction was determined by immunoblotting. Typical results from four independent experiments are shown.

chlorophyll, respectively, in good agreement with the degree of FNR loss from the membranes.

The levels of MV causing FNR solubilization were lower than those required for degradation (Fig. 1B). Most of the reductase released into the soluble fraction appeared to be functional, as indicated by the increase in FNR activity in extracts from treated plants (Fig. 4). Inhibitors of electron transport prevented FNR solubilization in isolated chloroplasts (Fig. 5A), whereas uncouplers had no effect (Fig. 5B).

Figure 4. FNR-dependent diaphorase activity of soluble fractions of wheat leaves treated with MV. Leaf soluble fractions were obtained for each MV treatment, as described in “Materials and Methods.” Lysates were incubated with protein A-Sepharose or with protein A-Sepharose bound to anti-FNR IgG for 2 h at 4°C. Suspensions were cleared by centrifugation, and diaphorase activity was determined in the supernatants by following potassium ferricyanide reduction (Serra et al., 1993). Values are the means of three replicate experiments. Open bars indicate total diaphorase activity of the lysates, striped bars indicate the activity remaining after immunoprecipitation, and closed bars indicate the FNR-specific diaphorase. Recombinant purified FNR was used as a control of immunoprecipitation. One diaphorase unit is defined as the amount of enzyme capable of catalyzing the reduction of 1 µmol of ferricyanide per min.

Figure 5. Effect of DCMU, H₂O₂, and uncouplers of photophosphorylation on FNR solubilization. Intact chloroplasts were isolated and incubated under the same conditions described in Figure 3, with the indicated additions. Soluble and thylakoid proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with FNR antisera. Typical results from three different experiments are shown. A, DCMU and H₂O₂ concentrations were 100 µM and 10 mM, respectively. B, The concentrations of carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) and the A23187 ionophore were 50 µM. MV concentration was 100 µM.

Treatment of the plastids with H₂O₂ instead of MV produced essentially the same effect (Fig. 5A). The results are consistent with the participation of active oxygen species generated after MV reduction by the photosynthetic electron transport chain.

Chloroplast FNR interacts with the thylakoid membrane through a 17-kD polypeptide (Vallejos et al., 1984). Solubilization of this protein accompanied that of FNR itself in MV-treated plants (Fig. 6A), suggesting that it may be the actual target of oxidative modification. Release of FNR and its binding protein was not caused by extensive disruption of the thylakoid membrane structure, since the status of the soluble fractions of other proteins, such as the peripheral coupling factor 1 of the H⁺-ATP synthetase (Fig. 6B) or the...
luminal electron carrier plastocyanin (not shown), were unaffected by MV treatment. Similar results were obtained when the herbicide was applied to isolated chloroplasts instead of whole plants (data not shown).

DISCUSSION

Experiments described in the present work were designed to provide insights on the eventual participation of FNR in the oxidative stress responses of plants. This hypothesis was formulated after the observation that increased levels of FNR protein and/or transcript in *E. coli* and *C. reinhardtii* were associated with MV resistance (Bianchi et al., 1995; Kitayama et al., 1995; Krapp and Carrillo, 1995). Protein extracts from leaves subjected to MV treatment showed a remarkable degradation of Rubisco at MV concentrations higher than 10 μM (Fig. 1, A and C). This phenomenon was very helpful as an indicator of the degree of oxidative stress suffered by the plants under analysis, since a direct relationship between the extent of Rubisco degradation and the intensity of the stress factor assayed has been established previously (Mehta et al., 1992; Landry and Pell, 1993; Desimone et al., 1996). Under these conditions, however, we failed to observe induction of FNR synthesis, either by measuring the levels of FNR protein (Fig. 1B) or transcripts (Fig. 2A).

Chloroplast FNR is encoded in the nuclear genome, and its expression is induced by light via phytochrome A, cGMP, and calmodulin (Bowler and Chua, 1994). Our results indicate that this FNR gene lacks oxidant-responsive elements, as demonstrated for the analogous flavoprotein of *E. coli* (Liochev et al., 1994) and postulated for the reductase of *C. reinhardtii* (Kitayama et al., 1995). A second root-type FNR has also been reported to occur in chloroplasts, providing a minor contribution to the overall FNR activity of photosynthetic tissues (Wada et al., 1997). This isoform is specifically induced by nitrate (Bowsher et al., 1993; Ritchie et al., 1994; Wada et al., 1997) and should not be recognized by the probes used in this study. The possibility of oxidant-induced expression of this nonphotosynthetic reductase cannot be ruled out by our experiments. Further research will be necessary to elucidate this question.

Although MV failed to induce FNR expression in wheat plants, it did promote solubilization of an active reductase into the stromal fraction (Figs. 3–5). The latter result was somehow surprising, since oxidative treatments are known to stimulate Rubisco association with the chloroplast insoluble fraction before proteolysis of the large subunit (Mehta et al., 1992; Landry and Pell, 1993; Desimone et al., 1996). Oxidants also cause aggregation of thylakoid proteins (Roberts et al., 1991), presumably resulting from an increase in protein hydrophobicity and the formation of covalent cross-linking. We showed that other thylakoid components, both luminal and peripheral, remained attached to the membranes under MV treatment (Fig. 6), indicating that FNR solubilization was a specific phenomenon. The combined effects of inhibitors of electron transport and uncouplers of photophosphorylation (Fig. 5) preclude a direct role of MV or membrane potential in the solubilization process, suggesting that FNR release was related to the accumulation of active oxygen species generated after MV reduction in the Mehler reaction. In agreement with this conclusion, we observed FNR solubilization in chloroplasts treated with H₂O₂ (Fig. 5A). The mechanisms by which these effects might occur are at present unknown, but, interestingly, the FNR-binding protein that links the flavoprotein to the thylakoid membrane (Vallejos et al., 1984) was released together with the reductase upon MV treatment (Fig. 6A), suggesting that the binding protein might be the actual site of oxidative modification.

The precise mechanism by which FNR exerts its protective action against oxidative stress remains obscure, even in *E. coli*, in which the reductase levels are increased up to 20 times after an oxidative challenge with MV (Liochev et al., 1994). The response is modulated by the *E. coli* soxRS regulon, an adaptive regulatory system specifically evolved to cope with superoxide toxicity. The redox-sensitive protein SoxR is activated during superoxide accumulation, promoting the de novo synthesis of the transcriptional activator SoxS, which in turn induces the expression of different target genes, including that encoding FNR (Hidalgo et al., 1997). Members of the regulon operate at various levels and at different stages of the antioxidant response: direct scavenging of toxic radicals (Mn-superoxide dismutase), DNA repair activities (endonuclease IV), and functional replacement of oxidant-sensitive enzymes by insensitive enzymes (fumarase C and aconitase A). Direct involvement of FNR in any of these activities seems unlikely, suggesting that the flavoprotein may be playing a more general role in the metabolic context of the stressed cell by modulating, through an as yet unknown mechanism, its overall redox status.

These considerations of the antioxidant role of FNR in bacterial systems may help in understanding its possible functions in plant cells. As already mentioned, FNR catalyzes the reversible electron transfer between NADP(H) and Fd. In illuminated chloroplast thylakoids, when Fd is photosynthetically reduced at the level of PSI, the reaction is driven toward NADP⁺ reduction, providing the NADPH required for biosynthetic pathways (Shin and Arnon, 1965). Soluble FNR is no longer able to reduce NADP⁺ at high rates, but it becomes an efficient NADPH consumer (Forti and Bracale, 1984). Fd and a number of adventitious electron acceptors, including MV, can be used in this reaction, leading to the so-called diaphorase activity (Shin and Arnon, 1965; Arakaki et al., 1997).

A plausible route by which soluble FNR may contribute to the elimination of toxic radicals is through the NADPH-driven reduction of an abundant scavenger, or its regeneration after detoxification has been accomplished (Fig. 7), as occurs with glutathione and dehydroascorbate reductases in chloroplasts (Asada, 1994; Foyer et al., 1994). Thiols, quinones, and unsaturated isoprenoids are among the scavengers that may interact with FNR in a productive manner, but identification of the eventual compound(s) has not been attempted.

Alternative mechanisms can be envisaged, assuming a more general role for soluble FNR in the stress metabolism
of the plastid. Oxidative conditions in chloroplasts are expected to cause Rubisco degradation (Fig. 1; see also Desimone et al., 1996), as well as oxidative inactivation of other enzymes of the reductive pentose phosphate pathway (Polle, 1996), resulting in an overall decrease of the NADPH demand by the Calvin cycle. A rise in the intraplasmidic NADPH/NADP⁺ ratio might in turn induce oxidative damage by at least two possible routes: (a) an increase in the Mehler reaction rates, attributable to NADP⁺ shortage (Biehler and Fock, 1996), and (b) the NADPH-driven reduction of Fe³⁺ and other transition metals involved in Oh⁻ formation in situ through the Fenton reaction (Keyer et al., 1995). The somehow paradoxical conclusion is that accumulation of a reductant such as NADPH could increase the risk of oxidative injury when the plastids are exposed to redox-cycling agents.

Within this context, the rapid release into the chloroplast stroma of a NADPH consumer such as FNR might help to prevent or alleviate the transient accumulation of abnormally high levels of NADPH, therefore decreasing the rates of the Mehler reaction and of the Fe²⁺-mediated OH⁻ production (Fig. 7). The NADP⁺ photoreduction activity of thylakoids from MV-treated plastids was reduced to 30 to 50% of that observed in membranes from untreated controls. NADPH can be potentially generated in chloroplasts by alternative sources such as Glc-6-P dehydrogenase or NAD⁺-malate dehydrogenase. The latter enzyme is inhibited by oxidants (Ocheretina and Scheibe, 1994), indicating that its activity in MV-treated plastids should be negligible. Conversely, Glc-6-P dehydrogenase is expected to be active under oxidative conditions (Brennan and Anderson, 1980), although its contribution to the NADPH pool in stressed chloroplasts is likely to be small compared with that of the photosynthetic electron transport. Therefore, partial FNR solubilization during oxidative stress should cause a significant decrease in the NADPH steady-state concentration. The MV tolerance provided to E. coli cells by a mutagenized FNR, which cannot reduce Fd but is still able to oxidize NADPH at normal rates in the presence of artificial electron acceptors (Krapp et al., 1997), is in agreement with the alleged function of the reductase in maintaining the NADPH/NADP⁺ homeostasis. This effect may also explain the remarkable constancy of the NADPH/NADP⁺ ratio in chloroplasts illuminated over a broad range of irradiances, while the ATP/ADP ratio and the phosphorylation potential increased steadily under these conditions (Gerst et al., 1994).

We propose that stromal accumulation of NADPH reductase at the onset of an oxidative challenge may play a protective, antioxidant role by keeping NADPH concentrations at tolerable levels. This regulatory mechanism would replace the need for an up-regulation of FNR synthesis and accumulation, as it occurs in the E. coli cytosol under similar stress conditions. Work is currently in progress to evaluate these possibilities.

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