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Forum Review

Redox Signaling in the Chloroplast: The Ferredoxin/Thioredoxin System

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ABSTRACT

Chloroplasts have developed a light-dependent system for the control of the activities of key enzymes involved in assimilatory (photosynthetic) and dissimilatory pathways, which allows a switch between these opposing pathways to prevent futile cycling. This regulatory system, known as the ferredoxin/thioredoxin system, consists of several proteins constituting a redox cascade that transmits the light signal perceived by chlorophyll to selected target proteins, thereby influencing their activity. A central component of the redox cascade is a novel enzyme, the ferredoxin:thioredoxin reductase, which is capable of reducing a disulfide bridge with the help of an iron-sulfur cluster. Recent developments on the elucidation of the structures of several implicated proteins and on the mechanism of signal transfer have greatly improved our understanding of this regulatory mechanism. This review describes the components of the redox cascade, the principal target proteins, and the mechanism of action of the light-signal transfer. *Antioxid. Redox Signal.* 5, 69–78.

INTRODUCTION

In oxygenic photosynthetic organisms, assimilatory, photosynthetic pathways coexist with dissimilatory pathways in the same cell compartment, using some of the same enzymes. For an efficient use of photosynthetic energy, these organisms needed to develop control mechanisms, which allow a switch between these opposing pathways. In the light, enzyme reactions associated with the assimilation of carbon dioxide have to be functional, whereas those leading to the degradation of photosynthetic storage products have to be halted. In the dark, this effect has to be reversed permitting degradation of photosynthetic storage products to provide metabolic energy and building blocks. An obvious signal for such a control is light. Light actually activates a number of biosynthetic enzymes and inhibits a key enzyme of carbohydrate degradation, the glucose 6-phosphate dehydrogenase. Light, sensed by the thylakoid pigments, acts through a redox signaling cascade known as the ferredoxin/thioredoxin (Trx) system on the activity of target enzymes, which are activated or deactivated by reduction of regulatory disulfides to sulfhydryl groups, causing some structural change that modifies their catalytic capacity. In the dark, this process is fully reversed by oxygen. As photosynthetic electron transport produces oxygen in the light, the redox equilibrium state of the target enzymes, and hence their activity, will depend on the "electron pressure" built up by the photosystems. Subsequent fine tuning of the Trx-activated enzymes depends on other cellular factors, such as pH and metabolite concentrations, both of which are linked to light (15, 27, 80).

During the last few years, several reviews presenting various aspects of the ferredoxin/Trx system have been published (23, 28, 44, 57, 58, 77, 82, 83). Here the essential elements of this chloroplast redox signaling pathway and its mechanism will be described with emphasis on the most recent developments.

COMPONENTS OF THE REDOX SIGNALING PATHWAY

The redox signal, which originates in the thylakoid membrane, is transmitted through a redox cascade involving

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ferredoxin, ferredoxin:thioredoxin reductase (FTR), and Trxs, all rather small stromal proteins, to the target enzymes.

Ferredoxin

Ferredoxin, the first soluble electron carrier in the stroma, receives electrons from photosystem I. Plant-type ferredoxins are soluble, acidic proteins of 12 kDa whose 2Fe-2S cluster with a redox potential of around −400 mV can carry one electron (Table 1). Three-dimensional structures, obtained for several ferredoxins, exhibit large similarities (23). They share the same fold and expose two patches of negative surface charges on either side of the Fe-S cluster, which are essential for the interaction with other proteins (25, 45). These surface charges confer a certain specificity to the interaction with electron acceptor proteins, resulting in clear differences in the affinities between ferredoxin and FTR from different organisms (P. Schürmann, unpublished observations).

FTR

This novel enzyme, found exclusively in oxygenic photosynthetic organisms, *i.e.,* in cyanobacteria and in chloroplasts of eukaryotes (23, 82), has been isolated and characterized from different sources, and its mechanism (86, 87) and structure (24) have been studied. The FTR is a colored protein of 20–25 kDa, composed of two nonidentical subunits of similar size. One of the subunits, the catalytic subunit, contains a 4Fe-4S cluster, responsible for the yellow-brownish color of the protein, and a redox-active disulfide bridge. The second subunit, known as variable subunit, seems to have only a structural function (23).

Catalytic subunits of FTR from different organisms have a constant size of ~13 kDa and a highly conserved primary structure. Among the strictly conserved residues are seven cysteines (Cys), six of them organized in two CPC and one CHC motifs. These six Cys are the functionally essential residues forming the redox-active disulfide bridge and ligating the Fe-S cluster. This ligation does not follow usual consensus motifs for 4Fe-4S centers, but shows a novel arrangement with the fingerprint: **C**PCX$_{16}$**C**P**C**X$_{8}$**C**HC (cluster ligands are in bold). In spinach FTR, the solvent-accessible Cys54 and the buried Cys84 form the active-site disulfide. The four remaining cysteines, Cys52, Cys71, Cys73, and Cys82, are ligands to the iron center positioning the cluster adjacent to the redox-active disulfide bridge (22), an essential structural feature of the FTR.

Variable subunits range in size from 8 to 13 kDa and show pronounced sequence variability. The size variability appears to be due to an N-terminal extension of variable length, present in the known eukaryotic enzymes, but absent from the prokaryotic counterparts.

Recombinant, perfectly active FTR, expressed in *E. coli,* has been obtained from spinach and *Synechocystis* sp. PCC 6803 (29, 85) and used for spectroscopic (87) and structural studies (24). The *Synechocystis* FTR crystallized as dark brown, well diffracting crystals allowing structural resolution to 1.6 Å. The FTR has a rather unique molecular structure. It is a flat, disk-like molecule, with the catalytic subunit sitting on top of the heart-shaped variable subunit. The main body of the variable subunit consists of a β-barrel, and the catalytic subunit is an overall α-helical structure. The Fe-S center and the active-site disulfide bridge are both located in the catalytic subunit, in the center of the heterodimer, where the molecule is only 10 Å thick. The 4Fe-4S cluster is positioned on one side of the flat molecule, close to the surface, containing three positive charges. The redox-active disulfide bridge is on the opposite side with a surface of more hydrophobic character. These surfaces are ideally suited for simultaneous docking of negatively charged ferredoxin on one side and Trx on the opposite side of the flat molecule.

Trxs

Trxs are ubiquitous, low-molecular-weight redox proteins capable of catalyzing thiol–disulfide exchange reactions through their redox-active disulfide bridge with the sequence -WCGPC-. Chloroplasts contain two different types of Trxs, the *f*-type and the *m*-type, differing in phylogenetic origin, primary structure, and target protein specificity. Both types are nucleus-encoded with the exception of the *m*-type in red algae, where its gene was found on the chloroplast genome (74). All eukaryotic, photosynthetic organisms that were analyzed possess both types of Trxs; however, photosynthetic prokaryotes lack the *f*-type.

Trx *f* was originally described as the specific activator protein for chloroplastic fructose 1,6-bisphosphatase (FBPase). The *f*-type Trxs are of eukaryotic origin, and their primary structures show extensive homologies. They are slightly longer than other Trxs due to additional amino acids at their N-termini. The C-terminal part of the sequences resembles classical animal Trx in containing a third, strictly conserved Cys.

Trx *m* was discovered as essential activator protein for the NADP-dependent malate dehydrogenase (NADP-MDH) of chloroplasts in C4 and C3 plants. It is found in cyanobacteria and in chloroplasts of algae, dicots, and monocots and resembles strongly the Trx from anoxygenic prokaryotes, both heterotrophic and photosynthetic. The *m*-type Trxs are, therefore, also known as bacterial-type Trxs. Due to their structural relatedness, the bacterial and *m*-type Trxs are functionally similar and can be used interchangeably. However, the primary structures of the various *m*-type Trxs have less sequence similarity than their *f*-type counterparts known to date.

Three-dimensional structures of spinach chloroplast Trxs *f* and *m* (16, 65) and of *Chlamydomonas* Trx *m* (55) have been determined. They have the same typical three-dimensional structures with a central five-stranded β-sheet surrounded by four α-helices as found in Trxs from prokaryotes or humans. The *m*-type Trxs are structurally very similar to *E. coli* Trx (48), and also their surfaces around the active site Cys are largely similar. This supports biochemical evidence showing that these proteins are functionally interchangeable. The overall structure of Trx *f* does also not differ markedly from a typical Trx, but its surface topography is distinct from that of others. Trx *f* is more positively charged. Some of these charges surround the active site and might be instrumental in orienting Trx *f* correctly with target proteins, whereas the hydrophobic residues, also prominent in the contact area, may be more important in the less specific interaction with FTR,

TABLE 1. PROPERTIES OF PROTEINS INVOLVED IN THE FERREDOXIN/THIOREDOXIN SYSTEM OF CHLOROPLASTS

Protein	*Organism*	*Redox-active disulfide*	$E_{m,7.0}$ *(mV)*	*Activator thioredoxin*	*References*
Ferredoxin	Spinach		−420		
Ferredoxin:thioredoxin reductase	Spinach	−**C54**X_{29}**C84**−	−320		22, 38
Thioredoxin *f*	Spinach	−**C46**GP**C49**−	−290		38, 47
Thioredoxin *m*	Spinach	−**C37**GP**C40;**−	−300		38, 47
Fructose 1,6-bisphosphatase	Spinach	−**C155**IVDSDHDDESQLSAEEQR**C174**−	−305	*f*	11
Sedoheptulose 1,7-bisphosphatase	Wheat	−**C52**GGTA**C57**−		*f*	26
	Tomato		−295		42
Phosphoribulokinase	Spinach	−**C16**X_{38}**C55**−	−295	*m/f*	37
Rubisco activase	*Arabidopsis*	−**C392**TDPVAENFDPTARSDDGT**C411**−	−290	*f*	95
ATP synthase γ-subunit	Spinach	−**C199**DINGK**C205**−	−280	*f*	42
NADP-malate dehydrogenase	Sorghum	−**C24**FGVF**C29**−	−280	*f/m*	39
		−**C365**VAHLTGEGNAY**C377**−	−330		
		−**C24—C207**−	−310		
Glucose 6-phosphate dehydrogenase	Potato	−**C149**RIDKRDN**C157**−		*m*	90
	Pea		−300		82
Glyceraldehyde 3-phosphate dehydrogenase	Tomato		−310	*f*	42
Peroxiredoxin	Barley	**C64—C185***	−315		97

The redox potentials have been normalized at pH 7.0 assuming a slope of E_m vs. pH of −59 mV/pH unit.
*The two Cys of the disulfide bridge in peroxiredoxin are located on separate homologous subunits.

which reduces both Trxs efficiently. A marked difference is the presence of a third conserved Cys (Cys73 in spinach), exposed on the surface, close to the active site. The structural analysis also shows that the active-site Cys with the lower sequence number (Cys46 in spinach) is solvent-accessible, whereas its partner, Cys49, is buried, confirming biochemical evidence that Cys46 forms the heterodisulfide bond with the target protein (12).

The surface properties of the different Trxs are responsible for the observed specificity in their interaction with target proteins, and these interactions are most specific in homologous systems. The published results suggest that Trx *f* is the primary activator protein of enzymes involved directly or indirectly in carbon assimilation (Table 1). Also NADP-MDH, originally thought to be exclusively reduced by Trx *m*, is more efficiently activated by Trx *f* (31, 40). Conversely, phosphoribulokinase was recently reported to be slightly better reduced by Trx *m* than *f* (30), an observation that could be explained by the endosymbiotic origin of this enzyme. Phosphoribulokinase is the only Trx-regulated Calvin cycle enzyme in cyanobacteria, which possess only *m*-type Trxs. The only carbon metabolism enzyme, reported to be exclusively reduced by Trx *m*, is so far glucose 6-phosphate dehydrogenase (90).

CHLOROPLAST TARGET ENZYMES

A number of chloroplast enzymes catalyzing a wide variety of reactions have been proposed as targets for redox regulation by the ferredoxin/Trx system. This is usually based on the observation that light activation can be mimicked by a dithiol compound like dithiothreitol. However, there is yet no convincing experimental evidence for some of these enzymes for an absolute requirement of reduced Trx and for the presence of an accessible, redox-active disulfide bridge involved in regulation. For seven enzymes, all directly or indirectly involved in carbon metabolism, regulatory disulfides have been located by site directed mutagenesis, their redox potentials determined, and a Trx specificity reported (Table 1). FBPase, sedoheptulose 1,7-bisphosphatase, and phosphoribulokinase are constituents of the Calvin cycle, whereas Rubisco activase and ATP synthase are indirectly involved in carbon assimilation. NADP-MDH operates in a shuttle for the export of reducing equivalents, and glucose 6-phosphate dehydrogenase is a key enzyme for the degradation of sugars in the dark metabolism of the chloroplast.

For another five enzymes, an involvement of Trx in regulation was reported, but regulatory disulfides have not yet been demonstrated. These enzymes include glyceraldehyde 3-phosphate dehydrogenase, ADP-glucose pyrophosphorylase, acetyl-CoA carboxylase, ferredoxin:glutamate synthase, and NiFe-hydrogenase.

Redox regulation through Trx was proposed to be implicated in the phosphorylation of light-harvesting antenna complex II (LHCII) of photosystem II (17) by disulfide bond reduction in a LHCII kinase (76). However, recent results suggest that the observed decreased phosphorylation *in vitro* may be due to the reductive activation of ATPase (18).

The ferredoxin/Trx system is involved not only in regulation, but also in detoxification of hydrogen peroxide via Trx peroxidase, or peroxiredoxin (6). Recently, chloroplast-specific peroxiredoxins have been reported (5, 34) that can be reduced via FTR and chloroplast Trxs (97).

The chloroplast **FBPase** is a well known redox-regulated enzyme. Compared with the cytosolic isoform, the chloroplast protein includes an insert of ~15 residues in the middle of its primary structure (71). Structural information revealed that this insert constitutes a rather flexible loop (20, 88) containing three conserved Cys of which the two more N-terminal form the redox-active disulfide bridge (11, 20). In oxidized FBPase, this disulfide appears to lock the active site in a catalytically incompetent configuration. The loop, containing the disulfide, is connected through two β-strands of an N-terminal β-sheet with the active site some 20 Å away. A superposition of the active sites of chloroplastic and gluconeogenic FBPases shows that most of the important catalytic residues occupy similar positions except for Glu105, which is a critical ligand for Mg^{2+} ions essential in catalysis. In the oxidized enzyme, this Glu105, which is on a loop connecting the two β-strands, is displaced by Val109, thereby preventing the coordination of Mg^{2+}. Through reduction of the disulfide bridge, the regulatory loop is destabilized, allowing the two β-strands to move some 8 Å away from the active site. This shifts Glu105 in a catalytically favorable position, thus restoring a functional active site (20, 23). These conclusions drawn from structural analysis are corroborated by recent biochemical results, which show that the opening of the regulatory disulfide bridge, by either reduction or mutation, greatly reduces the amount of Mg^{2+} needed for optimal activity of the enzyme (11). Another feature of the loop is the presence of several negative charges. They are probably instrumental in the interaction with Trx *f*, which exposes positive charges (36, 63, 89).

The **sedoheptulose 1,7-bisphosphatase** is unique to eukaryotic photosynthetic organisms, where it is present only in chloroplasts. Among a variable number of Cys found in the enzyme from different sources, four are at strictly conserved positions, in pairs in the N-terminal part. The two most N-terminal Cys, arranged in a **C**XXXX**C** motif, constitute the regulatory disulfide (26). Based on modeling, which suggested structural similarities with the FBPase, the disulfide is located on a flexible loop between the subunits of the dimeric enzyme. Oxidation or reduction of the regulatory disulfide may alter the conformation of the dimer and thereby change the activity of the enzyme (72).

The **phosphoribulokinase** of eukaryotes and cyanobacteria contains four Cys at conserved positions. The two Cys in the N-terminal part, Cys16 and Cys55, of the spinach protein form an intramolecular disulfide bond in the oxidized, inactive enzyme (68). Both Cys are located in the nucleotide binding domain of the active site, and Cys55 plays a facilitative role in catalysis (67, 69) by binding the sugar phosphate in the active site (62). Cys55 also forms the transient heterodisulfide with Cys46 of Trx *f* during reductive activation (13). Phosphoribulokinase is so far the only example of a Trx-linked enzyme with a regulatory Cys as part of its active site. As one of the reduced Cys residues is involved in substrate binding, the formation of a disulfide bridge very effectively blocks catalytic activity.

Several lines of evidence show that Rubisco is activated by light; however, a direct involvement of a redox regulation of

the enzyme via the ferredoxin/Trx system could not be established. The finding that **Rubisco activase**, which regulates the Rubisco activity by removing inhibitory sugar phosphates at the expense of ATP, can be specifically activated by Trx *f* at physiological ratios of ATP/ADP created finally the link to the observed light activation (94). In most plants, two isoforms of Rubisco activase coexist in the chloroplast. The redox regulation depends on the larger of the two isoforms, which has two conserved Cys residues located near its C-terminus. These two residues have been identified as Cys392 and Cys411 for the 46-kDa polypeptide from *Arabidopsis* (94). Further characterization of the regulation by Trx *f* demonstrated that reduction significantly lowered the $S_{0.5}$ of Rubisco activase for ATP. Reduced Trx *f* in the micromolar concentration range, the same as observed for other target enzymes, was sufficient to rapidly and fully activate the enzyme. A midpoint potential of −344 mV at pH 7.9 was determined, which is essentially isopotential to Trx *f*. Interestingly, mixing experiments with different ratios of 43-kDa to 46-kDa isoforms revealed that a 1:1 ratio of the two isoforms is required to completely inhibit activase activity after a reduction–oxidation cycle. These results, taken together with information from the primary structure, were used to propose a scheme for the redox regulation of both isoforms of Rubisco activase by the C-terminus of the larger isoform (95).

The chloroplast **ATP synthase** contains in the γ subunit of CF_1 the structural element with two Cys residues, which, in the oxidized enzyme, form a disulfide bond allowing for thiol modulation (66, 84). This structural element, originally described for the spinach enzyme (61), confers redox modulation when introduced into the subunit of the non–redox-regulated *Synechocystis* enzyme (53, 91). The regulatory disulfide appears to be inaccessible in the dark on the inactive enzyme and becomes exposed upon activation by the transmembrane potential difference. This has been confirmed in a recent study, which determined different kinetic parameters of the thiol modulation (35). In another study, the entire central region of the γ subunit of CF_1 from spinach, including the regulatory element, has been inserted in the γ subunit of a thermophilic bacterial F_1. The recombinant chimeric protein exhibited thiol modulation properties (7), and the rotation of the γ subunit was influenced by changes of the redox conditions (8).

The **NADP-MDH** is one of the best studied redox-modulated chloroplast enzymes and appears to have the most complex regulatory mechanism (1, 59, 60, 77). It is found in C4 and C3 plants and exhibits no activity when oxidized. The chloroplast enzyme differs in the primary structure from the NAD-dependent enzyme by the presence of N- and C-terminal extensions and eight strictly conserved Cys. Five of them are engaged in the regulatory process; two are present in the N-terminal extension in a CXXXXC motif, two are in the C-terminal extension separated by 11 residues, and the fifth Cys is located in the core part of the protein at the interface of the subunits of the dimeric enzyme.

The two regulatory disulfides exert differential effects on activation, which can be understood based on recent crystallographic analyses (19, 46). The C-terminal extension of ~13 residues is held by the disulfide bridge against the core structure of the protein. Its tip, carrying negative charges, dips into the active site, obstructing access to the substrate by intrasteric inhibition (50). The negative charges mimic the substrate oxaloacetate and can, in addition, interact with $NADP^+$ if present in the active site, which explains the observed inhibition of activation by $NADP^+$. Through reduction of the disulfide, the C-terminal extension gains more mobility (54), liberating the active site and permitting access to the substrate.

The N-terminal extension with its disulfide is located at the interface between the two subunits. It has interactions with the catalytic domain of one subunit and with the nucleotide-binding domain of the other subunit, which might maintain both in a catalytically unfavorable position. Reduction of the disulfide loosens the N-terminal extension, resulting in a more relaxed, catalytically efficient structure of the active site. A fifth internal Cys residue (Cys207 in sorghum) may also be involved in the regulatory events by forming a transient disulfide bridge, most probably with one of the N-terminal Cys (78).

The chloroplast isoform of **glucose 6-phosphate dehydrogenase** is redox-regulated. However, unlike the other enzymes, it is inactivated by reduction through Trx, thereby stopping the oxidative pentose phosphate cycle in the light. This is a logical consequence of its function as a regulatory enzyme in carbohydrate degradation. The two Cys residues responsible for redox regulation have been located in the N-terminal half of the potato enzyme, within the NADP-binding domain. Homology modeling using the crystal coordinates of the *Leuconostoc* enzyme positions the regulatory Cys on an exposed loop, close enough to enable the formation of a disulfide bridge, and freely accessible for interaction with Trx. Whereas no information is available about structural changes due to reduction, it was reported to increase the K_m for glucose 6-phosphate ~30-fold (90).

The activity of the chloroplast **glyceraldehyde 3-phosphate dehydrogenase** has for a long time been shown to be light-activated (96), but to date the evidence for an involvement of the ferredoxin/Trx system is not yet conclusive. This might be due to the complex behavior of the enzyme, which appears as a large, inactive hexadecameric complex of 600 kDa in the dark dissociating into 150-kDa active heterotetramers in the light. This transformation is brought about by the combined action of different effector molecules and a reductant (2, 3). Low concentrations of glutathione were as effective as dithiothreitol or Trx, suggesting that the required reductant need not necessarily be Trx (3). However, experimental conditions may be critical for the demonstration of an absolute Trx requirement (73). Indirect evidence suggests the involvement of a regulatory disulfide. The light-regulated enzyme possesses a C-terminal extension on subunit B, which contains two conserved Cys. Removal of the extension provides a recombinant, active, homotetrameric enzyme composed of only subunit B, which does not aggregate. This is taken as indication that the Cys in the extension are responsible for the aggregation and therefore involved in light activation (4, 81). Recently, these two Cys were shown to form the only disulfide bridge detected in the enzyme and proposed to make an interdomain disulfide cross-link (70). In addition, a redox potential of $E_{m,7.0} = -310$ mV has been measured for the reductive activation of the tomato enzyme (42), a value in

the range observed for the other redox-regulated enzymes (Table 1).

The **ADP-glucose pyrophosphorylase** catalyzes the first committed step in starch biosynthesis. The potato tuber enzyme is reduced by Trx *f* and half as efficiently by Trx *m*, thereby increasing its affinity for 3-phosphoglycerate, the principal activator. The reduction opens an intermolecular disulfide bridge between the two small subunits of the heterotetrameric enzyme providing probably better access to 3-phosphoglycerate. As the Cys in the small subunit is conserved in virtually all plant enzymes, it is proposed that in photosynthetic tissue the ferredoxin/Trx system is involved in this covalent mechanism of regulation of starch biosynthesis (9).

Three other chloroplast enzymes not involved in carbon metabolism have recently been reported to be redox-regulated, and a Trx specificity has been observed. However, there is no experimental evidence yet for a regulatory disulfide, although for one of these enzymes possible candidates have been proposed (92). **Acetyl-CoA carboxylase**, which catalyzes the first committed step in fatty acid biosynthesis, appears to be redox-regulated, and Trx *f* functions as the most efficient activator protein (51, 52, 79). The reaction velocity of **ferredoxin: glutamate synthase** was significantly stimulated by reduced Trx, with Trx *m* being the most efficient effector protein (56). And finally, the activity of a **NiFe-hydrogenase** from the green alga *Scenedesmus obliquus* was shown to be inhibited by reduced Trx *f* (92). This is, after the glucose 6-phosphate dehydrogenase, the second enzyme whose activity is reduced by reduction.

A comparison of the regulatory disulfides present in the target enzymes shows that the two Cys are not arranged in a consensus motif, as was proposed for some time, although in two of the enzymes a CXXXXC sequence is the responsible element (Table 1). The regulatory Cys are not necessarily close on the primary structure, but can be separated by many residues and are usually not positioned in the active site. In some of the enzymes, they are found on extensions or insertions of the primary structures compared with non–redox-regulated isoforms. The effects of reduction on the target enzymes are also diverse. These observations indicate that the regulatory disulfides have been added to the enzymes during their adaptation to photosynthetic function. The absence of any consensus motif suggests that this adaptation arose multiple times during evolution and that the mechanism of control was tailored to each particular enzyme.

THE MECHANISM OF REDOX SIGNAL TRANSFER

The redox signal produced by light-driven electron flow originates in the thylakoid membranes as an "electron signal" and is transformed on its way to target enzymes into a "thiol signal." This transformation is accomplished by FTR, which reduces a disulfide bridge with the help of its Fe-S cluster, a so far unique reaction.

Ferredoxin, reduced by photosystem I, passes its electron on to FTR where it cleaves the enzyme's disulfide bridge yielding reduced, solvent-accessible Cys54 and a Cys-based thiyl radical on the buried Cys84. This radical is stabilized by covalent attachment to the Fe-S cluster, which thereby becomes oxidized. This is possible due to the close proximity of cluster and disulfide bridge. The nucleophilic Cys54 disrupts the disulfide bridge of Trx, which results in a transient, covalent complex linking FTR and Trx through an intermolecular disulfide bridge. In this complex, Trx covers one side of the flat FTR molecule, whereas the opposite side stays free to accept another ferredoxin molecule delivering the second electron needed for complete reduction of Trx. This second electron reduces the cluster-ligated Cys84 and reestablishes the original oxidation state of the cluster. The reduced internal Cys84 cleaves the intermolecular disulfide, releasing fully reduced Trx and returning the active site of the FTR to the disulfide state (87). This sequence of events is entirely compatible with available structural data (23).

Similarly, Trx reduces regulatory disulfides of target enzymes via the formation of a transient heterodisulfide complex between the two reaction partners. The reactive, solvent-exposed Cys, which is the one closer to the N-terminus of Trx, cleaves the target disulfide by nucleophilic attack, thereby forming a covalently linked mixed disulfide between the two proteins. In a fast second step, the buried sulfhydryl attacks the mixed disulfide to produce oxidized Trx and reduced target enzyme (41). The existence of mixed disulfides between target enzymes and Trxs has been demonstrated for phosphoribulokinase (13), NADP-MDH (32, 33), and FBPase (10).

In order to transfer electrons through the regulatory redox chain, there has to be sufficient thermodynamic driving force, which will depend on the redox potentials of the individual components. Using the fluorescent probe monobromobimane and activity measurements, these have been determined for the members of the regulatory chain and for several target enzymes (Table 1) (11, 37–39, 42, 49). The potential differences between ferredoxin ($E_{m,7.0}$ = −420 mV), FTR (−320 mV), and Trx *m* or *f* (−300 or −290 mV, respectively) are large enough to keep both chloroplast Trxs essentially reduced in the light. However, the redox potentials of the target enzymes vary between more positive (−280 mV) and more negative (−330 mV) values. Disregarding other metabolic factors, which are known to influence the catalytic activity of Trx-regulated enzymes (15), these redox potentials suggest a sequential order in the redox activation by light. Target enzymes involved directly or indirectly in the regeneration of the CO_2 acceptor, phosphoribulokinase (−295 mV) and ATP synthase (−280 mV), and in the activation of the carboxylating enzyme, Rubisco activase (−291 mV), have the most positive potentials and would therefore be activated first. FBPase (−305 mV) and sedoheptulose 1,7-bisphosphatase (−295 mV), which together control the entry of carbon into the regenerative phase of the Calvin cycle and into starch synthesis, have slightly more negative potentials requiring more reduced Trx *f* and would follow next. NADP-MDH with the most negative potential (−330 mV) would be last, being kept inactive as long as there is no surplus of reducing equivalents.

As the members of the FTR system are located in the chloroplast stroma where the pH is increasing from ~7.0 in the dark to 8.0 in the light and the redox potentials of the disulfide/dithiol couples are expected to be pH-dependent

(21), the pH dependency for several of the components has been determined (11, 37–39). For all of them, except for phosphoribulokinase, the data obtained in the range of physiological pHs could be fitted to a straight line with a slope of −59 mV/pH, a value expected for a process in which two protons are taken up per disulfide reduced (21). This indicates that the driving force for the reduction of the enzymes does not change with the light-dependent pH changes in the stroma.

OUTLOOK

Whereas recombinant protein techniques enabled the elucidation of the structures of proteins involved in the redox cascade and of the mechanisms of signal transfer, there remains the puzzling fact that chloroplasts possess two types of Trxs and that their respective targets *in vivo* are not yet all well defined. Certain stress conditions induce even another Trx-like protein. In *Solanum tuberosum,* submitted to a water stress, a new stromal protein C CDSP 32, for chloroplastic drought-induced stress protein C, has been reported (75). This is a nuclear-encoded 32-kDa protein of 243 residues composed of two Trx domains, but only the C-terminal region, containing the typical **C**GP**C** motif, shows Trx activity. CDSP 32 is proposed to play a role in the preservation of chloroplastic structures against oxidative injury upon drought (14).

New approaches have recently been used to search for the *in vivo* targets interacting with specific Trxs. Heterologous complementation of a yeast mutant strain revealed that Trx *m*, but not *f,* affected the tolerance to oxidative stress (43). Specific fluorescence labeling of proteins in cellular extracts after their reduction with specific Trxs, followed by electrophoretic separation and biochemical analysis of the labeled spots, was used to identify new targets (93). Although this approach has been used with the cytoplasmic Trx system, it can be adapted to search for targets in chloroplasts. Another way to look for targets is by trapping interacting proteins with mono-Cys Trx mutants, possessing only the accessible active-site Cys and fixed on an affinity matrix (64). This approach already revealed some targets interacting with Trx *m*. These efforts might not only clarify the question of interaction specificity, but also reveal hitherto unknown target proteins.

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ABBREVIATIONS

Cys, cysteine; FBPase, fructose 1,6-bisphosphatase; FTR, ferredoxin:thioredoxin reductase; NADP-MDH, NADP-dependent malate dehydrogenase; Trx, thioredoxin.

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