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FORUM ORIGINAL RESEARCH ARTICLE

CcdA Is a Thylakoid Membrane Protein Required for the Transfer of Reducing Equivalents from Stroma to Thylakoid Lumen in the Higher Plant Chloroplast

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Abstract

In order to transfer reducing equivalents into the thylakoid lumen, a specific thylakoid membrane transfer system is suggested that mediates the disulfide bond reduction of proteins in the thylakoid lumen of higher plant chloroplasts. In this system, although stromal thioredoxin can supply the reducing equivalents to a thioredoxin-like protein HCF164 in the thylakoid lumen, a mediator protein for electron transfer in the thylakoid membranes is proposed to be required to link the two suborganellar compartments. CcdA is a candidate protein as a component for this transfer system since CcdA- and HCF164-deficient mutants in *Arabidopsis thaliana* show the same phenotype. We now show that CcdA is localized in the thylakoid membrane and that its redox state, as well as that of HCF164, is modulated in thylakoids by stromal *m*-type thioredoxin. Our results strongly suggest that CcdA may act as a mediator in thylakoid membranes by transferring reducing equivalents from the stromal to the lumenal side of the thylakoid membrane in chloroplasts. *Antioxid. Redox Signal.* 13, 1169–1176.

Introduction

The redox state of higher plant chloroplasts fluctuates widely under light and dark conditions. In the light, reducing equivalents are produced by the reduction of water molecules by photosystem II and reduced ferredoxin is produced by photosystem I. Ferredoxin-NADP reductase derives reducing equivalents from ferredoxin to produce the reductant NADPH (26). NADPH is further used for the reduction of CO_2 in the chloroplast stroma. However, a portion of the reducing equivalents transferred to ferredoxin is utilized for redox regulation of thiol-enzymes by stromal thioredoxins *via* ferredoxin-thioredoxin reductase (6, 13, 35, 45). Although the redox control of the activities of four Calvin cycle enzymes, glyceraldehyde dehydrogenase, fluctose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and phosphoribulokinase, and some thiol enzymes such as malate dehydrogenase and ATP synthase in chloroplasts, were uncovered in the 1980s, many more thiol enzymes, whose activities are regulated by stromal redox conditions, have been most recently identified by way of proteomic studies of the target enzymes of thioredoxin (Trx) (3, 14, 17, 31, 32, 37, 54). Consequently, Trx-dependent redox regulation of the activities of stromal proteins in various metabolic pathways, including the Calvin cycle (5, 8, 10, 29, 41, 51), starch synthesis (2), tetrapyrrol metabolism (20), lipid metabolism (44, 52), and protein folding (38) have also been characterized.

In contrast, knowledge pertaining to redox regulation on the lumenal side of the thylakoid membrane remains very limited (16). We previously demonstrated that HCF164, which was reported as a thioredoxin-like protein and an indispensable factor required for formation of the cytochrome b_6f complex (28), is located in the thylakoid lumen and can function as a reducing equivalent carrier to protein targets located in the lumen (36). The target proteins for HCF164 were comprehensively screened by Trx-affinity chromatography using a resin-immobilized single-cysteine mutant of HCF164 (17, 39). This strategy allowed identification of the N subunit of photosystem I (PSI-N) and components of the cytochrome b_6f complex as HCF164 targets in the thylakoid lumen, and confirmed that the redox state of PSI-N is modulated in an HCF164-dependent manner both *in vitro* and in thylakoids. In order to function as a carrier of reducing equivalents in the thylakoid lumen, HCF164 in turn must receive reducing equivalents. We therefore examined stromal Trx proteins as a possible source of such reducing equivalents and revealed *m*-type Trx to be specifically responsible for reduction of HCF164 in the thylakoid lumen. These results indicate that higher plant chloroplasts possess a reducing equivalent transfer system which operates across the thylakoid membrane from the stroma to the lumenal side.

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It is therefore evident that in order to better understand this reducing equivalent transfer system, thylakoid membrane component(s) relevant to electron transfer across the membrane should be identified. Page *et al.* reported that the *Arabidopsis ccda* mutant showed the same phenotype as the *hcf164* mutant [i.e., an inability to assemble the cytochrome b_6f complex in thylakoids (42)]. In addition, they showed that CcdA protein is a polytopic membrane protein which consists of six-transmembrane spanning regions. However, the precise localization of CcdA protein in chloroplasts could not be determined since they were unable to generate a specific antibody against this protein. Multiple amino acid sequence alignment of CcdA in the protein database indicates that plant CcdA is a homolog of the prokaryotic thiol disulfide transporter (21, 23, 33, 48). Taken together, CcdA represents a candidate protein which may act as a component in the reducing equivalent transfer system operating across the thylakoid membranes. In this study, we carried out a thorough investigation into the putative CcdA-mediated reducing equivalent transfer mechanism across the thylakoid membranes.

Materials and Methods

Cloning, expression and purification of CcdA protein

The gene for the mature form of CcdA (AT5G54290, amino acid residues 81 to 354) was obtained by PCR amplification from an *Arabidopsis* cDNA library (53), using the following oligonucleotides; 5′-cggaattcgcagacttgaagatgattgtc-3′ (*Eco*RI) and 5′-ccgctcgagcatgaccatagtagcagcagg-3′ (*Xho*I). The restriction sites for the enzyme shown in parentheses are underlined. The amplified DNA fragments were cloned into the *Eco*RI and *Xho*I sites of pASK-IBA2 (IBA, Göttingen, Germany) and the DNA sequences confirmed. The recombinant Strep-tag II fused CcdA protein was expressed in *Escherichia coli* C41(DE3) cells and purified as follows: *E. coli* cells were suspended into 25 m*M* HEPES-KOH (pH 7.6) containing 150 m*M* NaCl, and disrupted by French press (5501-M, Ohtake Works, Tokyo, Japan) at 4°C. The disrupted cells were centrifuged at 11,000 *g* for 15 min. The supernatant was then centrifuged at 100,000 *g* for 30 min and the membrane fraction obtained was washed. The membrane fraction was then homogenized, and solubilized in 25 m*M* HEPES-KOH (pH 7.6) containing 150 m*M* NaCl, 1 m*M* EDTA, 0.5% Triton X-100, and protease inhibitor cocktail Complete EDTA–free (Roche Diagnostics, Basel, Switzerland), with gentle stirring for 60 min on ice. The solubilized fraction was centrifuged at 100,000 *g* for 30 min and the supernatant applied to a Strep-Tactin sepharose column (IBA). The column was washed with 25 m*M* HEPES-KOH (pH 7.6) containing 150 m*M* NaCl, 1 m*M* EDTA, and 0.05 % *n*-dodecyl-*β*-D-maltoside, and eluted with 25 m*M* HEPES-KOH (pH 7.6) containing 150 m*M* NaCl, 1 mM EDTA, 0.05 % *n*-dodecyl-*β*-D-maltoside, and 2.5 m*M* D-desthiobiotin. The eluted fractions containing CcdA protein were collected and dialyzed against 25 m*M* HEPES-KOH (pH 7.6), 1 m*M* EDTA, and 0.05 % *n*-dodecyl-*β*-D-maltoside. The dialyzed CcdA fractions were applied to a DEAE-Toyopearl 650 M column (Tosoh, Tokyo) and eluted with a 0–250 m*M* linear gradient of NaCl in 25 m*M* HEPES-KOH (pH 7.6), 1 m*M* EDTA, and 0.05 % *n*-dodecyl-*β*-D-maltoside. The peak fraction containing CcdA was collected, and stored at −80°C.

Preparation of Trx-f and Trx-m

Recombinant Trx-*f* and Trx-*m* were expressed in *E. coli* using the plasmids constructed for spinach Trxs (49). Spinach Trx-*f* (36) and Trx-*m* (38) were purified as described previously in the absence of dithiothreitol (DTT).

Antibodies

Polyclonal anti-CcdA, anti-HCF164 and anti-stromal cyclophilin serum were raised in rabbits against purified CcdA (AT5G54290), soluble domain of HCF164 (AT4G37200) (36), and cyclophilin (AT3G62030, CYP20-3) (37, 38) proteins, respectively. Polyclonal anti-E37 was generously gifted from M. A. Block (4, 22, 50). Polyclonal anti-PsbA (D1) and anti-Toc75 were purchased from Agrisera (Vännäs, Sweden). Monoclonal anti-spinach rubisco large subunit (anti-RbcL) was purchased from Cosmo Bio (Tokyo, Japan). Antibodies for marker proteins of suborganellar compartments were selected according to their specificity for proteins from *Arabidopsis* and spinach lysates.

Fractionation of suborganelle compartments in chloroplasts from Arabidopsis *and spinach leaves*

Intact *Arabidopsis* chloroplasts were prepared as described previously (46, 47) with the following modifications. *Arabidopsis* (ecotype Columbia) rosette leaves from 3-to-4-week old plants were harvested, then rapidly homogenized in a Waring blender with ice-cold 400 m*M* sorbitol, 5 m*M* sodium ascorbate, 0.05% bovine serum albumin (fatty acid-free), 2 m*M* EDTA, 5 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 10 m*M* $NaHCO_3$, and 20 m*M* Tricine-KOH (pH 8.4), and filtered through Miracloth (Calbiochem, La Jolla, CA). Crude chloroplast pellets obtained by centrifugation at 2000 *g* for 10 min, were purified on a 40 and 70% (v/v) Percoll step gradient in 330 m*M* sorbitol, 5 m*M* sodium ascorbate, 3 m*M* $MgCl_2$, and 50 m*M* HEPES-KOH (pH 7.6). After a 5 min centrifugation at 4500 *g*, intact chloroplasts were recovered as a heavy green band between the 40% and 70% layers, and washed twice in 330 m*M* sorbitol, 5 m*M* sodium ascorbate, 3 m*M* $MgCl_2$, and 50 m*M* HEPES-KOH (pH 7.6).

Intact spinach chloroplasts were prepared as described previously (40, 55, 56) with the following modifications. Spinach leaves were rapidly homogenized in a Waring blender with ice-cold 330 m*M* sorbitol, 5 m*M* sodium ascorbate, 0.05% bovine serum albumin (fatty acid-free), 2 m*M* EDTA, 3 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, and 50 m*M* HEPES-KOH (pH 7.6), and filtered through Miracloth (Calbiochem). Crude chloroplast pellets obtained by centrifugation at 2000 *g* for 10 min, were purified on a 40% and 70% (v/v) Percoll step gradient in 330 m*M* sorbitol, 5 m*M* sodium ascorbate, 3 m*M* $MgCl_2$, and 50 m*M* HEPES-KOH (pH 7.6). After a 5 min centrifugation at 4500 *g*, intact chloroplasts were recovered as a heavy green band between the 40% and 70% layers, and washed twice with 330 m*M* sorbitol, 5 m*M* sodium ascorbate, 3 m*M* $MgCl_2$, and 50 m*M* HEPES-KOH (pH 7.6).

The intact chloroplasts of *Arabidopsis* and spinach were then fractionated to suborganelle compartments as follows (1, 30): The intact chloroplasts were incubated with 3 m*M* $MgCl_2$ and 25 m*M* HEPES-KOH (pH 7.6) for 10 min on ice to disrupt the envelope membranes. The obtained broken chloroplasts were then separated on a sucrose step gradient with 0.46 *M* (6 ml), 1.0 *M* (6 ml), and 1.2 *M* (3 ml) in 3 m*M* $MgCl_2$ and

25 m*M* HEPES-KOH (pH 7.6) by centrifugation at 70,000 *g* for 60 min. Stroma, envelope (a mixture of inner and outer membranes), and thylakoid fractions were retrieved from the supernatant, the 0.46 *M*/1.0 *M* sucrose interface, and the pellet, respectively.

Fractionation of Arabidopsis *intact thylakoids*

Intact thylakoids (80 μg chlorophyll/ml), which were prepared as described previously (9, 36), were sonicated for 3 min on ice by microtip of the sonifier (Branson Sonifier 250, output 2, duty cycle 30%; Branson, Danbury, CT) in 3 m*M* $MgCl_2$ and 25 m*M* HEPES-KOH (pH 7.6). The sonicated thylakoids were centrifuged at 100,000 *g* for 30 min at 4°C. Thylakoid lumen and thylakoid membrane fractions were retrieved as the supernatant and the pellet, respectively.

Trx-dependent CcdA reduction assay on thylakoid membranes

The sonicated thylakoid membrane fraction, which was prepared as described above, was incubated with or without Trx-*f* (final 1 μ*M*), or Trx-*m* (final 1 μ*M*) at each DTT concentrations in 0.1 *M* sorbitol, 5 m*M* $MgCl_2$, 10 m*M* NaCl, 20 m*M* KCl, and 30 m*M* Tricine-KOH (pH 8.0) for 60 min at 25°C. The experiment to measure the time-dependent reduction of CcdA on thylakoid membranes was performed under conditions described in the legend to Figure 5. Following completion of the reaction, the samples were precipitated with trichloroacetic acid (final 5%), washed with ice-cold acetone, and finally dissolved into buffer containing 125 m*M* Tris-HCl (pH 6.8), 4% SDS, and 10 m*M* 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid (AMS), to specifically modify free sulfhydryl groups (37,38). Reduced and oxidized CcdA proteins in the thylakoid membrane lysate were separated by nonreducing SDS-PAGE (13% (w/v)) and detected by Western blot analysis.

Results

CcdA and HCF164 proteins are localized in thylakoids of chloroplasts

As expected from the membrane topology of CcdA protein determined by PhoA and LacZ topological reporter assay (42), it was very difficult to generate specific antibodies to the polytopic membrane protein, CcdA. To overcome this problem, we purified CcdA protein from CcdA-expressed *E. coli* membrane fractions, but not from the inclusion bodies. By using this protein as an antigen, we succeeded in the preparation of specific antibody which can exclusively detect CcdA protein in whole thylakoids (Fig. 1). The molecular weight of the CcdA detected showed the same mobility as the mature recombinant CcdA protein on SDS-PAGE (Figs. 1B and 1C).

To examine the function of CcdA as a reducing equivalent mediator across the thylakoid membrane, we initially sought to determine the localization of CcdA protein in chloroplasts. For this purpose, intact chloroplasts of *Arabidopsis* and spinach were fractionated into envelope, stroma, and thylakoid fractions (1, 30): the CcdA protein was exclusively detected in the thylakoid fraction in *Arabidopsis* and spinach by Western blotting (Fig. 2). The purity of our suborganellar fractions was assessed by marker proteins, E37 (for envelope), CYP20-3 (for stroma) (15, 43), PsbA (for thylakoids) of *Arabidopsis*, and Toc75 (for envelope), Rubisco large subunit (for stroma), PsbA (for thylakoids) of spinach. The results clearly showed that

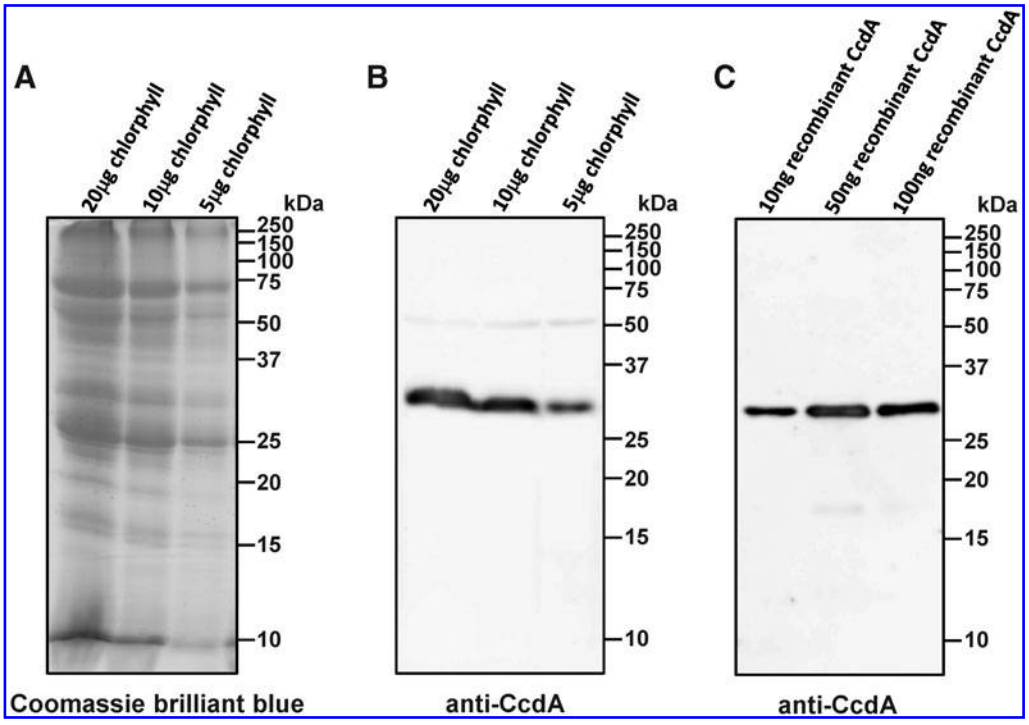

FIG. 1. Immunodetection of CcdA in *Arabidopsis* thylakoids lysate by specific antibody. (**A**) Coomassie brilliant blue-staining of thylakoids lysate proteins from *Arabidopisis* leaves. (**B**) Western blot analysis of CcdA in thylakoids lysate from *Arabidopisis* leaves by anti-CcdA serum. (**C**) Western blotting of purified recombinant CcdA protein by anti-CcdA serum.

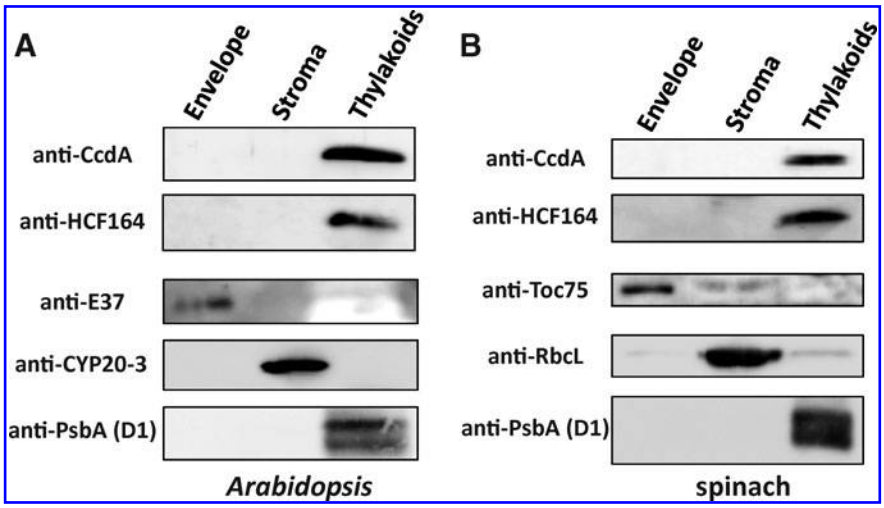

FIG. 2. Localization of CcdA and HCF164 proteins in chloroplasts of higher plants. Suborganellar compartments in chloroplasts from *Arabidopsis* and spinach leaves were fractionated into envelope, stroma, and thylakoids. CcdA and HCF164 were detected by Western blotting with specific antibodies, and each suborganellar marker protein was detected with anti-E37 (for *Arabidopsis* envelope), anti-Toc75 (for spinach envelope), anti-CYP20-3 (for *Arabidopsis* stroma) (15, 43), anti-RbcL (for spinach stroma), and anti-PsbA (for *Arabidopsis* and spinach thylakoids). (**A**) Fractionation of suborganellar compartments in chloroplasts from *Arabidopisis* leaves. (**B**) Fractionation of suborganellar compartments in chloroplasts from spinach leaves.

CcdA protein is certainly localized in the thylakoids. In parallel, we confirmed the localization of HCF164 in the chloroplasts by using the specific antibody for this protein. HCF164 was only detected in the thylakoid fraction in *Arabidopsis* and spinach, but not in the envelope and stromal fractions (Fig. 2).

CcdA protein is a thylakoid membrane protein and has a redox-responsive disulfide bond

Since CcdA protein was reported to be a polytopic membrane protein possessing two well-conserved cysteine residues (42), it is conceivable that CcdA protein is an integral thylakoid membrane protein. In order to validate this assumption, *Arabidopsis* thylakoid preparations were separated into lumen and membrane fractions as described in Materials and Methods. Following SDS-PAGE analysis, CcdA protein was only found in thylakoid membrane fractions in *Arabidopsis* (Fig. 3A). Accuracy of this fractionation was confirmed by the following marker proteins: plastocyanin (for the thylakoid lumen) and PsbA (for the thylakoid membranes). To clarify whether two cysteine residues of CcdA protein can form a disulfide bond under oxidizing conditions, we then investigated the redox state changes of CcdA protein on thylakoid membranes using a cysteine residue specific chemical modification with AMS. Without any prior reduction treatment, CcdA protein on *Arabidopsis* thylakoid membranes was detected as the oxidized form (Fig. 3B, left lane). When the thylakoid membranes were treated with high concentrations of DTT, CcdA protein became reduced (Fig. 3B, right lane). Judging from the extent of the shift of the protein bands on the gel, the CcdA protein most likely possessed a disulfide bond which can be modulated by redox conditions.

CcdA protein on the thylakoid membranes is reduced by stromal Trx-m

In a previous report, we confirmed that HCF164 present in the thylakoid lumen is reduced by reducing equivalents from the stromal side, and stromal *m*-type Trx is the unique source of reducing equivalents in this system (36). We therefore surmised that if both HCF164 and CcdA protein function in the same reducing equivalent transfer pathway, reduction of a

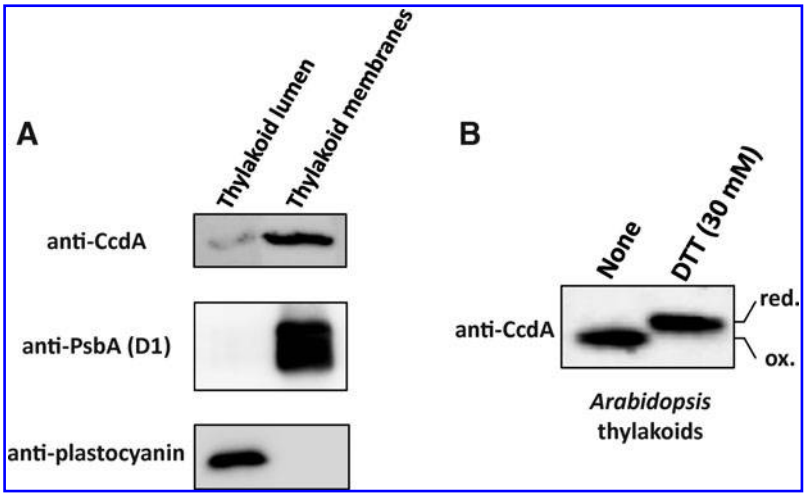

FIG. 3. Characterization of CcdA protetin on *Arabidopsis* thylakoid membranes. (**A**) Determination of the localization of CcdA protein in *Arabidopsis* thylakoids. Intact *Arabidopsis* thylakoids were fractionated into thylakoid lumen and membrane. The CcdA protein was detected by Western blotting with a specific antibody, and thylakoid marker proteins were detected with anti-PsbA (for thylakoid membranes), and antiplastocyanin (for thylakoid lumen). (**B**) Redox states of CcdA protein on thylakoid membranes. *Arabidopsis* thylakoid membranes were precipitated by trichloroacetic acid after none or 30 m*M* DTT treatment for 60 min at 25°C. Samples were modified with a maleimidyl reagent, AMS, and loaded on nonreducing SDS-PAGE (13% (w/v)). Redox states of CcdA protein were visualized by Western blotting.

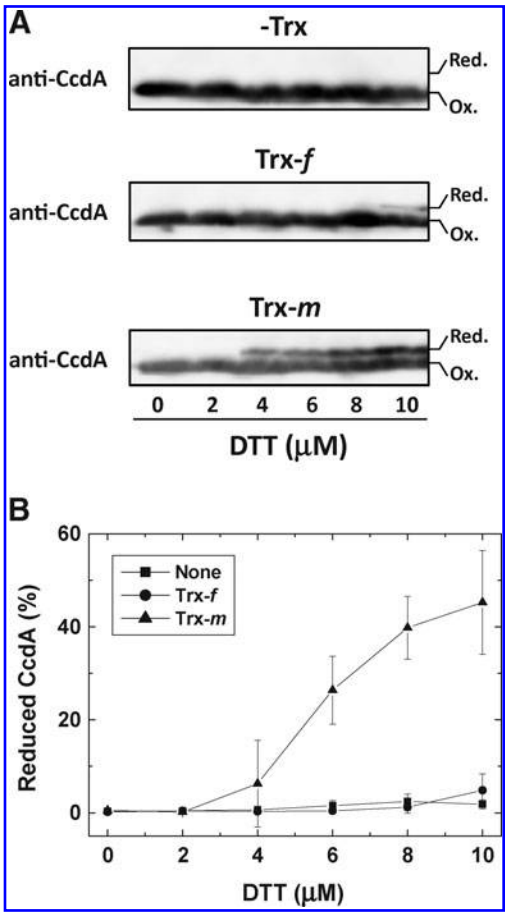

FIG. 4. Reduction of CcdA proteins on thylakoid membranes by stromal thioredoixn. (**A**) *Arabidopsis* sonicated thylakoid membranes were incubated with or without Trx-*f* (1 μM) or Trx-*m* (1 μM) at each concentration of DTT for 60 min at 25°C. Quenched samples were incubated with AMS (10 m*M*) to monitor redox states of CcdA, separated with nonreducing SDS-PAGE (13% (w/v)) and visualized by using an anti-CcdA specific antibody. (**B**) Reduced or oxidized bands of CcdA from (**A**) were quantified and the fractions of the reduced forms of CcdA determined.

disulfide bond in the CcdA molecule should be promoted by stromal Trx, especially by *m*-type Trx as well as HCF164. Trx-dependent reduction of CcdA was examined, using sonicated thylakoid membranes. As expected, CcdA protein was found to occur in the oxidized form in sonicated *Arabidopsis* thylakoid membranes (Fig. 3B, left lane). We monitored the redox state of CcdA on thylakoid membranes treated with an exogenous supply of reductant. CcdA was partially reduced when the membranes were incubated with the reduced form Trx-*m* (Fig. 4A, lower panel). The reduction of CcdA protein by Trx-*m* was observed to occur in a DTT-concentration dependent manner (Fig. 4B), but a low concentration of DTT (10 μM) was sufficient to reach a near maximal level. The reduction rate ($t_{1/2}$) of CcdA by Trx-*m* was found to occur at approximately 20 min upon incubation of membranes with 5 μM Trx-*m* and 10 μM DTT, and 45 min to plateau (Figs. 5A and 5B). The observed CcdA reduction time course on sonicated thylakoid membranes was similar to that of HCF164 in intact thylakoids (36).

Finally, we tested the transfer of reducing equivalents from Trx-*m* to CcdA using intact thylakoids instead of sonicated thylakoid membranes. However, reduction of CcdA was not observed in intact thylakoids (Fig. 5C). When intact *Arabidopsis* thylakoids were used, it is likely not to be possible to maintain the CcdA cysteine residues in the reduced form since HCF164 and other thylakoid lumen components function as a cysteine disulfide relay system downstream of CcdA.

Discussion

Plant CcdA proteins have been predicted to function in thylakoid membranes as an indispensable factor for the assembly of the cytochrome b_6f complex (42). Indeed Page *et al.* indicated that CcdA was imported into chloroplasts by way of the plastid targeting sequence, and the mature protein would be a polytotic membrane protein possessing six-membrane spanning region. In contrast, we successfully obtained direct

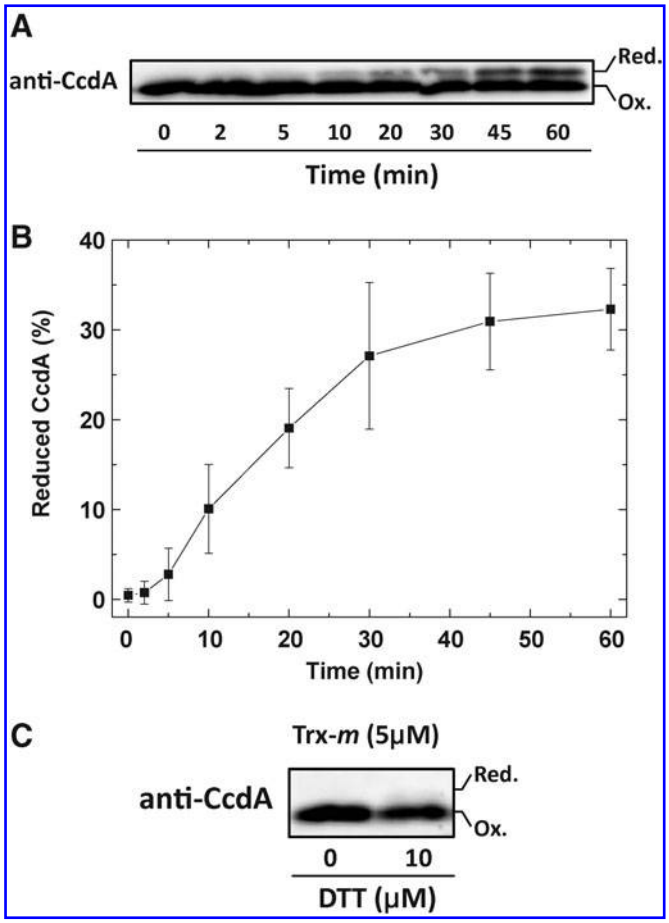

FIG. 5. Reduction rate of CcdA on thylakoid membranes by stromal Trx-*m*. (**A**) Sonicated thylakoid membranes were incubated for various periods with Trx-*m* (5 μM) and DTT (10 μM) at 25°C, and the redox states of CcdA were determined using AMS modification and Western blotting for an anti-CcdA specific antibody. (**B**) Reduced or oxidized bands of CcdA from (**A**) were quantified and the fractions of the reduced forms determined. (**C**) Intact thylakoids were incubated with Trx-*m* (5 μM) for 60 min at 25°C, and the redox states of CcdA determined by AMS modification and Western blotting using an anti-CcdA specific antibody.

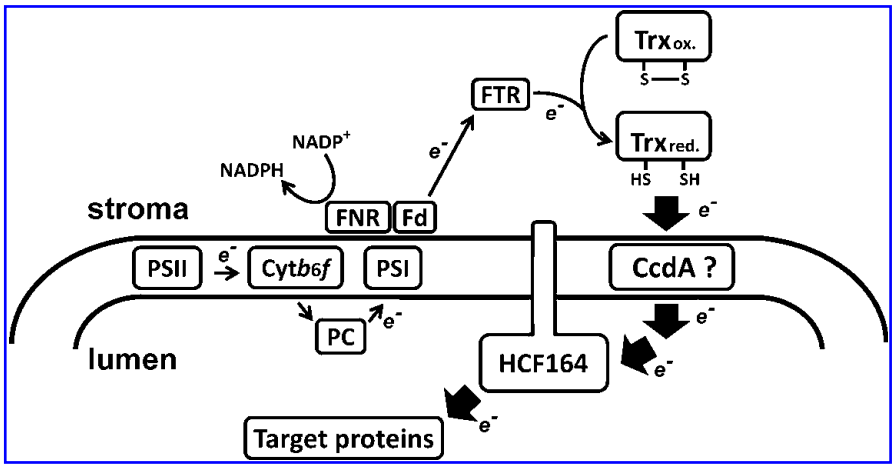

FIG. 6. Tentative model of a thioredoxin-dependent reducing equivalent transport pathway across thylakoid membranes. Cytb_6f, cytochrome b_6f complex; e^-, reducing equivalents; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ reductase; FTR, ferredoxin-thioredoxin reductase; PSI, photosystem I; PSII, photosystem II; PC, plastocyanin; Trx, thioredoxin.

evidence by fractionation of chloroplast proteins that CcdA protein is localized on thylakoid membranes. Moreover, we confirmed that HCF164, a downstream factor of the CcdA protein, is also localized in thylakoids alone, but is not present in the stroma and envelope fractions (Fig. 2).

Based on amino acid sequence analysis, bacterial CcdA is thought to be a homolog of the membrane spanning domain of the bacterial DsbD localized in the plasma membranes, the latter known as a reducing transfer protein from cytoplasm to periplasm (25). Though DsbD itself has three pairs of disulfide bonds, the membrane spanning domain contains only one cysteine pair, which is first reduced by cytosolic Trx (24). In the case of the bacterial CcdA, nothing is known to date on the reducing equivalent transfer system around this protein. One of the interesting findings in our former study was that HCF164 in the thylakoid lumen was reduced only by Trx-*m*, the chloroplast Trx considered to be of bacterial origin, but not by Trx-*f*, which is thought to be the Trx of eukaryotic origin. We expected the root cause of this specificity to be attributed to the evolutionary origin of the chloroplast. In this study, we suggest that the specificity of Trx in the reduction of HCF164 must be determined by CcdA, which previously interacts with stromal Trx-*m*.

A potential model describing the flow of reducing equivalents from the stromal to lumenal proteins is shown in Figure 6. In this study, electron flow downstream of CcdA on thylakoid membranes was not determined, since we were unable to detect direct interaction between CcdA and lumenal HCF164. As mentioned above, CcdA contains only one cysteine pair in the molecule and nothing is known about the molecular mechanism of reducing equivalent transfer from the stroma side Trx to the lumenal side proteins such as the catalytic moiety of HCF164. We therefore must postulate that there exist additional factor(s), which are likely to function in the transfer of reducing equivalent across the thylakoid membrane.

Based on the genome sequence project of *Arabidopsis thaliana*, the chloroplast type Trx-family proteins are categorized into four groups, Trx-*f*, Trx-*m*, Trx-*x*, and Trx-*y* (7, 27, 34). As shown above, we demonstrated that Trx-*m*, but not Trx-*f*, is the preferred candidate for the stromal source of reducing equivalents, which is used for reduction of proteins in the thylakoid lumen. Since Trx-*x* and Trx-*y*, which have been recently identified and characterized in *Arabidopsis* (11, 12), are also observed in cyanobacteria (18, 19), these Trx proteins may function as Trxs of bacterial origin in the reduction of CcdA pathway in addition to Trx-*m*. Whether Trx-*x* and Trx-*y* function within the system across thylakoid membranes is an important question whose answer would shed light on the evolutionary origin of this transfer system.

In order to obtain a full picture of the reducing equivalents transfer system in thylakoids, direct interaction of the multiple component(s) in the system should be confirmed by both biochemical and genetic approaches. Further biochemical analyses of the mechanism for the transfer of reducing equivalents between two proteins in which the distance of the active cysteines is not close enough for dithiol–disulfide exchange reaction is also required.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

AMS = 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid
ATP = adenosine triphosphate
DTT = dithiothreitol
EDTA = ethylenediaminetetraacetic acid
NADPH = nicotinamide adenine dinucleotide phosphate
PAGE = polyacrylamide gel electrophoresis
PCR = polymerase chain reaction
PSI = photosystem I
RbcL = rubisco large subunit
SDS = sodium dodecyl sulfate
Trx = thioredoxin

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