Dissipation of the proton electrochemical gradient in chloroplasts promotes the oxidation of ATP synthase by thioredoxin-like proteins

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Chloroplast F$_0$F$_1$-ATP synthase (CF$_0$CF$_1$) uses an electrochemical gradient of protons across the thylakoid membrane (Δ$\mu$H$^+$) as an energy source in the ATP synthesis reaction. CF$_0$CF$_1$ activity is regulated by the redox state of a Cys pair on its central axis, that is, the γ subunit (CF$_1$-γ). When the Δ$\mu$H$^+$ is formed by the photosynthetic electron transfer chain under light conditions, CF$_1$-γ is reduced by thioredoxin (Trx), and the entire CF$_0$CF$_1$ enzyme is activated. The redox regulation of CF$_0$CF$_1$ is a key mechanism underlying the control of ATP synthesis under light conditions. In contrast, the oxidative deactivation process involving CF$_0$CF$_1$ has not been clarified.

In the present study, we analyzed the oxidation of CF$_1$-γ by two physiological oxidants in the chloroplast, namely the proteins Trx-like 2 and atypical Cys-His-rich Trx. Using the thylakoid membrane containing the reduced form of CF$_0$CF$_1$, we were able to assess the CF$_1$-γ oxidation ability of these Trx-like proteins. Our kinetic analysis indicated that these proteins oxidized CF$_1$-γ with a higher efficiency than that achieved by a chemical oxidant and typical chloroplast Trxs. Additionally, the CF$_1$-γ oxidation rate due to Trx-like proteins and the affinity between them were changed markedly when Δ$\mu$H$^+$ for- mation across the thylakoid membrane was manipulated artificially. Collectively, these results indicate that the formation status of the Δ$\mu$H$^+$ controls the redox regulation of CF$_0$CF$_1$ to prevent energetic disadvantages in plants.

According to the chemiosmotic theory, chloroplast F$_0$F$_1$-ATP synthase (CF$_0$CF$_1$) plays a central role in photosynthetic energy conversion in green plants (1, 2). When photosynthetic electron transport reactions generate the electrochemical gradient of protons across the thylakoid membrane (Δ$\mu$H$^+$) under light conditions, the CF$_2$CF$_1$ embedded in this membrane synthesizes ATP using the gradient as a driving force (3). The regulation of CF$_0$CF$_1$ activity is important for two reasons. First, when the Δ$\mu$H$^+$ is insufficient to drive ATP synthesis, CF$_0$CF$_1$ can catalyze the reverse reaction, ATP hydrolysis, coupled with Δ$\mu$H$^+$ formation as a general mechanism of F$_0$F$_1$-ATP synthase (3). Second, the CF$_0$CF$_1$ in thylakoid membranes primarily controls proton efflux from the thylakoid lumen and is also involved in the maintenance of lumen side acidity. This acidification regulates the electron transfer activity of the cytochrome b$_6$f complex. In addition, it is also thought to be an essential signal for initiating the non-photochemical quenching required for photoprotection (4–6). Therefore, the activity of CF$_0$CF$_1$ seems to be controlled tightly according to the Δ$\mu$H$^+$ level across the thylakoid membrane, that is, under both light and dark conditions.

CF$_0$CF$_1$ is a molecular motor enzyme, and the c-ring portion of F$_0$ rotates during the proton translocation from the luminal side to the stroma side in the ATP synthesis reaction. This c-ring rotation leads to rotation of the central axis portion of F$_1$, which is composed of the γ and ε subunits, and induces conformational changes at the catalytic subunit β. In turn, a catalytic reaction occurs at three catalytic sites on each β subunit. CF$_0$CF$_1$ is also known as a thiol-modulated enzyme, and its rotation axis, that is, the γ subunit (CF$_1$-γ), has a plant-specific insertion sequence containing a redox-active Cys pair (Cys$^{199}$ and Cys$^{205}$ in spinach CF$_1$-γ) (7–9). In an early study in the field, Junesch and Gräber investigated the influence of redox regulation on CF$_0$CF$_1$ activity using isolated spinach thylakoid membranes, finding that the apparent $V_{\text{max}}$ was the same for the oxidized and reduced forms of the enzymes but that the Δ$\mu$H$^+$ threshold required to drive the reduced-form enzyme was lower than that required for the oxidized form (10). In other studies, the process of activating CF$_0$CF$_1$ via reduction at the dark-to-light transition has been clarified. Specifically, the disulfide bond of the oxidized form of CF$_1$-γ is reduced by reducing equivalents supplied via ferredoxin (Fd), Fd-thioredoxin reductase, and thioredoxin (Trx) from the photosynthetic electron transport chain (11–13). Trx is a small and ubiquitous redox-active protein that possesses a highly conserved amino acid sequence, WCGPC, at its active site and catalyzes a dithiol–disulfide exchange reaction with its target proteins (14, 15). In green plants, Trx constitutes a gene superfamily (e.g., 20 genes in Arabidopsis thaliana), which is classified into seven major classes based on amino acid sequences and their subcellular localization (16, 17). Five Trx subtypes exist in chloroplasts, namely Trx-f, Trx-m, Trx-x, Trx-γ, and Trx-z, which exhibit specific target selectivity.

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(18, 19). By in vitro and in vivo analyses, we showed that both Trx-f and Trx-m are involved in reducing CF₁–γ (20). It is also known that ΔμH⁺ formation across the thylakoid membrane is a prerequisite for the reduction of CF₁–γ (20, 21). Thus, CF₆₀CF₁ is active only under light conditions.

In contrast to the light-induced CF₆₀CF₁ activation process, the oxidative deactivation process under dark conditions has not been clarified. Indeed, the oxidation process related to all Trx-regulated enzymes in chloroplasts, not only CF₆₀CF₁, is poorly understood, and the proteins involved in this oxidation have not been assigned. In the 1980s, the in vivo redox state of CF₁–γ was assumed to be in equilibrium with the NADP⁺/NADPH pool via the Fd/Trx system or glutathione pool (22, 23). However, Mills and Mitchell found that CF₁–γ in lysed pea chloroplasts cannot be oxidized in vitro by Trx-f alone or by glutathione (24).

The recent studies of the protein oxidation system in chloroplasts have provided us with the important information. With advances in analysis technology, comparative genomic studies have revealed the presence of Trx-like proteins in chloroplasts as well as the typical Trxs (25, 26). Among these Trx-like proteins, Trx-like 2 (TrxL2) and atypical Cys-His–rich Trx (ACHT) from Arabidopsis were found to be the oxidizing factors for several Trx target proteins. Both TrxL2 isoforms (TrxL2.1 and TrxL2.2) have been shown to oxidize the proteins involved in the Calvin–Benson cycle (Rubisco activase, fructose 1,6-bisphosphatase (FBPase), and sedoheptulose 1,7-bisphosphatase (27)) and oxidative pentose phosphate pathway (glucose-6-phosphate dehydrogenase (28)), and glycolytic pathway (phosphofructokinase) (29). Among five ACHT isoforms, ACHT1 and ACHT2 can also oxidize FBPase (30). These proteins are oxidants with the following common features: (i) possession of a Trx-like motif of CxxC instead of the typical active site sequence, (ii) a higher midpoint redox potential than that of the typical Trxs and Trx target proteins (TrxL2.1: −258 mV, TrxL2.2: −245 mV, ACHT1: −252 mV, and ACHT2: −247 mV at pH 7.5), and (iii) a higher efficiency in terms of reducing 2-Cys peroxyiredoxin (2CP) (27, 30, 31). 2CP is responsible for detoxifying hydrogen peroxide (H₂O₂) reductively because it is the most abundant peroxiredoxin in chloroplasts (32). Thus, the oxidation process involving thiol-modulated enzymes in chloroplasts is now thought to be due to the transfer of reducing equivalents from the reduced-form enzymes to H₂O₂ via Trx-like proteins and 2CP. This oxidation process is assumed to be always functional under conditions where H₂O₂ is generated, such as in a photosynthetic environment. However, under light conditions, the reducing power supplied by the photosynthetic electron transfer system exceeds the final oxidizing power of H₂O₂, resulting in the reduction of various chloroplast enzymes. In contrast, as the transition from light to dark conditions occurs, the supply of reducing power decreases, and the oxidation process of these enzymes becomes dominant (33).

In terms of the redox regulation of CF₆₀CF₁, it is known that the dynamics of reduction occurring at the light-to-dark transition and those of oxidation occurring at the light-to-dark transition differ significantly in this enzyme relative to those in other stromal redox-regulated enzymes in chloroplasts (34). However, the molecular mechanisms underlying these dynamic responses are unclear. Therefore, a thorough analysis of this regulation system is needed to improve our understanding of how the ATP synthesis reaction in chloroplasts is regulated in response to fluctuations in light. In the present study, we focused on the capacity of Trx-like proteins and typical Trxs to oxidize CF₁–γ in vitro. We also examined the influence of ΔμH⁺ formation across the thylakoid membrane on the oxidation of CF₁–γ. Our results provide important insights into the relationship between the redox regulation of CF₆₀CF₁ and ΔμH⁺ formation in the thylakoid membrane.

Results
Thylakoid membranes from leaves infiltrated with reductants show clear H⁺ pump activity

We intended to prepare thylakoid membranes containing the reduced form of CF₆₀CF₁ from spinach leaves; however, CF₆₀CF₁ was oxidized entirely when the thylakoid membranes were isolated from untreated spinach leaves without light irradiation or reducing agent treatment (Fig. 1A, labeled as “Untreated”). For the reduction, spinach leaves were irradiated at 1000 to 1500 μmol photons m⁻² s⁻¹ for 10 min and infiltrated with 20 mM of reduced DTT (DTTred) under vacuum conditions. Thylakoid membranes were then prepared from the leaves, and DTTred was removed in a subsequent washing step. The redox state of CF₁–γ in the thylakoid membranes was confirmed using the thiol-modifying reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS). Using this method, we successfully isolated thylakoid membranes in which at least 80% of the contained CF₁–γ was in the reduced form [Fig. 1A, labeled as “+ DTTred (Vac).”]. In contrast, CF₁–γ was in the fully oxidized form when the infiltration of a DTTred-free solution was used without irradiation [Fig. 1A, labeled as “− DTTred (Vac).”].

Next, we assessed the ATP-driven H⁺ pump activity in each thylakoid membrane preparation (Fig. 1B). When thylakoid membranes are supplemented with ATP, proton translocation into the thylakoid lumen and the ATP hydrolysis reaction should occur simultaneously. The resulting proton gradient formed across the thylakoid membrane can be detected using the ΔpH indicator 9-amino-6-chloro-2-methoxyacridine (ACMA), the fluorescence of which is quenched by protonation (35). As shown in Figure 1B, when ATP was added to the thylakoid membranes, two quenching phases were observed: a rapid phase immediately after ATP addition and a subsequent gradual phase. The rapid phase can be attributed to a direct interaction between ATP and ACMA, whereas the gradual phase is thought to be due to H⁺ pump activity caused by CF₆₀CF₁ (36). Among the membrane preparations from leaves treated differently, only thylakoid membranes containing the reduced form of CF₆₀CF₁ exhibited high H⁺ pump activity and restored fluorescence intensity with the addition of an uncoupler, namely carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), implying that the H⁺ pump activity in
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Figure 1. Manipulation of the redox state of CF₁-γ in the thylakoid membrane. A, determination of the redox state of CF₁-γ in the thylakoid membrane. Thylakoid membranes were isolated from spinach leaves using three different methods (untreated, DTTred infusion, and infusion without DTTred) as described in the Experimental procedures. After the modification of the free thiols of thylakoid proteins with AMS, proteins were subjected to nonreducing SDS-PAGE, and the redox state of CF₁-γ was visualized by Western blotting. Unmodified samples dissolved in nonreducing SDS sample buffer without AMS were also loaded (labeled as "AMS−"). B, ATP-driven H⁺-pump activity measurements taken in the thylakoid membrane. Acidification of the thylakoid lumen was monitored using fluorescence quenching of ACMA (excitation at 410 nm, emission at 480 nm) at 25 °C. The reaction was initiated by the addition of 5 mM ATP, and the lumen acidification was dissipated by the addition of 5 μM FCCP to the thylakoid membrane. AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; ACMA, 9-amino-6-chloro-2-methoxyacridine; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Ox, oxidized form; Red, reduced form.

TrxL2 and ACHT oxidize CF₁-γ efficiently under uncoupled conditions

We reconstructed an in vitro oxidation assay system to evaluate the ability of Trx-like proteins to oxidize the CF₁-γ of CF₀CF₁ in thylakoid membranes. The thylakoid membranes containing reduced CF₁-γ was incubated with each isoform of TrxL2 and ACHT at various concentrations (0–500 nM) or for various incubation periods (0–900 s) in the presence of 50 μM of oxidized DTT (DTTox) as a final oxidation power (Figs. 2 and 3 and Fig. S1). To form the ΔµH⁺ across the thylakoid membrane, the reaction solution was supplemented with an artificial electron mediator, 1-methoxy-5-methylphenazinium methylsulfate, and the thylakoid membranes were irradiated at 600 to 650 μmol photons m⁻² s⁻¹ (13, 20, 37). To achieve control conditions under which no ΔµH⁺ was formed, the same experiments were performed under light conditions in the presence of FCCP or under dark conditions. We confirmed that the chemical oxidant DTTox could not oxidize CF₁-γ when used alone, especially under conditions in which the ΔµH⁺ was

Figure 2. CF₁-γ oxidation by DTTox in the presence and absence of the ΔµH⁺. A, visualization of CF₁-γ oxidation by DTTox. Thylakoid membranes (50 μg Chl/ml) were incubated with 50 μM DTTox for 15 min in the presence or absence of the ΔµH⁺. After the modification of the free thiols of the proteins residing on the thylakoid membrane with AMS, proteins were subjected to nonreducing SDS-PAGE, and the redox state of CF₁-γ was visualized by Western blotting. Unmodified samples dissolved in nonreducing SDS sample buffer without AMS were also loaded (labeled as "AMS−"). B, quantification of the redox state of CF₁-γ. The CF₁-γ reduction level shown in (A) was quantified as the ratio of the reduced form to the total and plotted against the reaction time. Each value represents the mean ± SD (n = 7–8). AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; Ox, oxidized form; Red, reduced form.
formed (Fig. 2). Conversely, all Trx-like proteins examined in this study, namely TrxL2.1, TrxL2.2, ACHT1, and ACHT2, were able to oxidize CF1-γ under the appropriate conditions; however, each protein showed a different affinity for CF1-γ oxidation (Fig. 3, A and B) and a different kinetic pattern (Fig. 3, C and D). Furthermore, these oxidation patterns were affected by the ΔμH⁺ formation conditions. Therefore, we determined the half concentration (S₁/₂) and...
Typical Trxs fail to oxidize CF1-γ

We also examined the ability of typical Trxs to oxidize CF1-γ using the same method described above (Fig. 4). First, we investigated CF1-γ oxidation by Trx-f, which is recognized as a major reducing mediator of many redox enzymes in chloroplasts, including CF1-γ (19). Trx-f oxidized CF1-γ slightly in the absence of the ΔμH⁺, but the kinetic parameters of this oxidation could not be determined due to its low efficiency (Fig. 4, A–D). In addition, we examined the effects of the following Trxs: Trx-m, which is the most abundant chloroplast Trx in Arabidopsis (38) and has been shown to reduce CF1-γ (20), and Trx-x and Trx-γ, which are thought to be a part of the antioxidant system because they efficiently reduce 2CP and peroxiredoxin Q, respectively (19, 39, 40). Trx-z was not considered in the present study because it is known to function as part of an RNA polymerase complex (41). As shown in Figure 4, E and F, Trx-m1, Trx-x, and Trx-y2 did not oxidize CF1-γ effectively. Thus, we conclude that the typical Trxs present in chloroplasts do not function in CF1-γ oxidation.

Discussion

Thiol-based reductive activation and oxidative deactivation of CFoCF1 are believed to play crucial roles in the efficient management of ATP production and ΔμH⁺ consumption during photosynthesis. However, the complete regulation mechanism has not been determined because biochemical analysis has been lacking, especially analysis of the CFoCF1 oxidation process. In the present study, we characterized the kinetics of CF1-γ oxidation by two physiological oxidants, namely the proteins Trxl2 and ACHT.

First, we established a procedure for preparing thylakoid membranes containing CFoCF1 for which the redox state is controlled artificially. Using our thylakoid membrane preparations, we confirmed that reduced CFoCF1 generates the ΔμH⁺ across the thylakoid membrane via ATP hydrolysis and H⁺ translocation (Fig. 1). Second, we performed oxidation experiments using the thylakoid membrane preparations because light irradiation enabled the formation of a steady ΔμH⁺ across the thylakoid membrane. CF1-γ was oxidized efficiently by Trxl2 and ACHT, especially under uncoupled conditions (Figs. 2 and 3). The formation of the ΔμH⁺ across the thylakoid membrane is known to be a prerequisite for reductive activation of CFoCF1 by Trx (20, 21); thus, the present results imply that oxidation and reduction of CFoCF1 are inversely dependent on the ΔμH⁺ formed across the thylakoid membrane. A similar result was reported previously in relation to the redox dynamics of CF1-γ in spinach intact chloroplasts when the chloroplasts were exposed to light, and adding an uncoupler to the chloroplasts induced the rapid oxidation of CF1-γ even under light conditions, although the reduction level of the stromal redox-regulated enzyme FBPase was not affected by adding FCCP to the chloroplasts (42). Differences

Table 1
Kinetic parameters of Trx-like protein-dependent oxidation in CF1-γ

| Species | Conditions | ΔμH⁺ (nM) | Significance by condition (p < 0.05) | [R] | t1/2 (s) | Significance by condition (p < 0.05) | | |
|---------|------------|-----------|---------------------------------|------|---------|---------------------------------|------|--------|------|--------|
| Trxl2.1| Light, −FCCP Formed | 7.1 ± 2.5 | a                           | 0.993 | 9.4 ± 1.1 | a                           | 0.986 |        |      |        |
|         | Light, +FCCP Not formed | 7.1 ± 3.8 | a                           | 0.865 | 8.4 ± 1.0 | a                           | 0.994 |        |      |        |
|         | Dark, −FCCP Not formed | 8.9 ± 1.0 | a                           | 0.966 | 9.9 ± 1.9 | a                           | 0.964 |        |      |        |
| Trxl2.2| Light, −FCCP Formed | 24.8 ± 10.6 | a                          | 0.976 | 114.9 ± 21.9 | a                           | 0.955 |        |      |        |
|         | Light, +FCCP Not formed | 8.1 ± 2.0 | a                           | 0.878 | 14.4 ± 3.0 | b                           | 0.995 |        |      |        |
|         | Dark, −FCCP Not formed | 12.5 ± 5.1 | a                           | 0.977 | 17.9 ± 2.1 | b                           | 0.991 |        |      |        |
| ACHT1   | Light, −FCCP Formed | 110.8 ± 43.4 | a                         | 0.973 | 433.2 ± 62.5 | a                           | 0.964 |        |      |        |
|         | Light, +FCCP Not formed | 22.7 ± 4.6 | b                           | 0.941 | 88.9 ± 37.5 | b                           | 0.990 |        |      |        |
|         | Dark, −FCCP Not formed | 48.1 ± 8.7 | ab                         | 0.987 | 625 ± 64 | b                           | 0.967 |        |      |        |
| ACHT2   | Light, −FCCP Formed | 272.9 ± 50.2 | a                         | 0.916 | 688.9 ± 385.9 | a                           | 0.855 |        |      |        |
|         | Light, +FCCP Not formed | 50.1 ± 8.0 | b                           | 0.920 | 70.3 ± 15.3 | b                           | 0.984 |        |      |        |
|         | Dark, −FCCP Not formed | 66.1 ± 6.4 | b                           | 0.974 | 59.2 ± 11.3 | b                           | 0.974 |        |      |        |

Data were analyzed using one-way ANOVA and Tukey’s honest significance difference test (p < 0.05). Different letters indicate significant differences among the same Trx-like proteins under different ΔμH⁺ formation conditions. The letters “a” and “b” mean that the values are significantly different, and “ab” means that the values are not significantly different from both “a” and “b.”
in the redox dynamics of membrane-embedded CF$_1$ and other stromal Trx target proteins in *Arabidopsis* plants were also observed under artificial light-controlled conditions mimicking those in the field (34). Interestingly, while other stromal redox-regulated enzymes were oxidized gradually as light intensity decreased, only CF$_1$-$\gamma$ maintained an almost fully reduced state even under weak light conditions and was eventually oxidized when light was turned off. This $\Delta$H$^+$-dependent oxidative deactivation of CF$_1$-CF$_1$ also likely functions *in vivo* to avoid wasteful ATP hydrolysis in plants under nonphotosynthetic conditions.

Using our *in vitro* assay system, we were able to estimate the apparent affinity between Trx-like proteins and CF$_1$-$\gamma$, finding that this affinity increased markedly under uncoupled conditions (Fig. 3 and Table 1). However, among the proteins examined in the present study, TrxL2.1 did not show this

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**Figure 4.** CF$_1$-$\gamma$ oxidation assisted by typical Trxs in the presence and absence of the $\Delta$H$^+$. A, Trx-f1 concentration-dependence of CF$_1$-$\gamma$ oxidation. Thylakoid membranes (50 μg Chl/ml) were incubated with the indicated concentrations of Trx-f1 and 50 μM DTTox for 15 min in the presence or absence of the $\Delta$H$^+$. The redox state of CF$_1$-$\gamma$ was visualized as described in the *Figure 2A* legend. B, quantification of the CF$_1$-$\gamma$ redox state. The CF$_1$-$\gamma$ reduction level shown in (A) was quantified as described in the *Figure 2B* legend. C, time course of CF$_1$-$\gamma$ oxidation by Trx-f1. Thylakoid membranes (50 μg Chl/ml) were incubated with 500 nM Trx-f1 and 50 μM DTTox for the indicated time in the presence or absence of the $\Delta$H$^+$, and the redox state of CF$_1$-$\gamma$ was visualized as described in the *Figure 2A* legend. D, quantification of the CF$_1$-$\gamma$ redox state. The CF$_1$-$\gamma$ reduction level shown in (C) was quantified as described in the *Figure 2B* legend. E, CF$_1$-$\gamma$ oxidation assisted by the other Trxs. Thylakoid membranes (50 μg Chl/ml) were incubated with 500 nM Trx isoforms and 50 μM DTTox for 15 min in the presence or absence of the $\Delta$H$^+$, and the redox state of CF$_1$-$\gamma$ was visualized as described in the *Figure 2A* legend. F, quantification of the CF$_1$-$\gamma$ redox state. The CF$_1$-$\gamma$ reduction level was quantified as described in the *Figure 2B* legend. Different letters indicate significant differences (p < 0.05; one-way ANOVA and Tukey’s honest significance difference test). A, C and E, Ox, oxidized form; Red, reduced form. B, D and F, each value represents the mean ± SD [n = 3 or 4 (B); n = 3 (D and F)].
tendency, and no $\Delta\mu H^+$-dependent change in $S_{1/2}$ values was observed in association with this protein. This may have been due to the higher affinity between TrxL2.1 and CF1-γ compared with that between the other Trx-like proteins and CF1-γ. Trxl2.1 and Trxl2.2 share a common atypical Trx motif, WCRK, but they show only about 53% identity. The difference in kinetic parameters between Trxl2 isoforms may be due to this amino acid sequence difference. We also found that none of the typical Trxs tested oxidized CF1-γ efficiently (Fig. 4), which is consistent with a previous study (24), whereas Trx-f and Trx-m have been shown to reduce CF1-γ under $\Delta\mu H^+$ formation conditions through physical interactions (20). Conversely, both Trx-f and Trx-m have been reported to oxidize their specific target proteins (30, 43). Therefore, further studies on the conformational changes in CF1-γ that regulate the affinity with Trx-like proteins and Trxs are required. The $\Delta\mu H^+$ formation across the thylakoid membrane is known to induce the conformational changes in CF1-γ (44–46). Komatsu-Takaki reported that these structural changes were achieved within about 1 to 30 s of light irradiation of the thylakoid membrane (45). However, the atomic-level structure of CFoCF1 when energized by $\Delta\mu H^+$ remains to be clarified. The conformational change of CF1-γ induced by the $\Delta\mu H^+$ formation may alter its midpoint redox potential more positively. In contrast, cryo-EM studies have revealed structural differences between the reduced and oxidized forms of spinach CFoCF1 in the nonenergizing state (47, 48). In the structures shown in (48), the short β hairpin loop of CF1-γ is destabilized when the disulfide bond formed by two Cys at the redox switch is reduced. In addition, three negatively charged amino acids (Glu$^{210}$, Asp$^{211}$, and Glu$^{212}$ in spinach CF1-γ) located at the end of the short β hairpin loop structure change their interactions with the surrounding amino acids depending on the redox state of the switch. The importance of these charged amino acids in redox regulation was reported in several studies (49–52). The redox state of CF1-γ may also regulate the affinity for Trxs; however, further structural analyses are needed to reveal the underlying mechanism more clearly.

Reverse-genetic studies in Arabidopsis have provided important insights into the oxidative regulation of CF1-γ by Trx-like proteins. Yokochi et al. generated separate Arabidopsis mutant plants deficient in TrxL2 and ACHT using the CRISPR/Cas9 system and tested the redox dynamics of various Trx target proteins in each mutant strain in response to a light-to-dark transition (53). They found that TrxL2 deficiency retarded the in vivo oxidation of CF1-γ, whereas ACHT deficiency did not have this effect. These results are consistent with our finding that TrxL2 has a high affinity for CF1-γ (Fig. 3 and Table 1). However, CF1-γ was almost fully oxidized in dark-adapted TrxL2-deficient plants (53), suggesting that other oxidation factors, such as ACHT, may also be involved in CF1-γ oxidation in vivo. Although TrxL2 and ACHT are about 10-fold less abundant in vivo than typical Trxs and their target proteins (27, 53), they may more efficiently oxidize CF1-γ if the affinity of CF1-γ with Trx-like proteins and typical Trxs changes due to $\Delta\mu H^+$ dissipation. In 2CP-knockdown mutant plants generated by T-DNA insertion, CF1-γ was found to be oxidized with a delay (27). Collectively, these results indicate that CF1-γ oxidation in vivo is accomplished primarily via the Trxl2/2CP cascade.

Based on the present findings, we propose a new schematic model of the redox regulation of CFoCF1 (Fig. 5) with reference to the model proposed previously by Junesch and Gräber (10). In the oxidation process, $\Delta\mu H^+$ dissipation causes a conformational change at CF1-γ, and Trx-like proteins then act to oxidize the redox switch on CF1-γ. Overall, our study provides an overview of the specific mechanism underlying the inactivation of CFoCF1 as well as insights into the energetic strategy of plants in response to fluctuations in light conditions.

**Experimental procedures**

**Preparation of thylakoid membranes containing the reduced form of CFoCF1**

Fresh market spinach was washed well and left overnight in the dark at 4 °C. Harvested leaves (about 10 g in fresh weight) were irradiated with 1000 to 1500 μmol photons m$^{-2}$ s$^{-1}$ using a LED illuminator for 10 min at room temperature. The leaves were then immediately immersed in a reducing solution containing 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM MgCl$_2$, 10 mM NaCl, and 20 mM DTT$_{red}$ and vacuum-infiltrated for 3 min in this solution. Subsequently, the leaves were homogenized three times for 3 s in a mixer with 200 ml of the reducing solution at 4 °C. The homogenate was then filtered through four layers of gauze and centrifuged at 3000 g and 4 °C for 10 min. The pellet was suspended using the reducing solution and centrifuged at 300g and 4 °C for 1 min. The supernatant was collected and centrifuged at 3000 g and 4 °C for 10 min. The abovementioned wash step was performed twice with a non-reducing wash solution containing 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM MgCl$_2$, and 2-Cys peroxiredoxin; H$_2$O$_2$, hydrogen peroxide.

**Fig. 5. Scheme of the redox regulation of CFoCF1 adapted from that of Junesch and Gräber** (10). Oxidative deactivation of CFoCF1 by Trx-like proteins promoted by $\Delta\mu H^+$ dissipation is included in the scheme. Straight arrows indicate the direction of change in CFoCF1 activation states. Curved arrows indicate the flow of reducing power. Triangles labeled with ‘$H^+$’ indicate the formation of $\Delta\mu H^+$ across the thylakoid membrane. 2CP, 2-Cys peroxiredoxin; H$_2$O$_2$, hydrogen peroxide.
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10 mM NaCl at 4 °C. The resulting pellet was suspended in the nonreducing wash solution with a chlorophyll (Chl) concentration of 0.5 mg/ml. The preparation was left in the dark on ice for at least 1 h before use.

Preparation of recombinant Arabidopsis Trx and Trx-like proteins

All expression plasmids for Trx-like proteins and typical Trxs used in this work (TrxL2.1, At5g06690; TrxL2.2, At5g04260; ACHT1, At4g26160; ACHT2, AT4G29670.1; Trx-3, At3g02730; Trx-At1g03680; Trx-x, At1g50320; and Trx-γ2, At1g43560) were constructed as described previously (19, 27, 30, 54). Each expression plasmid was transformed into Escherichia coli strain BL21(DE3), and transformed cells were cultured at 37 °C. The desired protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside followed by a further culture at 21 °C overnight. The cells were then disrupted using sonication and centrifuged at 125,000g for 40 min, after which the resulting supernatant was used in protein purification. TrxL2.1 and TrxL2.2 proteins were purified using a Ni-nitrilotriacetic acid affinity chromatography step conducted using a Superdex 75 column 10/300 (GE Healthcare). ACHT1 and ACHT2 proteins were purified using a combination of Ni-nitrilotriacetic acid affinity chromatography, cation-exchange chromatography, and size-exclusion chromatography as described previously (30). The other Trx proteins contained no affinity tag and were purified using a combination of anion-exchange chromatography, cation-exchange chromatography, or hydrophobic-interaction chromatography as described previously (19, 20, 54). The concentrations of purified proteins were determined using a BCA protein assay (Pierce).

Measurement of H+ pump activity in thylakoid membranes

The ATP-driven H+ pump activity of CFoCF1 in the thylakoid membranes was measured according to the fluorescence quenching of ACMA based on a previous method with some modifications (36, 55). The emitted fluorescence of ACMA (excitation at 410 nm, emission at 480 nm) was measured using a FP-8500 spectrofluorometer (Jasco), in which the temperature in the sample chamber was 25 °C. At 180 s after the initiation of the measurement, 5 mM ATP was added to the reaction solution containing 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM MgCl2, 10 mM NaCl, 0.3 μg ml⁻¹ ACMA, and 20 μg Chl/ml of thylakoid membranes. After the measurement was continued for 15 min, 5 μM FCCP was added, and the stable fluorescence intensity following the addition of FCCP was taken as 1.0. The reaction solution was stirred continuously during the measurement.

Determination of the CF1-γ redox state in thylakoid membranes

To quantify the redox state ratio of CF1-γ in thylakoid membranes, a thylakoid membrane solution was mixed with an equal volume of 20% trichloroacetic acid (TCA) and left on ice for 30 min. The TCA precipitants were then washed with ice-cold acetone, and the resulting precipitated proteins were labeled with AMS using the following procedure. The precipitants were suspended in nonreducing SDS sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 7.5% (v/v) glycerol, and 0.01% (w/v) bromophenol blue] containing 2 mM of AMS. After incubation for 30 min at room temperature, protein samples were boiled for 5 min at 95 °C. Proteins were then separated using SDS-PAGE and transferred to a PVDF membrane. Antibodies against CF1-γ (34) were used to perform Western blotting. Chemiluminescence was detected using horseradish peroxidase–conjugated secondary antibodies and ECL Prime (GE Healthcare) and visualized on an LAS 3000 Mini Imaging System (Fuji Film). The resultant band intensities were quantified using ImageJ.

In vitro assay of CFoCF1 oxidation by Trx-like proteins and Trxs

The nonreducing wash solution used in the preparation of thylakoid membranes was degassed for 1 h at room temperature, and the following reactions were performed in this solution. Prior to the oxidation assay, 150 to 5000 nM of Trx-like proteins or Trx were incubated for 10 min at 25 °C with a DTTox mixture containing 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM MgCl2, 10 mM NaCl, and 500 μM DTTox. Subsequently, 100 μl of the DTTox mixture containing Trx-like proteins or Trx was added to 900 μl of a thylakoid solution to initiate the oxidation reaction. The composition of the final reaction mixture was 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM MgCl2, 10 mM NaCl, 100 μM 1-methoxy-5-methylphenazinium methylsulfate, 50 μg Chl/ml of thylakoid membranes, 50 μM DTTox, and 15 to 500 mM Trx-like proteins or Trx. The oxidation reaction was performed for a specific time (0–900 s) at 25 °C and terminated by adding 10% TCA. For the formation of a ΔμH+ across the membrane, the thylakoid solution was irradiated with 600 to 650 μmol photons m⁻² s⁻¹ using a LED illuminator while stirring 5 min before the start of the reaction. The irradiation was then continued during the reaction. The followings were examined to assess uncoupled conditions: 5 μM FCCP was added to the reaction mixture or the reaction tube was wrapped in aluminum foil and placed in a dark room.

Data availability

All data are contained within the article and can be shared upon request (thisabor@res.titech.ac.jp).

Supporting information—This article contains supporting information.

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