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

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Chloroplast F_oF_1-ATP synthase (CF_oCF_1) uses an electrochemical gradient of protons across the thylakoid membrane ($\Delta\mu H^+$) as an energy source in the ATP synthesis reaction. CF_oCF_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 $\Delta\mu H^+$ is formed by the photosynthetic electron transfer chain under light conditions, CF_1-γ is reduced by thioredoxin (Trx), and the entire CF_oCF_1 enzyme is activated. The redox regulation of CF_oCF_1 is a key mechanism underlying the control of ATP synthesis under light conditions. In contrast, the oxidative deactivation process involving CF_oCF_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_oCF_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 $\Delta\mu H^+$ formation across the thylakoid membrane was manipulated artificially. Collectively, these results indicate that the formation status of the $\Delta\mu H^+$ controls the redox regulation of CF_oCF_1 to prevent energetic disadvantages in plants.

According to the chemiosmotic theory, chloroplast F_oF_1-ATP synthase (CF_oCF_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 ($\Delta\mu H^+$) under light conditions, the CF_oCF_1 embedded in this membrane synthesizes ATP using the gradient as a driving force (3). The regulation of CF_oCF_1 activity is important for two reasons. First, when the $\Delta\mu H^+$ is insufficient to drive ATP synthesis, CF_oCF_1 can catalyze the reverse reaction, ATP hydrolysis, coupled with $\Delta\mu H^+$ formation as a general mechanism of F_oF_1-ATP synthase (3). Second, the CF_oCF_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_6f 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_oCF_1 seems to be controlled tightly according to the $\Delta\mu H^+$ level across the thylakoid membrane, that is, under both light and dark conditions.

CF_oCF_1 is a molecular motor enzyme, and the c-ring portion of F_o rotates during the proton translocation from the lumen 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_oCF_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_oCF_1 activity using isolated spinach thylakoid membranes, finding that the apparent V_{max} was the same for the oxidized and reduced forms of the enzymes but that the $\Delta\mu H^+$ 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_oCF_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-*y*, 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 CF_1-γ (20). It is also known that $\Delta\mu H^+$ formation across the thylakoid membrane is a prerequisite for the reduction of CF_1-γ (20, 21). Thus, CF_oCF_1 is active only under light conditions.

In contrast to the light-induced CF_oCF_1 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 CF_oCF_1, is poorly understood, and the proteins involved in this oxidation have not been assigned. In the 1980s, the *in vivo* redox state of CF_1-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 CF_1-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), 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 peroxiredoxin (2CP) (27, 30, 31). 2CP is responsible for detoxifying hydrogen peroxide (H_2O_2) 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 H_2O_2 *via* Trx-like proteins and 2CP. This oxidation process is assumed to be always functional under conditions where H_2O_2 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 H_2O_2, 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 CF_oCF_1, it is known that the dynamics of reduction occurring at the dark-to-light 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 CF_1-γ *in vitro*. We also examined the influence of $\Delta\mu H^+$ formation across the thylakoid membrane on the oxidation of CF_1-γ. Our results provide important insights into the relationship between the redox regulation of CF_oCF_1 and $\Delta\mu H^+$ 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 CF_oCF_1 from spinach leaves; however, CF_oCF_1 was oxidized entirely when the thylakoid membranes were isolated from untreated spinach leaves without light irradiation or reducing agent treatment (Fig. 1*A*, 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 (DTT_{red}) under vacuum conditions. Thylakoid membranes were then prepared from the leaves, and DTT_{red} was removed in a subsequent washing step. The redox state of CF_1-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 CF_1-γ was in the reduced form [Fig. 1*A*, labeled as "+ DTT_{red} (Vac.)"]. In contrast, CF_1-γ was in the fully oxidized form when the infiltration of a DTT_{red}-free solution was used without irradiation [Fig. 1*A*, labeled as "– DTT_{red} (Vac.)"].

Next, we assessed the ATP-driven H^+ pump activity in each thylakoid membrane preparation (Fig. 1*B*). 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 1*B*, 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 CF_oCF_1 (36). Among the membrane preparations from leaves treated differently, only thylakoid membranes containing the reduced form of CF_oCF_1 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
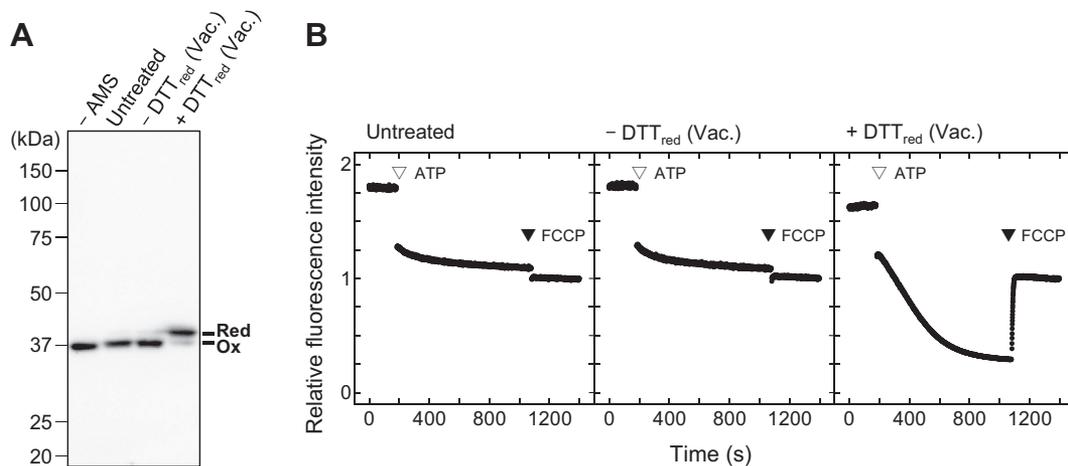
Figure 1. Manipulation of the redox state of CF$_1$-γ in the thylakoid membrane. *A*, determination of the redox state of CF$_1$-γ in the thylakoid membrane. Thylakoid membranes were isolated from spinach leaves using three different methods (untreated, DTT$_{red}$ infusion, and infusion without DTT$_{red}$) 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 CF$_1$-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.

thylakoid membranes depends on the CF$_1$-γ redox state. Therefore, we used the thylakoid membranes isolated using this reduction method in the following *in vitro* oxidation assay.

TrxL2 and ACHT oxidize CF$_1$-γ efficiently under uncoupled conditions

We reconstituted an *in vitro* oxidation assay system to evaluate the ability of Trx-like proteins to oxidize the CF$_1$-γ of CF$_o$CF$_1$ in thylakoid membranes. The thylakoid membranes containing reduced CF$_1$-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 (DTT$_{ox}$) 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 DTT$_{ox}$ could not oxidize CF$_1$-γ when used alone, especially under conditions in which the ΔμH$^+$ was
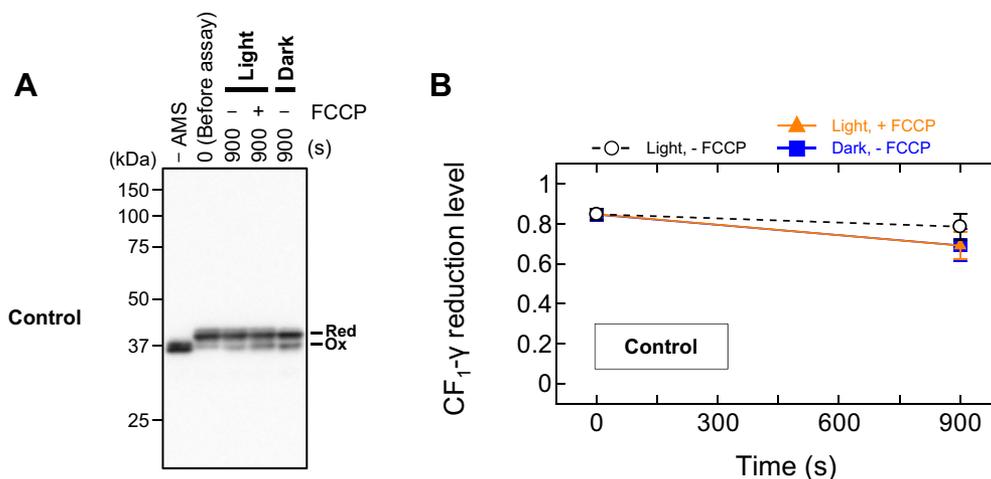

Figure 2. CF$_1$-γ oxidation by DTT$_{ox}$ in the presence and absence of the ΔμH$^+$. *A*, visualization of CF$_1$-γ oxidation by DTT$_{ox}$. Thylakoid membranes (50 μg Chl/ml) were incubated with 50 μM DTT$_{ox}$ for 15 min in the presence or absence of the ΔμH$^+$. After the modification of the free thiols of the proteins residing on the thylakoid membrane with AMS, proteins were subjected to nonreducing SDS-PAGE, and the redox state of CF$_1$-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 CF$_1$-γ. The CF$_1$-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.
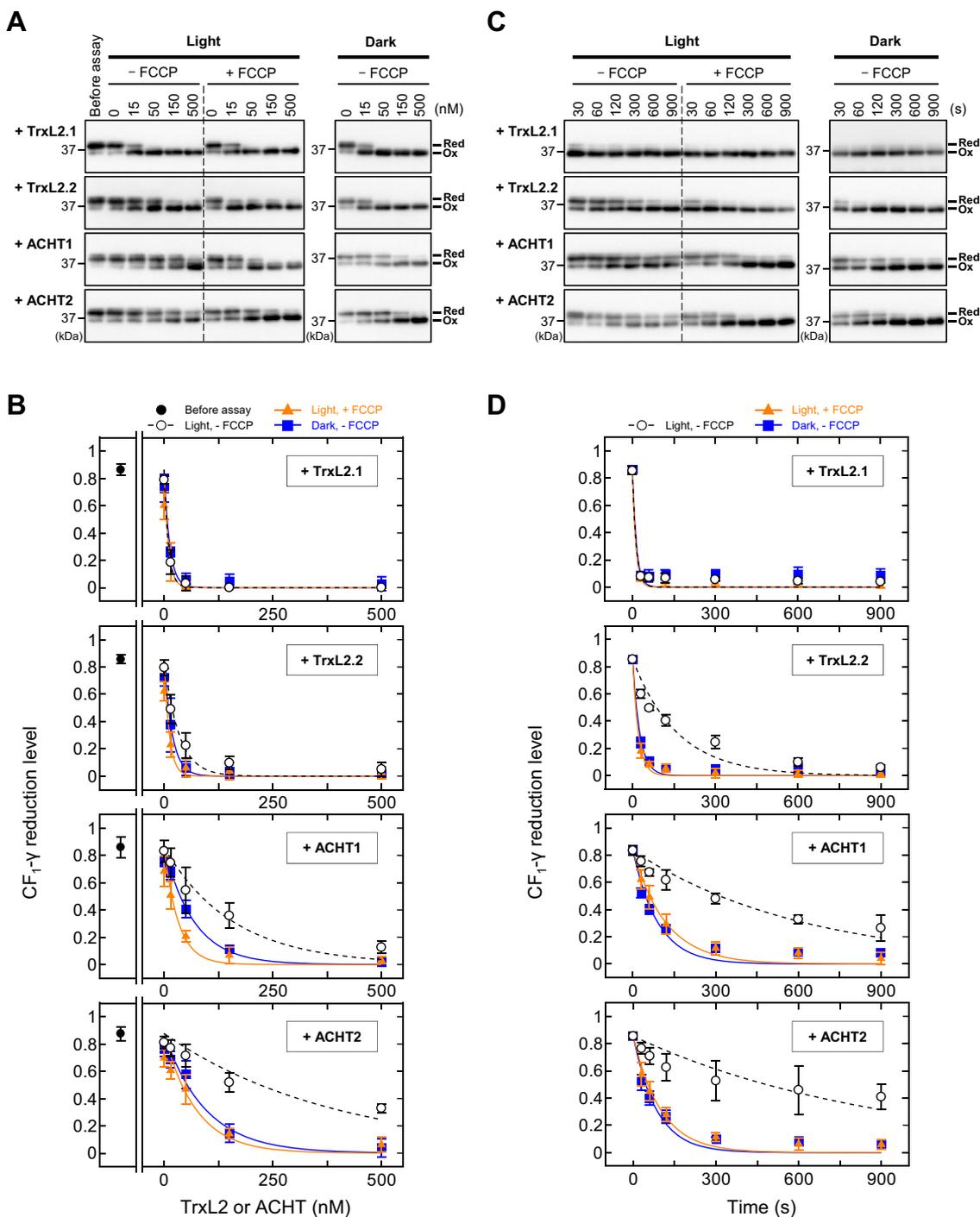
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Figure 3. CF$_1$-γ oxidation assisted by TrxL2 and ACHT in the presence and absence of the ΔμH$^+$. *A*, Trx-like protein concentration-dependence of CF$_1$-γ oxidation. Thylakoid membranes (50 μg Chl/ml) were incubated with the indicated concentrations of Trx-like proteins and 50 μM DTT$_{ox}$ for 15 min in the presence or absence of the ΔμH$^+$, and the redox state of CF$_1$-γ was visualized as described in the Figure 2A legend. *B*, quantification of the redox state of CF$_1$-γ. The CF$_1$-γ reduction level shown in (*A*) was quantified as described in the Figure 2B legend. *C*, time course of CF$_1$-γ oxidation by Trx-like proteins. Thylakoid membranes (50 μg Chl/ml) were incubated with 500 nM Trx-like proteins and 50 μM DTT$_{ox}$ for the indicated time in the presence or absence of the ΔμH$^+$, and the redox state of CF$_1$-γ was visualized as described in the Figure 2A legend. *D*, quantification of the CF$_1$-γ redox state. The CF$_1$-γ reduction level shown in (*C*) was quantified as described in the Figure 2B legend. *A* and *C*, whole images are also shown in Fig. S1. *C* and *D*, each value represents the mean ± SD (n = 3). The exponential fitting curves for the mean values are also shown. 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 CF$_1$-γ under the appropriate conditions; however, each protein showed a different affinity

for CF$_1$-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 ($S_{1/2}$) and

Table 1
Kinetic parameters of Trx-like protein-dependent oxidation in CF$_1$-γ

Species	Conditions	ΔμH$^+$	$S_{1/2}$ (nM)	Significance by condition ($p < 0.05$)	|R|	$t_{1/2}$ (s)	Significance by condition ($p < 0.05$)	|R|
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	0.976	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	62.5 ± 6.4	b	0.967
ACHT2	Light, – FCCP	Formed	272.4 ± 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".

half oxidation time ($t_{1/2}$) of reduction of CF$_1$-γ by fitting the following equation to the plotted data:

$$\{CF_1-\gamma_{red}\} = \{CF_1-\gamma_{red}\}_0 \times e^{-kv}$$

where $\{CF_1-\gamma_{red}\}$ is the reduction level of CF$_1$-γ, $\{CF_1-\gamma_{red}\}_0$ is the reduction level of CF$_1$-γ before the assay, k is the rate constant determined by each curve fitting, and v is the variable (reaction time or concentration of the Trx-like protein). When the ΔμH$^+$ was canceled through the addition of FCCP or not formed under dark conditions, TrxL2.2 oxidized CF$_1$-γ rapidly; however, this rapid oxidation was suppressed by the ΔμH$^+$ formed under light conditions. These differences were evident in the analysis of $t_{1/2}$ values (Table 1). For ACHT1 and ACHT2, changes in oxidation kinetics were similar to those of TrxL2.2, but changes in $S_{1/2}$ values were also observed in a ΔμH$^+$-dependent manner, implying that the affinity between ACHT and CF$_1$-γ is altered according to ΔμH$^+$ formation. In contrast, the oxidation kinetics of CF$_1$-γ by TrxL2.1 differed from those by the other three Trx-like proteins, and a ΔμH$^+$-dependent change in the efficiency of CF$_1$-γ oxidation was not detected due to the rapid oxidation reaction. Therefore, we measured the $t_{1/2}$ values again using TrxL2.1 at a lower concentration, 50 nM (Fig. S2 and Table S1), finding similar ΔμH$^+$-dependent oxidation of CF$_1$-γ. These results suggest that the oxidation of CF$_1$-γ by Trx-like proteins is strongly affected by the formation/dissipation of the ΔμH$^+$ across the thylakoid membrane, and the affinity of Trx-like proteins to CF$_1$-γ may also be affected by the ΔμH$^+$.

Typical Trxs fail to oxidize CF$_1$-γ

We also examined the ability of typical Trxs to oxidize CF$_1$-γ using the same method described above (Fig. 4). First, we investigated CF$_1$-γ oxidation by Trx-*f*, which is recognized as a major reducing mediator of many redox enzymes in chloroplasts, including CF$_1$-γ [19]. Trx-*f*1 oxidized CF$_1$-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 CF$_1$-γ

[20], and Trx-*x* and Trx-*y*, 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-*m*1, Trx-*x*, and Trx-*y*2 did not oxidize CF$_1$-γ effectively. Thus, we conclude that the typical Trxs present in chloroplasts do not function in CF$_1$-γ oxidation.

Discussion

Thiol-based reductive activation and oxidative deactivation of CF$_o$CF$_1$ 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 CF$_o$CF$_1$ oxidation process. In the present study, we characterized the kinetics of CF$_1$-γ oxidation by two physiological oxidants, namely the proteins TrxL2 and ACHT.

First, we established a procedure for preparing thylakoid membranes containing CF$_o$CF$_1$ for which the redox state is controlled artificially. Using our thylakoid membrane preparations, we confirmed that reduced CF$_o$CF$_1$ 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. CF$_1$-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 CF$_o$CF$_1$ by Trx [20, 21]; thus, the present results imply that oxidation and reduction of CF$_o$CF$_1$ are inversely dependent on the ΔμH$^+$ formed across the thylakoid membrane. A similar result was reported previously in relation to the redox dynamics of CF$_1$-γ in spinach intact chloroplasts when the chloroplasts were exposed to light, and adding an uncoupler to the chloroplasts induced the rapid oxidation of CF$_1$-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
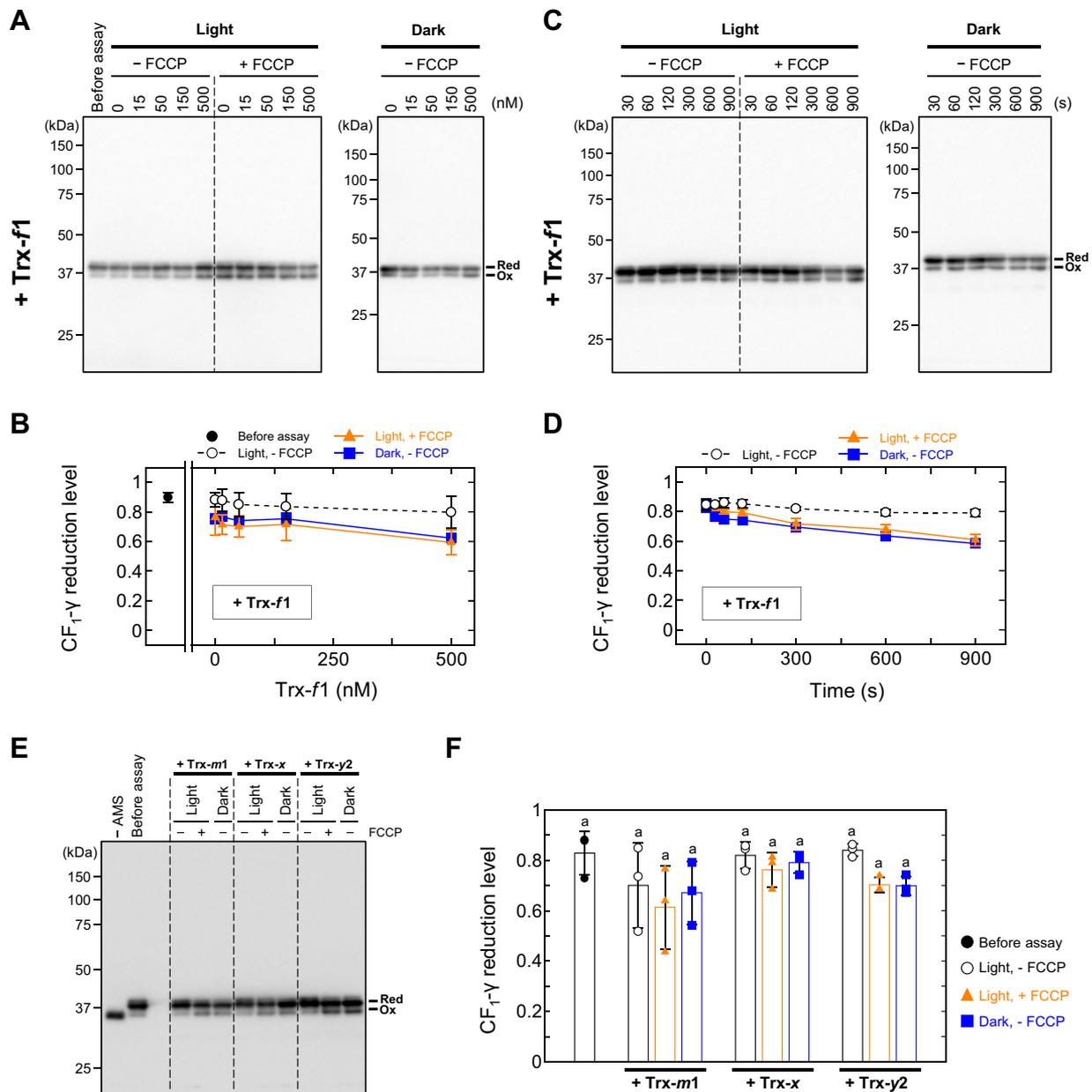
Figure 4. CF$_1$-γ oxidation assisted by typical Trxs in the presence and absence of the ΔμH$^+$. *A*, Trx-*f*1 concentration-dependence of CF$_1$-γ oxidation. Thylakoid membranes (50 μg Chl/ml) were incubated with the indicated concentrations of Trx-*f*1 and 50 μM DTT$_{ox}$ for 15 min in the presence or absence of the ΔμH$^+$, and the redox state of CF$_1$-γ was visualized as described in the Figure 2A legend. *B*, quantification of the CF$_1$-γ redox state. The CF$_1$-γ reduction level shown in (*A*) was quantified as described in the Figure 2B legend. *C*, time course of CF$_1$-γ oxidation by Trx-*f*1. Thylakoid membranes (50 μg Chl/ml) were incubated with 500 nM Trx-*f*1 and 50 μM DTT$_{ox}$ for the indicated time in the presence or absence of the ΔμH$^+$, and the redox state of CF$_1$-γ was visualized as described in the Figure 2A legend. *D*, quantification of the CF$_1$-γ redox state. The CF$_1$-γ reduction level shown in (*C*) was quantified as described in the Figure 2B legend. *E*, CF$_1$-γ oxidation assisted by the other Trxs. Thylakoid membranes (50 μg Chl/ml) were incubated with 500 nM Trx isoforms and 50 μM DTT$_{ox}$ for 15 min in the presence or absence of the ΔμH$^+$, and the redox state of CF$_1$-γ was visualized as described in the Figure 2A legend. *F*, quantification of the CF$_1$-γ redox state. The CF$_1$-γ 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*)].

in the redox dynamics of membrane-embedded CF$_o$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$_1$-γ maintained an almost fully reduced state even under weak light conditions and was eventually oxidized when light was turned off. This

ΔμH$^+$-dependent oxidative deactivation of CF$_o$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$_1$-γ, 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

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 CF_1-γ compared with that between the other Trx-like proteins and CF_1-γ. TrxL2.1 and TrxL2.2 share a common atypical Trx motif, WCRKC, 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 CF_1-γ efficiently (Fig. 4), which is consistent with a previous study (24), whereas Trx-*f* and Trx-*m* have been shown to reduce CF_1-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 CF_1-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 CF_1-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 CF_oCF_1 when energized by $\Delta\mu H^+$ remains to be clarified. The conformational change of CF_1-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 CF_oCF_1 in the nonenergizing state (47, 48). In the structures shown in (48), the short β hairpin loop of CF_1-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 CF_1-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 CF_1-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 CF_1-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 CF_1-γ, whereas ACHT deficiency did not have this effect. These results are consistent with our finding that TrxL2 has a high affinity for CF_1-γ (Fig. 3 and Table 1). However, CF_1-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 CF_1-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 CF_1-γ if the affinity of CF_1-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, CF_1-γ was found to be oxidized with a delay (27). Collectively, these results indicate that CF_1-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 CF_oCF_1 (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 CF_1-γ, and Trx-like proteins then act to oxidize the redox switch on CF_1-γ. Overall, our study provides an overview of the specific mechanism underlying the inactivation of CF_oCF_1 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 CF_oCF_1

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 filtrated 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 300*g* 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 nonreducing wash solution containing 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM $MgCl_2$, and
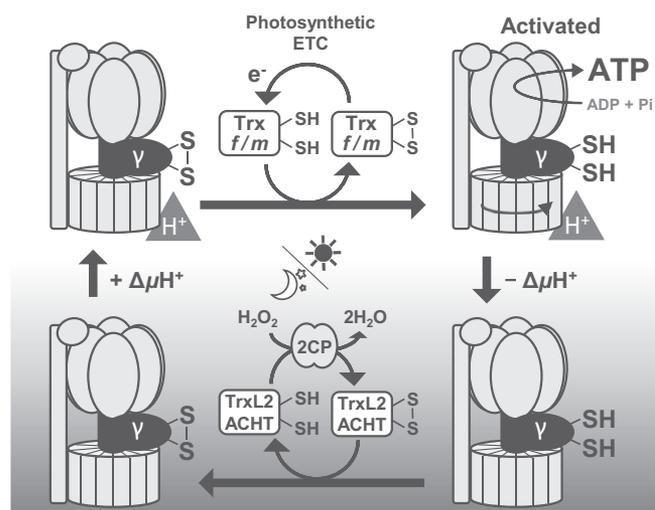

Figure 5. Scheme of the redox regulation of CF_oCF_1 adapted from that of Junesch and Gräber (10). Oxidative deactivation of CF_oCF_1 by Trx-like proteins promoted by $\Delta\mu H^+$ dissipation is included in the scheme. *Straight arrows* indicate the direction of change in CF_oCF_1 activation states. *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_2O_2, hydrogen peroxide.

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-*f*1, At3g02730; Trx-*m*1, At1g03680; Trx-*x*, At1g50320; and Trx-*y*2, 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 125,000*g* for 40 min, after which the resulting supernatant was used in protein purification. TrxL2.1 and Trx2.2 proteins were purified using a Ni-nitrilotriacetic acid affinity column as described previously ([27](#)) following an additional size-exclusion 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 CF_oCF_1 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 CF_1-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 CF_1-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 CF₀CF₁ 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 DTT_ox mixture containing 50 mM Tricine–NaOH (pH 7.5), 0.4 M sucrose, 5 mM MgCl₂, 10 mM NaCl, and 500 μM DTT_ox. Subsequently, 100 μl of the DTT_ox 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 DTT_ox, and 15 to 500 nM 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 $\Delta\mu H^+$ 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).

Abbreviations—The abbreviations used are: 2CP, 2-Cys peroxiredoxin; ACMA, 9-amino-6-chloro-2-methoxyacridine; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; DTT_{red}, reduced DTT; DTT_{ox}, oxidized DTT; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; TCA, trichloroacetic acid.

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