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Significance of the ε subunit in the thiol modulation of chloroplast ATP synthase☆

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Abstract

To understand the regulatory function of the γ and ε subunits of chloroplast ATP synthase in the membrane integrated complex, we constructed a chimeric F_oF_1 complex of thermophilic bacteria. When a part of the chloroplast F_1 γ subunit was introduced into the bacterial F_oF_1 complex, the inverted membrane vesicles with this chimeric F_oF_1 did not exhibit the redox sensitive ATP hydrolysis activity, which is a common property of the chloroplast ATP synthase. However, when the whole part or the C-terminal α-helices region of the ε subunit was substituted with the corresponding region from CF_1-ε together with the mutation of γ, the redox regulation property emerged. In contrast, ATP synthesis activity did not become redox sensitive even if both the regulatory region of CF_1-γ and the entire ε subunit from CF_1 were introduced. These results provide important features for the regulation of F_oF_1 by these subunits: (1) the interaction between γ and ε is important for the redox regulation of F_oF_1 complex by the γ subunit, and (2) a certain structural matching between these regulatory subunits and the catalytic core of the enzyme must be required to confer the complete redox regulation mechanism to the bacterial F_oF_1. In addition, a structural requirement for the redox regulation of ATP hydrolysis activity might be different from that for the ATP synthesis activity.

Keywords: F_oF_1; γ Subunit; ε Subunit; Redox regulation; Chloroplast ATP synthase

F_oF_1 ATP synthase synthesizes ATP from ADP and inorganic phosphate by using an electrochemical proton gradient, which is generated across the cytoplasmic membranes of bacteria, thylakoid membranes of chloroplasts, and inner membranes of mitochondria [1–3]. The enzyme consists of the membrane-embedded sector F_o and the extrinsic sector F_1. F_1 is composed of five different subunits designated α to ε according to their molecular weights with the stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ [4]. The minimum catalytic core, which shows the ATP hydrolysis activity, is $\alpha_3\beta_3\gamma$ [5,6]. The catalytic sites reside on each of the three β subunits at the interface with the α subunits [7]. F_o is composed of at least three different subunits, a, b, and c with the stoichiometry of $a_1b_2c_{10-14}$ [8–11] and constitutes the proton translocating device.

The rotation of the γ subunit of *Escherichia coli* F_1 coupled with ATP hydrolysis was suggested by

☆ *Abbreviations:* F_oF_1, ATP synthase complex; F_1, soluble subcomplex of ATP synthase; F_o, membranous sector of ATP synthase; CF_1, chloroplast F_1; CF_oCF_1, ATP synthase on chloroplast thylakoid; TF_oTF_1 and TF_1, F_oF_1 and F_1 obtained from thermophilic *Bacillus* PS3; γ_{TCT}, the chimeric TF_1-γ of which the central 111 amino acid resides were replaced by the counterpart 148 residues derived from CF_1-γ; ACMA, 9-amino-6-chloro-2-methoxy-acridine; DTT, dithiothreitol; AMS, 4-acetamido-4′-maleimidyl-stilbene-2, 2′-disulfonate; FCCP, *p*-(tri-fluoromethoxy)phenyl-hydrazone; ε_{CC}, the entire ε subunit from CF_1; F_oF_1-γ_{TCT}, F_oF_1-ε_{CC}, F_oF_1-γ_{TCT}/ε_{CC}; TF_oTF_1 in which γ, ε, or both subunits were substituted with γ_{TCT}, ε_{CC}, or γ_{TCT} and ε_{CC}, respectively; ε_{CT}, the ε subunit of which N-terminal 85 amino acid residues (Met^1–Asp^{85}) are from CF_1 and C-terminal 48 amino acid residues (Ile^{87}–Lys^{134}) from TF_1; ε_{TC}, the ε subunit of which N-terminal 86 amino acid residues (Met^1–Asp^{86}) are from TF_1 and C-terminal 49 amino acid residues (Ile^{86}–Ser^{134}) from CF_1; Trx-*f*, chloroplast thioredoxin-*f*; $\Delta\mu H^+$, electrochemical proton gradient.

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cross-link experiment of the β and γ subunits [12]. Thereafter, rotation of the γ subunit was directly observed by a single molecule observation technique using fluorescent actin filament attached to the γ subunit in the $\alpha_3\beta_3\gamma$ subcomplex of F_1 from the thermophilic bacterium *Bacillus* PS3 (TF_1) [13], the *E. coli* F_1 (EF_1) [14,15], and spinach chloroplast F_1 (CF_1) [16]. In 2002, Nishio et al. [17] reported DCCD-sensitive rotation of EF_oEF_1 in the membrane fragments by using the single molecule observation method.

Chloroplast ATP synthase (CF_oCF_1) is a latent enzyme, which requires activation by the proton gradient across the thylakoid membrane. It is furthermore modulated by the formation and the reduction of the disulfide bridge between two cysteine residues (Cys^{199} and Cys^{205}, the numbers are for spinach CF_1-γ) in the γ subunit [18,19]. Reduction shifts the threshold for ATP synthesis towards lower proton gradients [20,21]. This redox regulation is called thiol-modulation. The preferred reductant in vivo is thioredoxin-*f* (Trx-*f*) [22], which is reduced by the photosynthetic electron transport chain via ferredoxin and ferredoxin–thioredoxin reductase [23,24]. In CF_1, two cysteines are located on an additional amino acid stretch, the 'regulatory region' (from Ser^{193} to Leu^{241}) of CF_1-γ [25] which is absent in the non-modulated ATP synthases of mitochondria and bacteria. Accessibility of the regulatory dithiol/disulfide group of γ in CF_oCF_1 on the thylakoid membrane is affected by electrochemical proton gradient across the membrane [20].

The molecular mechanism of this redox regulation is not well understood. Several chimeric enzymes combining the whole or a part of the γ subunit from CF_1 with the rest of the enzyme complex from other species are available [26–32]. Using one of these chimera complexes, which was prepared from the recombinant CF_1-γ subunit and the α and β subunits of recombinant TF_1, we constructed a series of mutants to identify which amino acid residues in addition to the regulatory cysteines are important for the regulation [28,29]. Remarkably the deletion of amino acids Glu^{210}–Asp^{211}–Glu^{212} of CF_1-γ reversed the regulatory function, i.e., reduction caused decrease of the ATP hydrolysis activity. Another chimeric complex, $\alpha_3\beta_3\gamma_{TCT}$, in which the central 111 amino acid resides of the γ subunit of TF_1 were replaced by the respective 148 residues derived from CF_1-γ including the regulatory region [30] enabled us to observe redox-mediated changes of the rotation of this hybrid γ [33,34]. These findings suggest that the regulatory region of CF_1-γ can work as transferable functional unit in the various F_1-complexes.

In bacterial F_1 and CF_1, the ε subunit is known as an endogenous inhibitor and to suppress the ATP hydrolysis activity [35–37]. We already obtained the results that underscore the importance of the combination of γ and ε for their own functions. The ATP hydrolysis activity of the chimeric $\alpha_3\beta_3\gamma_{TCT}$ complex or the chimeric $\alpha_3\beta_3$ complex with CF_1-γ was strongly inhibited by CF_1-ε but not by TF_1-ε [27,30]. Hence, the subunit–subunit interaction between the regulatory region of CF_1-γ and CF_1-ε seems to be critical for the regulation in F_oF_1 complex. In the present study, we further investigated the function and the relevance of the regulatory region of CF_1-γ and CF_1-ε for the redox regulation of F_oF_1 complex on the membranes.

Materials and methods

Materials

9-Amino-6-chloro-2-methoxy-acridine (ACMA), DTT, and carbonyl cyanide *p*-(tri-fluoromethoxy) phenyl-hydrazone (FCCP) were from Sigma (St. Louis, MO, USA). 4-acetamido-4′-maleimidyl-stilbene-2, 2′-disulfonate (AMS) was from Molecular Probes (Eugene, OR, USA). Other chemicals were of the highest grade commercially available.

Methods

Plasmid construction for the chimeric ATP synthase complexes. A plasmid pTR19-ASDS, which was constructed for the functional expression of the TF_oTF_1 complex within the *E. coli* membrane [38], was used as an original vector for the expression of the chimeric complexes. We constructed the plasmids for five different chimeric complexes as shown in Fig. 1. To obtain the F_oF_1 complex containing γ_{TCT} (F_oF_1-γ_{TCT}), the *Bsi*wI–*Age*I fragment obtained from the expression plasmid for the $\alpha_3\beta_3\gamma$ sub-complex [30] containing a portion of α, whole γ_{TCT}, and a portion of β was ligated into pTR19-ASDS. To introduce CF_1-$\varepsilon(\varepsilon_{CC})$ into the complex, a DNA fragment encoding the ε subunit of spinach CF_1 was amplified using the polymerase chain reaction with primers; 5′-ccgccgcccgggattatgaaggagattaacatatgaccttaaatctttgtgtactg-3′ (*Sma*I) and 5′-gaggctagcaatacgatttcttcgtaagcggccgcctgcaggcccg-3′ (*Pst*I) with a vector containing the gene for CF_1-ε [27] as a template. The sites for the restriction enzymes shown in parentheses are underlined. The *Sma*I–*Pst*I fragment of the product was then ligated into pTR19-ASDS and thus the gene for TF_1-ε was substituted with one for ε_{CC}. For the introduction of the chimeric ε subunit into the F_oF_1-γ_{TCT} complex, polymerase chain reaction was carried out with F_oF_1-γ_{TCT} or F_oF_1-$\gamma_{TCT}/\varepsilon_{CC}$ as templates. In the present study, two chimeric ε subunits; ε_{CT}, which consists of the N-terminal β-sandwich domain from CF_1-ε (Met^1–Asp^{85}) and the C-terminal α-helix part (Ile^{87}–Lys^{134}) from TF_1-ε, and ε_{TC}, which consists of the N-terminal β-sandwich domain (Met^1 to Asp^{86}) from TF_1-ε and the C-terminal α-helix part (Ile^{86} to Ser^{134}) from CF_1-ε, were designed. The N-terminal portion of the ε subunit was then amplified using primers; 5′-ccgcggcccgggattatgaaggagattaacatatgaccttaaatctttgtgtactg-3′ (*Sma*I) and 5′-cgactctccccatcactgtagctaattcgaaccc-3′ (*Cla*I) for CF_1-ε, and 5′-ccgcggcccgggatagggggattggacaatgaaaacgatccacgtgagcg-3′ (*Sma*I) and 5′-cgccgctttggcgcggaggacatcgatgtcctccgcccgttcagc-3′ (*Cla*I) for TF_1-ε, respectively, and unique sites for the restriction enzymes *Sma*I and *Cla*I were introduced. The C-terminal portion of the ε subunit was amplified using primers; 5′-ccatcgatccccaagaagcccaaca-3′ (*Cla*I) and 5′-cgggcctgcaggcggccgcttacgaagaaatcgtattgctagcctc-3′ (*Pst*I) for CF_1-ε, and 5′-gctgaacgggcggaggacatcgatgtcctccgcgccaaagcggcg-3′ (*Cla*I) and 5′-ggctgaaaatcttctctcatccgccaaaacagccaagcttgcatgcctgcaggcg-3′ (*Pst*I) for TF_1-ε, respectively, and the *Cla*I and *Pst*I sites were introduced. To obtain the complex containing ε_{TC}, the *Sma*I–*Cla*I fragment for the N-terminal portion of TF_1-ε and the *Cla*I–*Pst*I fragment for the C-terminal portion of CF_1-ε were directly ligated into pTR19-ASDS containing γ_{TCT}. The complex containing ε_{CT} was prepared using the same way with the *Sma*I-*Cla*I

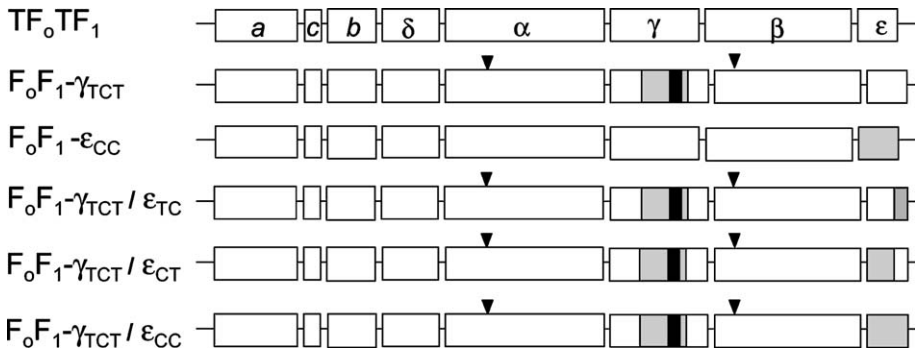

Fig. 1. Plasmids for the chimeric TF_oTF_1 complexes. The plasmids for the wild-type TF_oTF_1 and the chimeric TF_oTF_1 complexes are schematically shown. In the chimeric complexes, the shaded and filled parts have been substituted by the counterpart from the chloroplast ATP synthase genes. The regulatory region derived from CF_1-γ was shown as filled box. The portion of the plasmid sandwiched in two closed triangles was carried on from the plasmid for $\alpha_3\beta_3\gamma_{TCT}$ [30].

fragment for the N-terminal portion of CF_1-ε and the *Cla*I-*Pst*I fragment for the C-terminal portion of TF_1-ε.

Preparation of inverted membrane vesicles from E. coli. *E. coli* strain DK-8 [bglR, thi-1, rel-1, HfrPO1, Δ(uncB-uncC), ilv::Tn10], which lacks EF_oEF_1 [39], was used for the expression of TF_oTF_1 complex. DK-8 cells containing the desired plasmids were cultivated in 2× YT medium at 37 °C for 20 h. The cells harvested by centrifugation were disrupted twice by French-Pressure cell. The plasma membrane was then collected by ultra-centrifugation and the inverted membrane vesicles containing the chimeric F_oF_1 complex together with the respiratory chain were prepared by the method described previously [38,40].

Purification of the recombinant Trx-f. Spinach chloroplast Trx-*f* was expressed in *E. coli* and isolated as described [41]. The concentration of purified Trx-*f* was calculated from the absorbance at 278 nm using the published molar absorption coefficient value of 16,830 M^{-1} cm^{-1} [42].

Determination of redox states of the γ subunit. Inverted membrane vesicles were diluted to 10 mg protein/ml with 10 mM Hepes–NaOH (pH 7.5), 5 mM $MgCl_2$, and 10 % (w/v) glycerol. Vesicles with 100 μg of protein were incubated at 30 °C for 10 min with 50 μM $CuCl_2$ for oxidation or 90 μM DTT, 10 μM 2-mercaptoethanol, and 5 μM Trx-*f* for reduction in 100 μl solution. The oxidation reaction was terminated by adding 7.5 mM EDTA (pH 8.0). The redox state of γ_{TCT} in the chimeric F_oF_1 complex was then assessed by using AMS as described [43]. AMS-labeled proteins were separated by the 9% (w/v) polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS (SDS–PAGE) without 2-mercaptoethanol.

ATP hydrolysis activity. Inverted membrane vesicles were reduced or oxidized as described above and the ATP hydrolysis activity was measured in the presence of an ATP-regenerating system [44]. The assay mixture containing 50 mM Hepes–NaOH (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 100 μg/ml pyruvate kinase, 100 μg/ml lactate dehydrogenase, 5 mM phospho*enol*pyruvate, and 0.2 mM NADH was previously incubated at 37 °C. The reaction was then initiated by the addition of the membrane vesicles (10 μg as a protein) following the addition of 5 mM KCN and 1 μM FCCP to the vesicle suspension. The activity was measured by monitoring the decrease of NADH absorption at 340 nm using U-3100 spectrophotometer (Hitachi, Tokyo, Japan).

Proton translocation activity. Inverted membrane vesicles were oxidized or reduced as described above and were mixed with the reaction mixture containing 10 mM Hepes–NaOH (pH 7.5), 5 mM $MgCl_2$, and 100 mM KCl, and the solution was incubated at 42 °C for 1 min. The change of the internal pH of the inverted membrane vesicles was monitored as a change of the fluorescence from ACMA [40] and was measured by a FP-6300DS fluorescence spectrophotometer (JASCO, Tokyo, Japan). The ATP-driven proton translocation reaction was initiated by adding 0.5 mM ATP to the solution. In case of NADH-driven proton translocation, 0.2 mM NADH was used. The excitation and emission wavelengths for ACMA fluoresce measurement were 410 and 480 nm, respectively.

ATP synthesis activity. Ninety microliters (90 μg as a protein) of the inverted membrane vesicles, which were previously oxidized or reduced as described, was mixed with 810 μl reaction mixture containing 50 mM Hepes–KOH (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl, 5 mM Na–phosphate, and 5 mM ADP with or without 1 μM FCCP for 2 min at 42 °C. The ATP synthesis reaction was initiated by adding 10 mM NADH. 50 μl of the sample was then taken at 1, 2, and 3 min after the addition of NADH and mixed with 16 μl of 4 % (w/v) trichloroacetic acid on ice. The quenched solution was neutralized by adding 165 μl of 2 M Tris–acetate (pH 7.7), and the amount of ATP in the solution was determined using luciferin/luciferase assay system [45].

Results

Introduction of γ_{TCT} into the TF_oTF_1 complex

First, we introduced γ_{TCT} [30], the γ subunit containing the redox sensitive regulatory region of CF_1-γ, into the TF_oTF_1 complex (see Fig. 1, the second construct). The plasmid for this chimeric complex was transformed into *E. coli* cell and the inverted membrane vesicles containing the desired complex were prepared. We employed the thiol modifier AMS to visualize the redox state of the regulatory region as a change of the SDS–PAGE mobility of the polypeptide [43]. This way we determined whether the introduced regulatory region within the γ subunit in this bacterial F_oF_1 complex was sensitive to the redox conditions or not (Fig. 2A, lanes 3 and 4). The identity of the AMS-modified γ subunits in the reduced or oxidized state was further confirmed by western blotting using an antibody raised against CF_1-γ (Fig. 2B, lanes 3 and 4). As expected, the wild-type TF_1-γ in the F_oF_1 complex, which does not have any cysteines, was insensitive to the redox conditions (data not shown).

As previously reported, ATP hydrolysis activity of the reduced form $\alpha_3\beta_3\gamma_{TCT}$ complex was about 2-fold higher than that of the oxidized form [30]. Therefore, we measured change of the ATP hydrolysis activity of F_oF_1-γ_{TCT} on the membranes by reduction or oxidation.

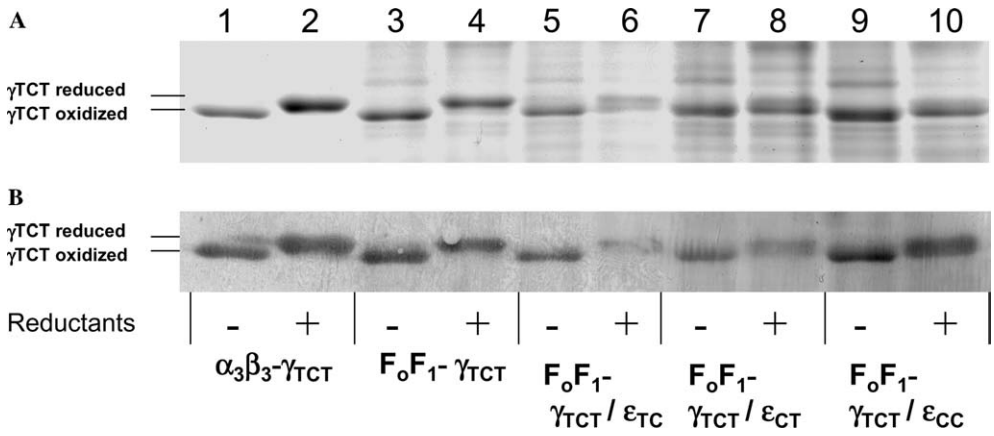

Fig. 2. Gel electrophoresis of the oxidized and reduced γ subunit. Oxidation and reduction of the regulatory region of γ_{TCT} in the TF_oTF_1 complex were visualized by AMS-labeling. (A) The inner membranes containing four different TF_oTF_1 complexes were oxidized or reduced as described under "Materials and methods," labeled with AMS, and then analyzed by 9% (w/v) SDS–PAGE without 2-mercaptoethanol. $\alpha_3\beta_3\gamma_{TCT}$ complex was used as a control. The protein bands were visualized with Coomasie brilliant blue R-250 (CBB) staining. (B) The γ_{TCT} bands in (A) were visualized by Western blotting method using the polyclonal antibody raised against spinach CF_1-γ.

The ATP hydrolysis activity of the membrane bound F_oF_1-γ_{TCT} complex was not affected very much by the redox states of the regulatory region, although the catalytic core of the complex was identical to the subcomplex $\alpha_3\beta_3\gamma_{TCT}$ (Table 1). Upon addition of 0.3 % (w/v) lauryldimethylamine oxide (LDAO), which is supposed to release F_1 from F_o, the ATP hydrolysis activity of this F_oF_1-γ_{TCT} complex became redox sensitive. The activity of the reduced form complex was 1.8-fold higher than that of the oxidized complex in the presence of LDAO (Table 1). This implies that the configuration of the F_1 portion was very similar to that of the previously studied soluble $\alpha_3\beta_3\gamma_{TCT}$ subcomplex [30].

Using the fluorescence quenching of ACMA we examined the redox regulation property of the ATP-driven proton translocation activity in the chimeric complex. Like for the membrane vesicles containing wild-type TF_oTF_1 complex, we could not observe any remarkable changes in the maximum velocity of proton translocation in the inverted membrane vesicles containing F_oF_1-γ_{TCT} by reduction or oxidation (Fig. 3A and B).

We then investigated the ATP synthesis activity of this F_oF_1-γ_{TCT} complex under redox conditions. The formation of the electrochemical proton gradient driven by NADH and the membrane tightness were confirmed as indicated (Fig. 4B, inset). As well as ATP hydrolysis activity and the proton translocation activity, we could not observe any change in the ATP synthesis activity by reduction or oxidation (Fig. 4B).

Introduction of the CF_1-ε subunit into the chimeric F_oF_1 complex

As stated, just the introduction of the redox sensitive regulatory region of the CF_1-γ subunit into F_oF_1 could not confer the complete redox regulation property into

Table 1
Thiol modulation of ATP hydrolysis activity of the chimeric complex on the membrane

Complex	LDAO	ATP hydrolysis (U/mg)		Reduced/oxidized (%)
		Reduced	Oxidized	
TF_oTF_1	−	0.79 ± 0.06	0.76 ± 0.02	103.9
F_oF_1-γ_{TCT}	−	1.50 ± 0.08	1.20 ± 0.06	125.0
F_oF_1-ε_{CC}	−	0.57 ± 0.02	0.53 ± 0.03	107.5
F_oF_1-$\gamma_{TCT}/\varepsilon_{TC}$	−	1.40 ± 0.10	0.90 ± 0.02	155.6
F_oF_1-$\gamma_{TCT}/\varepsilon_{CT}$	−	0.80 ± 0.03	0.65 ± 0.03	123.1
F_oF_1-$\gamma_{TCT}/\varepsilon_{CC}$	−	0.41 ± 0.01	0.22 ± 0.02	186.4
TF_oTF_1	+	4.50 ± 0.42	4.46 ± 0.55	102.0
F_oF_1-γ_{TCT}	+	5.37 ± 0.64	2.79 ± 0.42	193.5
F_oF_1-ε_{CC}	+	3.52 ± 0.12	3.31 ± 0.09	106.2
F_oF_1-$\gamma_{TCT}/\varepsilon_{TC}$	+	4.14 ± 0.22	2.42 ± 0.29	171.4
F_oF_1-$\gamma_{TCT}/\varepsilon_{CT}$	+	1.93 ± 0.08	1.42 ± 0.02	135.8
F_oF_1-$\gamma_{TCT}/\varepsilon_{CC}$	+	1.15 ± 0.11	0.62 ± 0.10	187.6

The inverted membrane vesicles containing the wild-type TF_oTF_1 or the five different chimeric complexes were reduced or oxidized as described under "Materials and methods" and the ATP hydrolysis activity was measured in the absence or the presence of 0.3% (v/v) LDAO. The standard errors were calculated from three independent experiments.

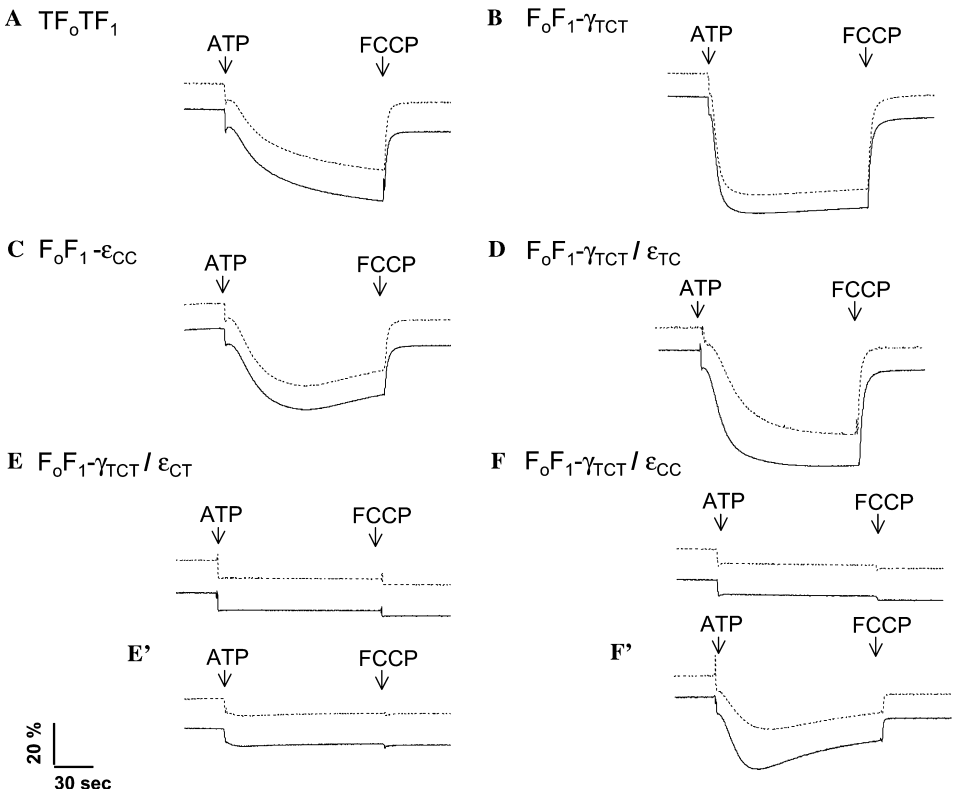

Fig. 3. Thiol modulation of the ATP-driven proton translocation activity of the chimeric complexes. The formation of a pH gradient across the membrane vesicles was determined by monitoring the decrease in the fluorescence intensity of ACMA. The inverted membrane vesicles with (A) wild-type TF_oTF_1, (B) F_oF_1-γ_{TCT}, (C) F_oF_1-ε_{CC}, (D) F_oF_1-γ_{TCT}/ε_{TC}, (E) F_oF_1-γ_{TCT}/ε_{CT}, and (F) F_oF_1-γ_{TCT}/ε_{CC} were treated with DTT, 2-mercaptoethanol, and Trx-*f* for reduction (solid lines) or $CuCl_2$ (dashed lines) for oxidation as described under "Materials and methods." 200 μg of protein was then used for each of the measurements. The reaction was initiated and terminated by adding ATP and FCCP. (E′) and (F′) show the measurements with the same inverted membrane vesicles as for (E) and (F), respectively, but 600 μg protein was used.

the bacterial complex. We therefore constructed another three chimeric complexes containing a whole CF_1-ε subunit or a part of it (Fig. 1). Those chimeric complexes were successfully expressed in *E. coli*, and the redox sensitivities of the γ subunit in these complexes were again confirmed by AMS labeling (Fig. 2).

We then examined the redox sensitivity of the ATP hydrolysis activity of these complexes (Table 1). In the case of F_oF_1-γ_{TCT}/ε_{CT}, partial activation by reduction was observed. In contrast, the ATP hydrolysis activity of membrane-bound F_oF_1-γ_{TCT}/ε_{TC} and F_oF_1-γ_{TCT}/ε_{CC} was more sensitive to reduction/oxidation irrespective of the addition of LDAO (Table 1), suggesting that the C-terminal region of the ε subunit of CF_1 can assist the redox regulation of F_oF_1 by the introduced γ_{TCT}. Exclusive substitution of the ε subunit with the entire CF_1-ε, ε_{CC} in the TF_oTF_1 complex (F_oF_1-ε_{CC}) showed—as to be expected—no redox sensitivity. Unlike the inverted membrane vesicles containing F_oF_1-γ_{TCT} (Fig. 3B) or F_oF_1-ε_{CC} (Fig. 3C), the maximum velocity of proton translocation in vesicles containing F_oF_1-γ_{TCT}/ε_{TC} (Fig. 3D) and F_oF_1-γ_{TCT}/ε_{CC} (Fig. 3F′) was affected by redox conditions. However, the vesicles with F_oF_1-γ_{TCT}/ε_{CC} were relatively leaky and larger amounts of membrane proteins were required to detect the activity (see Figs. 3F and F′). The membrane vesicles containing F_oF_1-γ_{TCT}/ε_{CT} were especially leaky and we could not observe any proton translocation activity (Figs. 3E and E′, and Fig. 4E, inset). Finally, we investigated the ATP synthesis activity of the vesicles containing these complexes under redox conditions. As ATP synthesis is promoted by the electrochemical proton gradient formed by NADH oxidation, the ATP synthesis activity was strongly related with the membrane tightness. For example, the inverted membrane vesicles containing F_oF_1-γ_{TCT}/ε_{CT} were very leaky (Fig. 4E, inset) and the complex could not synthesize any ATP. In contrast, F_oF_1-γ_{TCT}/ε_{TC} complex could synthesize ATP (Fig. 4D) with the equivalent rate as F_oF_1-γ_{TCT}, which was much faster than that by TF_oTF_1. However again we could not observe any change in the activity by reduction or oxidation with all chimeric complexes constructed in this study.

Discussion

In the present study, we prepared new chimeric complexes of TF_oTF_1 expressed in *E. coli* to focus on the

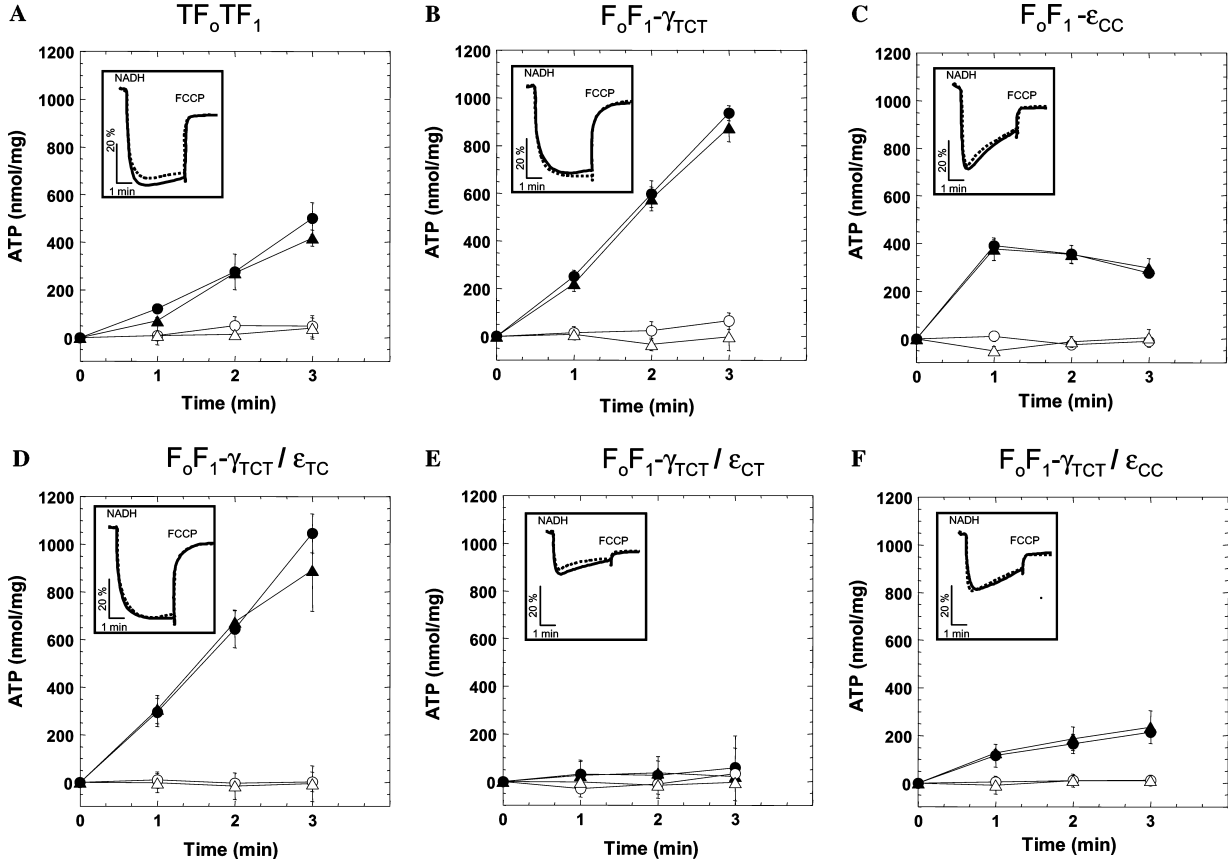

Fig. 4. Thiol modulation of ATP synthesis activity. The inverted membrane vesicles with (A) wild-type TF_oTF_1, (B) F_oF_1-γ_{TCT}, (C) F_oF_1-ε_{CC}, (D) F_oF_1-γ_{TCT}/ε_{TC}, (E) F_oF_1-γ_{TCT}/ε_{CT}, and (F) F_oF_1-γ_{TCT}/ε_{CC} were reduced (circle) or oxidized (triangle) and the ATP synthesis activity was determined in the absence (closed) and the presence (open) of 1 μg/ml FCCP. The results of three independent experiments were averaged. Inset, NADH-driven proton pump activity was measured to check the tightness of inverted membrane vesicles. One hundred and ten microgram of protein was used for each measurement. The *dashed* and *solid lines* show NADH-driven proton pump activity under oxidized and reduced conditions, respectively. The reaction was initiated and terminated by adding 0.2 mM NADH and 1 μg/ml FCCP.

significance of the subunit–subunit interaction for the redox regulation of the F_oF_1 complex. Just the introduced regulatory region from CF_1-γ did not work well as a redox modulator for ATP hydrolysis activity on the F_oF_1 complex (Table 1). This result was evidently different from our previous reports on the redox regulation of the chimeric F_1 complex. We have previously introduced the regulatory region of spinach CF_1-γ into the corresponding part of the TF_1 $\alpha_3\beta_3\gamma$ subcomplex [30]. Using this chimeric complex, we successfully observed redox-regulation of the ATP hydrolysis activity [30] and redox-regulation of rotation of the enzyme [33,34]. In contrast, redox regulation of ATP hydrolysis activity of F_oF_1 complex was only observed when the whole part or the C-terminal α-helix region of the ε subunit was also substituted with the corresponding region from CF_1-ε (Table 1). Thus, we found that the redox regulation in the F_oF_1 complex is not accomplished only by reduction or oxidation of the regulatory region of the γ subunit. The ε subunit, mainly its C-terminal α-helix region, must have a significant role for this modulation.

To understand the function of the C-terminal α-helix region of the ε subunit, it is also notable that the nucleotide dependent conformational change of this region of ε in the complex is important for the inhibitory effect of this subunit [46]. Furthermore, on the role of the ε subunit in the membrane bound CF_oCF_1 complex, a close relationship between the electrochemical proton gradient and the conformational change of this subunit had been reported [47,48]. Recently, Johnson and McCarty [49] reported that the C-terminal α-helix region of CF_1-ε changes the conformation in the complex dependent on the electrochemical proton gradient. Suzuki et al. [50] also reported that the drastic conformational change of TF_1-ε in the TF_oTF_1 complex occurs dependent on the electrochemical proton gradient. It had also been shown that the ε subunit is necessary to prevent leak of proton through F_o [51] and the β-sandwich domain of ε subunit bounds on the cytoplasmic surface of the *c*-subunit ring of F_o [52]. Indeed, the introduction of ε_{CT} or ε_{CC}, whose β-sandwich domain is derived from CF_1, into the TF_oTF_1 complex made the vesicles more

leaky (Figs. 3E and F). As shown in the insets of Figs. 4C–F, the electrochemical proton gradient formed by NADH was easily released through these chimeric F_oF_1 complexes. Consequently, these complexes exhibited no or very weak ATP synthesis activity. In contrast, F_oF_1-$\gamma_{TCT}/\varepsilon_{TC}$ complex exhibited significant ATP synthesis activity (Fig. 4D). Although the ATP hydrolysis activity of this complex was nicely modulated by the redox regulation like F_oF_1-$\gamma_{TCT}/\varepsilon_{CC}$ (Table 1), the ATP synthesis activity was not. One may claim that the lack of the redox sensitivity of the ATP synthesis activity of the chimeric complex is due to the insufficient formation of the electrochemical proton gradient, which is caused by the membrane leakiness. However, the results obtained from F_oF_1-$\gamma_{TCT}/\varepsilon_{TC}$ complex clearly eliminate this possibility. Thus, our results suggest that the further unknown but important subunit–subunit interaction is required to evoke the redox regulation of ATP synthesis activity on the bacterial F_oF_1 complex. The unsolved structural information on the subunit–subunit interaction in the complex finally provides the critical information to understand the entity of those molecular devices for redox regulation of chloroplast ATP synthase.

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