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Original Article

A Role for the Disulfide Bond Spacer Region of the *Chlamydomonas reinhardtii* Coupling Factor 1 γ-Subunit in Redox Regulation of ATP Synthase

Stuart A. Ross,[1,2] Mike X. Zhang,[1] and Bruce R. Selman[1]

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The γ-subunit of chloroplast coupling factor 1 contains a disulfide bond which is involved in the redox regulation of the enzyme. In all the sequenced plant γ-subunits this disulfide bond is separated by a five amino acid spacer region. To investigate the regulatory significance of this region genetic transformation experiments were performed with *Chlamydomonas reinhardtii. C. reinhardtii* strain *atpC*1 (*nit1*-305, cw 15, mt^-), which does not accumulate the CF_1 γ-subunit polypeptide, was independently transformed with two constructs, each bearing mutations within the disulfide bond spacer region between Cys^{198} and Cys^{204} of the γ-subunit. Successful complementation was confirmed by phenotypic selection, Northern blot analysis, and reverse transcription polymerase chain reaction. Whereas wild-type thylakoid membrane particles catalyze *in vitro*, PMS-dependent photophosphorylation that is stimulated 2-fold by the addition of DTT, similar particles from each of the mutant strains exhibit rates of ATP synthesis that are independent of DTT. Consistent with these results, wild-type CF_1 ATPase activity is stimulated by DTT which is in contrast to the ATPase activities of both the mutant strains which are independent of DTT addition. These results suggest a role for the γ-subunit disulfide bond spacer region in the redox regulation of chloroplast ATP synthase.

KEY WORDS: CF_0CF_1; *Chlamydomonas reinhardtii*; γ-subunit; redox regulation.

INTRODUCTION

The majority of ATP synthesis within cells is carried out by bipartite structured enzymes that are located in the inner membranes of mitochondria, the plasma membranes of bacteria, and the thylakoid membranes of chloroplasts. These so-called ATP synthases are composed of an F_0 sector which mediates proton transfer through the enzyme and an F_1 sector which contains the catalytic portion of the enzyme (McCarty and Moroney, 1985; Merchant and Selman, 1985; Ort and Oxborough, 1992). In plants, the majority of ATP synthesis is carried out by chloroplast coupling factor 1 (CF_0CF_1).[3] During periods of illumination the initiation of photosynthetic electron transfer leads to concomitant, unidirectional deposition of protons into the lumen of the thylakoid membrane. The free energy of this transmembrane pH gradient is utilized by coupling factor to synthesize ATP from ADP and P_i (Mitchell, 1961).

While it is now well established that the protonmotive is the major determinant in the activation

[1] Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706.

[2] To whom correspondence should be addressed at: Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755.

[3] Abbreviations: CF_0, coupling factor 0; CF_1, coupling factor 1; DTT, dithiothreitol; RT-PCR, reverse transcription-polymerase chain reaction; PMS, phenazine methosulfate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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of chloroplast coupling factor (Ryrie and Jagendorf, 1971, 1972; McCarty and Moroney, 1985; Ort and Oxborough, 1992), there are nonetheless a few modes of regulation which may serve to modulate enzymatic activity under various growth conditions (for example, light intensity). The reductive activation of coupling factor is a phenomenon that was first identified thirty years ago (Vambutas and Racker, 1965). Reductive activation of CF_1 appears to be mediated by a disulfide bond localized within the γ-subunit (Nalin and McCarty, 1984; Ketcham *et al.*, 1984; Mills and Mitchell, 1984; Shahak, 1985; Junesch and Graber, 1987; Hangarter *et al.*, 1987; Nocter and Mills, 1988; Kramer *et al.*, 1990, Ross *et al.*, 1995), the only disulfide bond within the entire multisubunit CF_1 sector (Nalin and McCarty, 1984). Sequence comparisons of γ-subunits from a variety of species demonstrate the existence of an extra peptide within the protein of the chloroplast enzymes which contain a disulfide bond that is not found in its mitochondrial or bacterial counterparts (Mason and Whitfield, 1990; Inohara *et al.*, 1991). For all the plant enzymes, the γ-subunit disulfide bond is separated by a spacer region of five amino acids, of which two are absolutely conserved while the other three are similar (see Fig. 1). While there have been many elegant biochemical studies concerning the CF_1 γ-subunit disulfide bond comparatively little attention has focused on this spacer region, which is perhaps due to the lack of sufficient technology with which to do so. Most photosynthetic mutations in higher plants are lethal (Goodenough, 1992) and furthermore, reconstitution of the five-subunit CF_1 has yet to be achieved. Because of these limitations, we have utilized the green

	198 204
E. coli	LPLP----------AS
Bostaurus mitochondria	FSLDT----------ISSA
Synechocystis 6803	FPLSPQG---LEAPDDEIFR
Spinacia oleracea	LPLSPKGEICDINGKCVDAA
Arabidopsis C1	LPLSPKGEICDINGTCVDAA
Arabidopsis C2	LPLSPKGEICDVKGECVVAI
C. reinhardtii	LPMTPMGEICDVDGKCVDAA
Plant Consensus	LPXXPXGEICDXXGXCVDAX
D199K/K203D	---------CKVDGDC----
D199A	---------CAVDGKC----

Fig. 1. Sequence comparisons of various F_1 γ-subunits and construct generation. This figure is adapted from papers by Mason and Whitfield (1990) and Inohara *et al.* (1991). Plant γ-subunits contain an additional region within the γ-subunit which contains a disulfide bond that is not found in the analogous subunit in either mitochondria, bacteria, or even a photosynthetic bacterium *Synechocystis* 6803. Two constructs, designated D199K/K203D and D199A, were engineered into the *Chlamydomonas reinhardtii* CF_1 γ-subunit gene and transformed into strain *atpC*1 (*nit1*-305, cw 15, mt^-).

algae *Chlamydomonas reinhardtii* as a model test system.

The primary advantage of using *C. reinhardtii* is its ability to grow exclusively on acetate as a reduced carbon source (Harris, 1989) which renders the photosynthetic apparatus nonessential. This allows for the isolation of conditionally lethal photosynthetic mutants. This property was previously exploited in our laboratory in order to obtain a strain which fails to synthesize the CF_1 γ-subunit (Smart and Selman, 1991). Here we describe nuclear transformation of *C. reinhardtii* strain *atpC*1 with γ-subunit constructs bearing mutations within the disulfide bond spacer region and the characterization of the enzymatic activities of these mutant enzymes. The aim of the present work was to test the regulatory significance of the spacer region between the disulfide bond of the CF_1 γ-subunit on the enzymatic activity of ATP synthase.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii strain *nit1*-305 (cw 15, mt^-) was the generous gift of Dr. Pete Lefebvre (University of Minnesota), and it served as the wild-type strain referred to in this study. With respect to coupling factor this strain behaves like a wild-type strain such as 137c (Smart and Selman, 1993; Ross *et al.*, 1995). The mutant *atpC*1 strain was previously generated from *nit1*-305 (Smart and Selman, 1991). Cells were maintained under constant illumination (120 $\mu mol/m^2 \cdot sec$) at 20°C on Sager-GranickII-NH_4^+ (SGII) (Sager and Granick, 1953; Harris, 1989) which contained 1% agar. Cells were cultured in liquid SGII-NH_4^+ in 50-ml and 2-liter flasks with constant stirring. They were allowed to reach mid-logarithmic phase which corresponds to a chlorophyll absorbance at 652 nm of approximately 0.7–0.9. The cells were then harvested by centrifugation at 4,000 × *g* for experimentation.

Generation of CF_1 γ-Subunit Constructs

Site-directed mutants were generated within the CF_1 γ-subunit by using a PCR mutagenesis strategy described elsewhere (Ross *et al.*, 1995). Briefly, The disulfide bond region of the γ-subunit is located within a 168-bp *Bgl* II cassette within the genomic *C. rein-*

hardtii CF_1 γ-subunit. Two mutant constructs were created by removal of this region by *Bgl* II digestion and replacement with fragments generated by PCR. Table I lists the strain designations and the primer sequences used to obtain the mutated PCR products. The numbers indicate the position(s) of the γ-subunit that were mutated. The letter preceding the number is the wild-type amino acid while the letter to the right of the number indicates the amino acid substitution. Proper sequence and orientation of each construct were confirmed by dideoxy sequencing (Sanger *et al.*, 1977).

Cotransformation of Strain *atpC*1 with Constructs D199K/K203D and D199A

Because of the possibility that the mutant γ-subunit constructs would not restore photoautotrophic growth, we employed a cotransformation protocol. Strain *atpC*1 is also null for nitrate reductase and it has been previously demonstrated that nitrate prototrophy can be restored to these cells by transformation with plasmid PMN24, the wild-type *C. reinhardtii* nitrate reductase gene (Kindle *et al.*, 1990; Ross *et al.*, 1995). *AtpC*1 cells were cotransformed with PMN24 and each of the γ-subunit constructs in separate experiments using a modified form of the transformation procedure described by Kindle (1990). The details are described elsewhere (Ross *et al.*, 1995). Approximately 50 nit^+ colonies were isolated, of which 5–8 were positive for photoautotrophic growth in the absence of acetate. These cells were designated as putative cotransformants and two colonies, each representing one of the γ-subunit constructs, were utilized for further characterizations.

Table I. Strain Designations and Antisense Primers[a]

Strain	Primer
D199K/K203D	5′-GCTTGAAGATCTCGTCGTCAGCGGCG TCCACGCAGTCGCCGTCCACCTTGCA CAGCTCG-3′
D199A	5′-GCTTGAAGATCTCGTCGTCAGCGGCG TCCACGCACTTGCCGTCCACGGCGCA CAGCTCG-3′

[a] The sense strand primer was 5′-GGAGGAAGGGGGTGTTGCG-GGGAGGTCCTG-3′.

DNA/RNA Isolation and Blotting

DNA/RNA isolation, gel electrophoresis, and Southern and Northern Blotting were all performed as described previously (Sambrook *et al.*, 1989; Smart and Selman, 1991). All blots were probed with [^{32}P] randomly primed *C. reinhardtii* γ-subunit cDNA previously excised from its host plasmid by *Eco*RI digestion (Yu and Selman, 1988).

Reverse Transcription-PCR

Five micrograms of total RNA were reverse transcribed using random hexamers as primers by following the manufacturer's procedure (Gibco/BRL; Bethesda, Maryland). The cDNA products were phenol/chloroform extracted, precipitated, and resuspended in 80 μl of TE (pH 8.0). Two microliters were used for each PCR reaction. The PCR reactions were set up as described previously (Smart and Selman, 1991). The reaction conditions were 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 2 min, 57°C for 1 min, and 72°C for 2 min.

Isolation of Thylakoid Particles

Chloroplast thylakoid particles were isolated as described (Selman-Reimer *et al.*, 1981) with some modifications. Briefly, cells were washed once with 50 mM Tricine-NaOH (pH 8.3). For photophosphorylation experiments cell suspensions were adjusted to an equivalent concentration of 1–2 mg chlorophyll/ml in a buffer that contained 50 mM Tricine-NaOH (pH 8.3), 40 mM NaCl, 5 mM $MgCl_2$, and 0.25 M sucrose. Cell suspensions were sonicated at room temperature, in 3-ml batches, in a bath-type sonicator (Laboratory Supplies Co., Inc., power output 80 kHz, 80 W) for 15 sec. For ATPase measurements, cells were sonicated for 1 min in 50 mM Tricine-NaOH (pH 8.3). After sonication, the cell suspensions were centrifuged at 200 × *g* for 15 sec. The supernatant was centrifuged again for 20 sec at the same speed to ensure the removal of the majority of unbroken cells. Finally, the thylakoid particles were pelleted by centrifugation at 30,000 × *g* for 10 min.

ATPase Assays

ATPase reactions were carried out at 37°C in a total volume of 0.1 ml which contained the following

components: 20 mM Tricine-NaOH (pH 8.0), 1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 10 mM [γ-^{32}P]ATP ($\sim 2 \times 10^5$ cpm), 25 mM dithiothreitol, and 0.5–1.5 μg of protein. The reactions were terminated as previously described (Selman, 1976) and released [^{32}Pi] was counted in a Packard 460C liquid scintillation counter.

Phenazine Methosulfate (PMS)-Dependent Photophosphorylation

Thylakoid particles were suspended to an equivalent chlorophyll concentration of 1–2 mg/ml. The suspensions were kept in the dark on ice for no more than 1 h prior to each experiment. The reaction mixture, in a total volume of 0.1 ml, contained the following: 50 mM Tricine-NaOH (pH 8.3), 10 mM NaCl, 0.5 mM $MgCl_2$, 2 mM [^{32}P]-phosphate, ($\sim 2.5 \times 10^6$ cpm/ml), 2 mM ADP, and 0.08 mM PMS. Five microliters of dark-adapted thylakoid particle membranes (corresponding to 5–10 mg chlorophyll/ml) were equilibrated with the reaction mixture on ice for 2 min prior to the start of the reactions. Reactions were initiated by the addition of 5 μl of 0.5 M DTT or ddH_2O immediately before illumination. The reaction mixtures were then illuminated at 20°C with white light isolated from a 150-W halogen projection lamp (light intensity $\sim 2.5 \times 10^3$ μmol/m^2 · sec) for 0, 15, 30, 45, and 60 sec. Esterified phosphate was extracted as described previously (Selman, 1976) and counted in a Packard 460 C liquid scintillation counter.

RESULTS

Generation of *atpC*1 Cotransformants

Figure 1 shows the CF_1 γ-subunit constructs that were cotransformed along with plasmid PMN24 (Kindle *et al.*, 1990), the *C. reinhardtii* nitrate reductase gene, into strain *atpC*1 (background *nit1*-305, cw 15, mt$^-$). The cells were selected first on SGII-NO_3^- plates. Nit$^+$ colonies were picked and placed into 96-well microtiter plates containing SGII-NO_3^- media and then selected for photoautotrophic growth in 96-well microtiter plates containing SGII-NH_4^+ without acetate. Starting from 2 μg of transforming DNA, about 50 nit$^+$ colonies were obtained, of which 5–8 were also positive for photoautotrophic growth and were thus labeled as putative cotransformants.

Total DNA was isolated from *nit1*-305, *atpC*1, and each of putative cotransformants and subjected to Southern blot analysis after *Pst* I digestion. In each case, the photoautotrophic colonies contained bands that hybridized to randomly primed [^{32}P]-labeled CF_1 γ-cDNA. There were numerous hybridization bands that were in addition to the endogenous *atpC*1 bands. These results indicate that the transforming γ-subunit constructs integrated in the *atpC*1 genome. In no case was there any indication of a reversion event (data not shown).

Northern Blot Analysis/RT-PCR from Cotransformed *atpC*1 Cells

Five-microgram samples of total RNA isolated from *nit1*-305, *atpC*1, and each of the cotransformant strains were subjected to Northern blot analysis and probed with randomly primed [^{32}P]-labeled CF_1 γ-cDNA (Fig. 2). Both D199K/K203D and D199A strains (Lanes 3 and 4, respectively) contain a hybridization product which co-migrates with the same product from the parental *nit1*-305 strain (Lane 1). In comparison, this product is absent in total RNA isolated from *atpC*1 (Lane 2).

To determine whether the cotransformed strains expressed the construct sequences we engineered and transformed into them, an RT-PCR procedure was utilized. Samples of RNA (5 μg) were reverse transcribed, and the cDNA products were then amplified by PCR through a region that contains intron 4 of the γ-subunit gene. The purpose of this was to rule out the possibility of obtaining a PCR product from genomic DNA which may have co-precipitated during the RNA

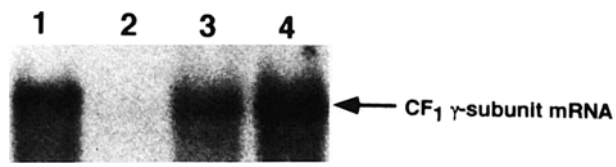

Fig. 2. Northern blot analysis of total RNA from *nit1*-305, *atpC*1, D199K/K203D, and D199A cotransformants. Samples of total RNA (5 μg) were separated in a 1% agarose gel, transferred to a nylon membrane, and probed with randomly primed [^{32}P]-labeled CF_1 γ-subunit cDNA. Lane 1, *nit1*-305; Lane 2, *atpC*1; Lane 3, D199K/K203D; Lane 4, D199A. RNA loading was monitored by probing for the CF_1 α-subunit mRNA which was of equal amount in each lane (not shown). This is in agreement with previously published results from our laboratory (Ross *et al.*, 1995). A repetition of this entire experiment gave identical results.

preparations. Figure 3A shows a cartoon of the area subjected to PCR. A PCR product arising from cDNA would be 308 bp and this would encompass the area of the γ-subunit where the mutations were introduced. Figure 3B shows that for *nit1*-305 and each of the two cotransformant strains there is a 308-bp product which co-migrates with the product amplified from the cloned γ-subunit cDNA (compare Lanes 3, 5, and 6 with Lane 2). Consistent with the Northern results, the 308-bp product is absent in the *atpC*1 lane (Lane 4), further suggesting that this strain is devoid of CF_1 γ-subunit messenger RNA.

Within the 308-bp product there is a 168-bp stretch which is flanked by *Bgl*II restriction sites and contains the DNA sequence for the γ-subunit disulfide bond spacer region. The RT-PCR products were gel purified, restricted with *Bgl* II, subcloned into the *Bam* HI site of pBluescript SK^+, and screened by blue/white detection. Plasmid DNA was isolated from numerous white colonies and track sequenced with dideoxycytosine. Figure 3C shows the dideoxycytosine track sequences for each of the two cotransformed strains in comparison to the wild-type γ-subunit sequence from *nit1*-305. The arrows indicate the sites where the mutations were introduced into this region. These results demonstrate that each of the cotransformed strains express the mutated γ-subunit genes we engineered and transformed into the *atpC*1 background.

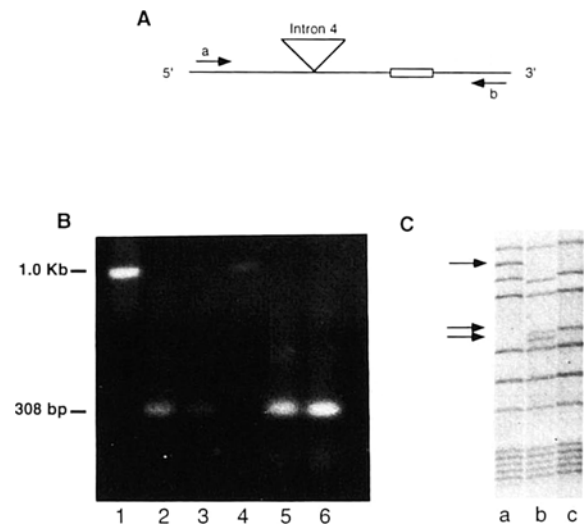

Fig. 3. Reverse transcription-PCR/partial cDNA sequencing of *nit1*-305, *atpC*1, D199K/K203D, and D199A. Samples of RNA (5μg) were reverse transcribed and amplified by PCR by following the manufacturer's protocol. (A) Map depicting the area of the generated CF_1 γ-subunit cDNAs subjected to PCR. Primers A and B were designed to amplify through intron 4 of the γ-subunit gene. The box represents the region which contains the γ-subunit disulfide bond. (B) Ethidium bromide stained 1% agarose gel which illustrates the RT-PCR results. Lane 1, γ-subunit genomic clone; Lane 2, γ-subunit cDNA; Lane 3, *nit1*-305; Lane 4, *atpC*1; Lane 5, D199K/K203D; Lane 6, D199A. For *nit1*-305 (Lane 3), D199K/K203D (Lane 5), and D199A (Lane 6) there is a 308-bp segment which co-migrates with the same product amplified from the γ-subunit cDNA (Lane 2). Note that this product is not found in *atpC*1 (Lane 4). (C) Partial cDNA sequence analysis which depicts only the dideoxycytosine track. The arrows indicate the mutated γ-subunit sequences which were introduced into strain *atpC*1 by transformation. A, D199A; B, D199K/K203D; C, *nit1*-305 (compare panel C with A and B). These data are representative of two separate but identical experiments.

Phenotype Assessment by Measurements of Heterotrophic and Photoautotrophic Growth

Approximately 10^3 cells of parental *nit1*-305, *atpC*1, and each of the two cotransformed strains were plated into 250 ml of either SGII-NH_4^+ for nonselective (heterotrophic) growth or SGII-NH_4^+ acetate minus media for measurements of photoautotrophic growth. For nonselective growth, the cells were cultured in continuous light of 120 μmol/m^2 · sec. For photoautotrophic growth, the cells were cultured in an 8-h light/16-h dark photoperiod for several days. During the 8-h illumination period the light intensity was also 120 μmol/m^2 · sec. At the indicated time points, two 1-ml samples of cells were harvested, immobilized in a solution of 1 mM iodine in 95% ethanol, and counted with a hemacytometer. The results from both sets of experiments indicated that strains D199K/K203D and D199A grew with identical kinetics to that of the parental *nit1*-305 strain. However, in both heterotrophic and photoautotrophic growth experiments the D199K/K203D strain reached a slightly lower final cell density than either D199A or *nit1*-305 (data not shown). Under photoautotrophic growth conditions *atpC*1 cells died within 12 h after plating because this strain is an obligate acetate auxotroph. This result is in agreement with previous findings from our laboratory (Smart and Selman, 1993; Ross *et al.*, 1995).

Measurements of Soluble ATPase Activities

Soluble, thylakoid-associated protein fractions enriched in CF_1 (0.5–1.5 μg of protein) were incubated in reaction mixtures with or without 25 mM DTT at 37°C for 0, 5, 10, and 15 min. The catalytic activities of the enzymes from *nit1*-305, D199K/K203D, and

D199A were measured as Mg^{2+}-ATPases. Figure 4 (panel A) illustrates that the presence of 25 mM DTT in the reaction mixture stimulates the ATPase activity on *nit1*-305, which is in contrast to the results of ATPase measurements from both strains D199K/K203D and D199A (Fig. 4, panels B and C, respectively). For these strains the rates are identical, regardless of the presence of DTT. Furthermore, these strains exhibit rates of ATP hydrolysis in the presence or absence of DTT which are similar to that of the *nit1*-305 strain in the presence of DTT (compare the slopes of ATP hydrolysis of each mutant to *nit1*-305 plus DTT). These experiments measured the manifest ATPase activity of the enriched CF_1 fractions. The *C. reinhardtii* enzyme, unlike its counterpart from spinach (Nalin and McCarty, 1984; Ketcham *et al.*, 1984), is isolated with manifest activity of 5–8 units, where a unit is defined as μmol P_i released/mg protein · min (Selman-Reimer *et al.*, 1981). Complete inhibition of enzymatic activity was achieved by the presence of 330 μg of a *Dunaliella salina* antiserum against the CF_1 holoenzyme in the reaction mixture but not a preimmune serum at the same concentration (data not shown). The antibody inhibition results are consistent with previous findings (Smart and Selman, 1993; Ross *et al.*, 1995).

PMS-Dependent Photophosphorylation

Figure 5 depicts the results from *in vitro* PMS-dependent photophosphorylation experiments from thylakoid particles isolated from the strains *nit1*-305, D199K/K203D, and D199A. Strain *atpC*1 was not

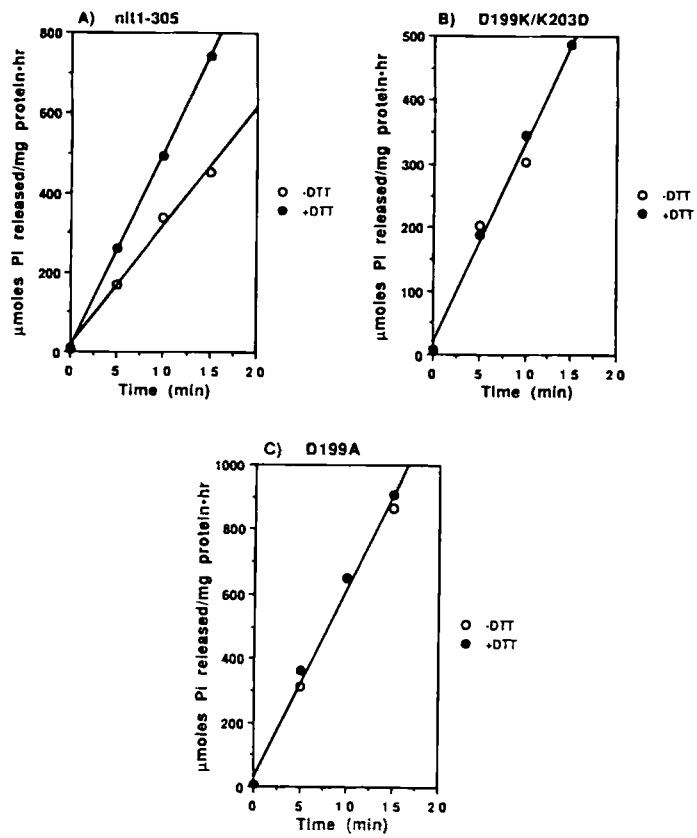

Fig. 4. Time course for soluble ATPase activity of partially purified CF_1 from strains *nit1*-305, D199K/K203D, and D199A. Protein fractions enriched in CF_1 were isolated from *nit1*-305, D199K/K203D, and D199A and assayed as Mg^{2+}-dependent ATPases over the course of 0, 5, 10, and 15 min. The data points are the means of duplicate experiments, each with a standard deviation of not greater than 10%. A repetition of this entire experiment gave similar results.

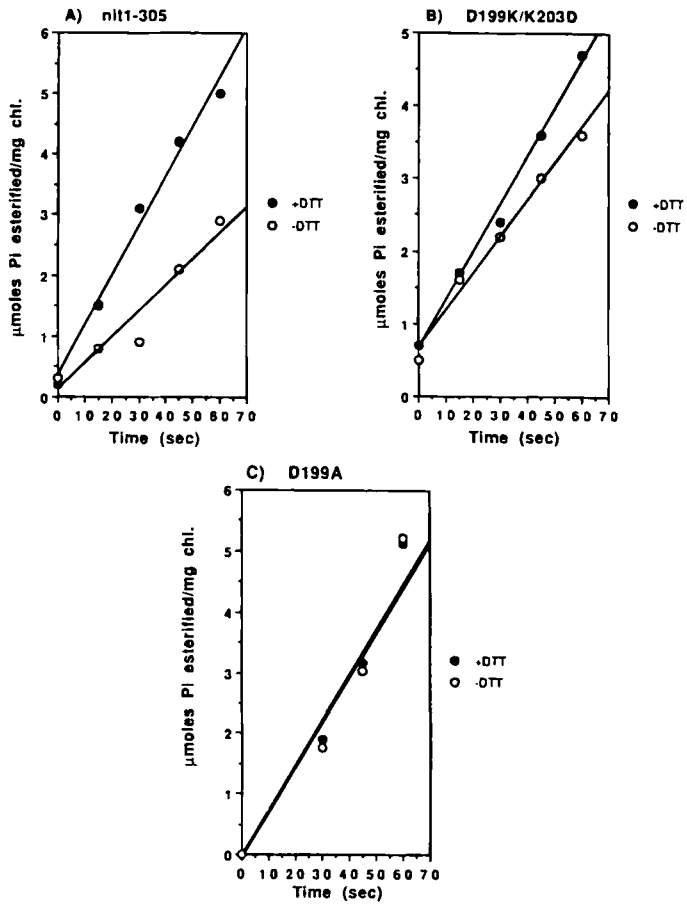

Fig. 5. PMS-dependent photophosphorylation of *nit1*-305, D199K/K203D, and D199A thylakoid particles. Dark relaxed thylakoids (5–10 µg of chlorophyll) were assayed in the presence or absence of 25 mM DTT for ATP synthesis from ADP and ^{32}P labeled phosphate for 0, 15, 30, 45, and 60 sec. The data points are the means of duplicate experiments, each with a standard deviation of not greater that 10%. A repetition of this entire experiment gave similar results.

tested because these cells do not assemble CF_1 complexes (Smart and Selman, 1993). Panel A indicates that the rate of photophosphorylation for *nit1*-305 thylakoid particles is increased approximately 2-fold by the addition of 25 mM DTT to the reaction mixture. Panels B and C demonstrate that the DTT stimulatory effect disappears for both the D199K/K203D and D199A strains, although for the former, the disappearance is not as pronounced as the latter. However, it should be noted that the loss of a DTT stimulatory effect is consistent with similar photophosphorylation experiments which were performed where ascorbate/dichloroindophenol/methyl viologen (ASC/DCIP/MV) were used as electron donors/acceptors instead of PMS. Similar to the data from PMS-dependent photophosphorylation experiments, the ASC/DCIP/MV results demonstrated that the DTT stimulation of *nit1*-305 photophosphorylation was again absent for both D199K/K203D and D199A (data not shown).

DISCUSSION

Recent advances in *Chlamydomonas* technology have made it possible to carry out structure/function relationship studies *in vivo* by transformation of one's gene of interest into either the nuclear or chloroplast genomes. In the work described here we have used nuclear transformation to address questions concerning the involvement of the CF_1 γ-subunit disulfide bond

spacer region in the regulation of ATP synthase. We utilized *C. reinhardtii* strain *atpC*1 which was isolated and characterized in our laboratory (Smart and Selman, 1991). This strain fails to synthesize the γ-subunit polypeptide and, consequently, does not assemble functional CF_1 complexes. We used a cotransformation protocol which takes advantage of the fact that *atpC*1 cells are also devoid of the nitrate reductase gene product. Because the possibility existed that the mutant constructs would not complement the defect in *atpC*1, cotransformation would allow for the isolation of strains which may harbor copies of the mutant γ-subunits, but would not be viable under photoautotrophic growth conditions. Such colonies could be easily detected by Southern blot analysis. Fortunately, the acquisition of cotransformed *atpC*1 cells turned out to be straightforward.

Three lines of evidence demonstrate that the cotransformed *atpC*1 cells harbor and express the mutant γ-subunits we engineered and transformed into them. First, the fact that the photoautotrophic colonies were obtained after transformation experiments of strain *atpC*1 with the γ-subunit constructs suggests that the transformed cells expressed the γ-subunit genes. This in turn would restore photoautotrophic growth. Second, Northern blot analysis (Fig. 2) demonstrated that each of the cotransformed strains express a γ-subunit mRNA which co-migrates with the same from product from the parental *nit1*-305 strain. In contrast, *atpC*1 cells do not contain this gene transcript. Third, results from the RT-PCR reactions coupled with the partial cDNA cloning and sequencing procedures (Fig. 3) demonstrate that each of the cotransformed strains express the engineered γ-subunit genes we introduced into the *atpC*1 background by transformation.

Taken together, the data from the ATPase and PMS-dependent photophosphorylation experiments suggest a role for the γ-subunit disulfide bond spacer region in the redox regulation of coupling factor. In both types of assays, the effect of DTT stimulation of enzymatic activity for the wild-type enzyme was not seen in either of the mutant strains tested. Curiously, both the ATPase and photophosphorylation activities of the double mutant D199K/K203D are slightly lower that of the D199A and *nit1*-305 strains. This finding may somehow reflect the observation that strain D199K/K203D took slightly longer to reach log phase growth which subsequently leveled off at lower cell density than the other strains.

In a sense, these results are somewhat surprising given that the mutations do not directly affect the cysteine residues involved in the formation of the CF_1 γ-subunit disulfide bond. We and others have demonstrated the involvement of these residues in redox control of chloroplast ATP synthase (Nalin and McCarty, 1984; Ketcham *et al.*, 1984; Mills and Mitchell, 1984; Shahak, 1985; Junesch and Graber, 1987; Hangarter *et al.*, 1987; Nocter and Mills, 1988; Kramer *et al.*, 1990, Ross *et al.*, 1995). The involvement of the spacer amino acids between the cysteine residues suggests that this entire region (spacer plus cysteines) is involved in the redox regulation of CF_1. One logical reason for the biochemical behavior of the two mutant enzymes discussed in this study is that the introduced mutations somehow prevent the formation of a γ-subunit disulfide bond and, therefore, coupling factor in these mutant strains would not be regulated by a redox event. However, based on Western blotting results from our laboratory, this seems unlikely. Wild-type thylakoid particles isolated in the presence of DTT yield an ε-subunit-deficient CF_1 isozyme upon purification (Duhe and Selman, 1990). The exclusion of DTT in the purification step leads to the isolation of CF_1 containing ε-subunit from wild type cells. These data indicate that an oxidized γ-subunit disulfide bond leads to the purification of CF_1 isozyme that contains ε-subunit. Western blotting of partially purified CF_1 from strains D199K/K203D and D199A isolated from thylakoid particles in the absence of DTT reveal the presence of the ε-subunit (M. X. Zhang, S. A. Ross, and B. R. Selman, unpublished observations). In contrast, Western blotting of partially purified CF_1 from *C. reinhardtii* strains harboring mutations in the cysteine residues of the γ-subunit disulfide bond (C198S, C204S, and C198S/C204S) results in an ε-deficient isotype regardless of the presence of DTT during the purification procedure (S. A. Ross and B. R. Selman, unpublished observations). It appears therefore that the reduction of the γ-subunit disulfide bond or its disruption by site-directed mutagenesis of the cysteine residues that comprise the bond results in an isotype devoid of any ε-subunit. However, since partially purified CF_1 isolated in the absence of DTT from strains D199/K203D and D199A has ε-subunit associated with it (as seen for wild type isolated without DTT), this argues that the γ-subunit disulfide bond is intact in these cells. An alternative explanation for the lack of a redox effect is that subunit–subunit interactions may have been perturbed by the introduction of site-directed mutants into the γ-subunit disulfide spacer region such that the regulatory disulfide is not accessible to reducing agents.

Two main conclusions can be drawn from these results. First, strain *atpC*1 can be complemented with mutated γ-subunit genes. We have recently demonstrated that *atpC*1 cells can be complemented with mutant γ-subunit constructs (Ross *et al.*, 1995). This was the first demonstration in *C. reinhardtii* of complementation of a known defect with a mutant gene. The work discussed herein further extends our previous findings and solidifies the strategy of using mutant genes as the complementing agents of known genetic effects in order to carry out structure/function studies. Second, biochemical analysis of the mutant CF_1 enzymes demonstrated an as yet unknown role for the γ-subunit disulfide bond spacer region in the redox regulation of the enzyme. The current view of the regulatory scheme of CF_1 that has emerged is that the disulfide bond of the γ-subunit disulfide bond is involved in the redox regulation of the enzyme. The data presented here suggest that the spacer region between the disulfide bond is also involved in redox regulation of coupling factor in plants.

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