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Characterization of *atpA* and *atpB* deletion mutants produced in *Chlamydomonas reinhardtii* cw15: electron transport and photophosphorylation activities of isolated thylakoids

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Abstract

We produced *atpA* and *atpB* deletion mutants of *C. reinhardtii* cw15 in order to investigate the role of certain domains of the α and β subunits of the chloroplast ATP synthase (CF_0CF_1) by site-directed mutagenesis. The deletion mutants were obtained by transformation with constructs containing the *aadA* cassette inserted into the *atpA* or *atpB* genes in place of the corresponding open reading frames. Homoplasmic strains were obtained by continued selection under heterotrophic growth conditions in the presence of spectinomycin. The deletion mutants could be complemented by transformation with the wild-type *atpA* or *atpB* genes, respectively. In both deletion mutants none of the subunits of CF_1 and of CF_0 were detected by electrophoretic analysis. Since the mother strain cw15 as well as the deletion mutants are cell wall-deficient, we were able to prepare photosynthetically active thylakoids and to study the photosynthetic characteristics of cw15 thylakoids and mutant thylakoids lacking the ATP synthase. Electron transport measurements showed that the PSII and PSI activities were not affected in the deletion mutants. Thylakoids from the deletion mutants were able to maintain a significantly higher light-induced proton gradient than thylakoids from cw15, confirming the absence of a functional proton channel in these deletion mutants. When substrates of photophosphorylation were added, the transmembrane proton gradient in wild-type thylakoids decreased and ATP was formed, while in the deletion mutant thylakoids the proton gradient was not affected and no ATP was formed. The phosphorylation/ΔpH relationship in *C. reinhardtii* thylakoids was shifted to lower ΔpH values by reduction with dithiothreitol, indicating that the *C. reinhardtii* enzyme is also similar to the higher plant chloroplast ATP synthase in this respect.

Keywords: CF_0CF_1; *atpA*; *atpB*; Deletion mutant; Photophosphorylation; Thiol modulation; Photosystem I; Photosystem II; (*Chlamydomonas reinhardtii* cw15)

1. Introduction

The green alga *Chlamydomonas reinhardtii* constitutes an ideal model organism for the study of plant photosynthesis. *C. reinhardtii* can be easily cultivated, the components of its photosynthetic apparatus are very similar to those of higher plants and many mutants affecting the photosynthetic apparatus have been isolated and genetically mapped [1]. *C. reinhardtii* can be transformed in the nuclear and chloroplast genome [2,3] and suitable selection markers and transformation techniques for both compartments are

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available [4–7]. The chloroplast genome of *C. reinhardtii* has been mapped in detail [8] and about 80% of its DNA sequence has been determined. Although the arrangement of the genes in the plastid genome of *C. reinhardtii* deviates from that in higher plants, the genes coding for chloroplast proteins are almost identically distributed between the plastid and the nuclear genomes and the gene products show high sequence similarities. The sequences of photosynthetic proteins of *C. reinhardtii* are more closely related to the corresponding higher plant sequences than to those of cyanobacteria.

Since *C. reinhardtii* can grow heterotrophically, it is possible to delete genes for components of the photosynthetic apparatus. Upon chloroplast transformation, the incorporation of the donor gene into the chloroplast genome occurs by homologous recombination [3]. Therefore, specific chloroplast sequences can easily be interrupted or deleted using the *aadA* cassette expressing spectinomycin resistance in the chloroplast [5]. In this paper, we report on two mutants deleted in the genes *atpA* or *atpB* [9,10], plastid genes encoding the α and β subunit, respectively, of the chloroplast proton translocating ATPase (ATP synthase, CF_0CF_1). The mutants were designed and produced as recipients for mutated *atpA* or *atpB* genes to investigate the role of specific amino acids of the two CF_1 subunits by site-directed mutagenesis.

The suitability of *C. reinhardtii* as a model organism for studying photophosphorylation depends on the complete molecular and functional characterization of its chloroplast ATP synthase. Recently we have isolated CF_0CF_1 of *C. reinhardtii* and identified all nine subunits by their N-terminal sequences [11]. So far photophosphorylation in *C. reinhardtii* was studied with thylakoids prepared by sonication of cell wall containing strains [12,13]. The detailed enzymatic characterization of wild-type and mutant ATP synthase activities and their regulation *in membrana* requires more stable and reproducible preparations. For this reason we have employed the *C. reinhardtii* strain cw15 which can be broken by mild mechanical treatment [14]. This allowed to develop a convenient protocol to isolate well coupled thylakoids [15]. Here we report on the characterization of electron transport, $\Delta\mu H^+$ formation and ATP synthase activity in thylakoids of *C. reinhardtii* strain cw15 and of the two deletion mutants.

2. Materials and methods

C. reinhardtii cw15 was cultivated in high salt (HS) medium [1]. Selection for transformants was carried out in high salt medium containing 0.4% acetate and 150 μg/ml spectinomycin (HSHA/spec) at low light. All cloning procedures were done using commercially available restriction endonucleases and DNA modifying enzymes according to the suppliers' instructions. DNA fragments were isolated from agarose gels using the 'Glass Select' DNA isolation kit (5 Prime-3 Prime, Boulder, CO) or by freezing the sliced agarose fragments in the presence of 80% phenol followed by subsequent chloroform extraction and ethanol precipitation of the DNA from the resulting supernatant. Transformation into *E. coli* JM109 or DH5α was done according to the instructions supplied in the pAlter site-directed mutagenesis kit (Promega Biotech). Plasmid DNA was isolated by alkaline miniprep as described in the T7 DNA sequencing protocol (Promega Biotech) or by the Qiagen plasmid kit (Qiagen GmbH, Hilden, Germany). DNA sequencing was carried out using 'Sequenase version 2' (USB) and denatured plasmid DNA as template.

Chloroplast transformation was done by vortexing in the presence of glass beads [7] or by particle gun bombardment using the Biorad PDS1000-He apparatus. Total DNA was isolated as described [16] except that cells scraped from agar plates were resuspended in the lysis buffer rather than grown in liquid medium.

Photosynthetically active thylakoids were prepared from cw15 and from the deletion mutants cw15ΔatpA and cw15ΔatpB according to the following protocol: cw15 was grown at 22°C in high salt medium [1] containing 0.2% acetate at a light intensity of 900 lux in a 14 h light–10 h dark cycle, in 400-ml cultures bubbled with sterile air (mixotrophic culture). The deletion mutants were cultivated in HS medium containing 0.4% acetate and 150 μg/ml spectinomycin in dim light at 22°C. The cultures were harvested at a cell density of 6×10^6 cells/ml, washed with medium I (300 mM sucrose, 10 mM Tricine, pH 8.0, 50 mM NaCl and 5 mM $MgCl_2$) and resuspended in 15 ml of the same medium at 4°C. All further steps were carried out at this temperature. The cells were passed two times through a Yeda Press at 5×10^5 N/m^2 and 5.5×10^5 N/m^2, respectively. The pellet

gained by 1 min centrifugation at $3000 \times g$ was washed in medium I. Intact chloroplasts were broken osmotically by resuspension of the pellet in medium II (2 mM Tricine, pH 8.0, 50 mM NaCl and 1 mM $MgCl_2$) and thylakoids were collected by centrifugation.

CF_0CF_1 was isolated from *C. reinhardtii* cw15 as reported recently [11]. For analysis of membrane proteins, thylakoids were isolated by resuspension of cells in sonication buffer (10 mM Tricine/NaOH; pH 8, 1 mM dithiothreitol (DTT)) followed by ultrasonication for five seconds and centrifugation for 10 min at $12\,000 \times g$. The pellet was washed twice with washing buffer. Membrane proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie R250, or transferred to nitrocellulose and immunostained using a CF_1 specific rabbit antiserum and proteinA alkaline phosphatase. The protein content was determined according to Lowry [17]. The chlorophyll content and the chl *a*/chl *b* ratio were determined as in Ref. [18].

Proton gradient formation was monitored at 20°C by means of 9-aminoacridine fluorescence quenching in a fluorimeter as described [19]. Thylakoids (final chlorophyll concentration of 25 μg/ml) were suspended to a final volume of 2.5 ml in reaction medium consisting of 25 mM tricine buffer (pH 8.0), 50 mM KCl, 50 nM valinomycin, 5 mM $MgCl_2$, 50 μM phenazine methosulphate (PMS) and 5 μM 9-aminoacridine. For thiol-modulated thylakoids reaction mixes contained in addition 10 mM DTT. The 9-aminoacridine fluorescence quench signal was calibrated by the ΔpH dependency of electron transport [20]. For the quantitative evaluation under our assay conditions the following empirical formula was found:

$$\Delta\mathrm{pH} = \log\left[(F_{\max} - F)/F\right] + 2.81$$

($F_{\max}$, F: fluorescence of 9-aminoacridine in the dark and light, respectively).

When ATP formation was measured simultaneously, 1 mM ADP plus 5 mM [^{32}P]P_i, together with an ADP-regenerating system (12 U/ml hexokinase and 10 mM glucose), were added after 3 min of preillumination. In samples taken after 10, 20, 30 and 40 s, the organic [^{32}P]phosphate formed was assayed as described [21].

Photosynthetic electron transport was measured by oxygen consumption using a Clark-type electrode (Hansatech; Bachhofer, Reutlingen, Germany) at a light intensity of 1200 μE m^{-2} s^{-1} (red light $>$ 630 nm) and a temperature of 20°C. The assay medium contained 25 mM tricine (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 1 mM NaCN and 0.5 mM methylviologen. The final volume was 2 ml and the final chlorophyll concentration was 25 μg/ml. Photosystem I-dependent electron transport from reduced dichlorophenolindophenol ($DPIPH_2$) to methylviologen was assayed in the same medium containing 4 μM DCMU ((3,4-dichlorophenyl)-1,1-dimethyl urea), 0.2 mM DPIP and 2 mM ascorbate instead of DTT.

3. Results and discussion

3.1. Preparation of atpA and atpB deletion mutants

The *atpA* deletion mutant, cw15ΔatpA, was produced using plasmid pUC-atpX-AAD [5]. This plasmid contains the bacterial *aadA* gene supplied with a mRNA stabilizing 3′ untranslated sequence derived from the *C. reinhardtii rbcL* gene, fused to the *C. reinhardtii atpA* promotor sequence of 0.7 kb. This *aadA* expression cassette expresses spectinomycin and streptomycin resistance in the chloroplast. We added a 2.1 kb *Pst*I-*Xba*I fragment encoding the C-terminal part of the α subunit and its 3′ untranslated domain to the plasmid pUC-atpX-AAD opened downstream of the expression cassette. The resulting construct (Fig. 1A) contains the 5′- and 3′ untranslated regions of *atpA*, which are necessary to incorporate the *aadA* expression cassette into the chloroplast genome in place of the *atpA* gene upon chloroplast transformation. This construct was used to transform *C. reinhardtii* cw15 by vortexing in the presence of glass beads [7]. Integration of the *aadA* gene into the chloroplast genome led to deletion of 1110 bases encoding the N-terminal 370 amino acids of the α subunit. Spectinomycin-resistant colonies were regrown on HSHA/spec agar plates in dim light for several months. Four of the resulting colonies that did not grow photoautotrophically were further analyzed.

The *atpB* deletion mutant cw15ΔatpB was also generated using the *aadA* cassette. Plasmid pT7atpB [22] was digested with *Bsm*I. This restriction endonuclease cuts the plasmid only twice, and the remaining

vector fragment (lacking the *atpB* open reading frame from codon Pro-2 to codon Ala-473) was gel-purified. The restriction overhangs were filled in and the *aadA* expression cassette, extracted from plasmid

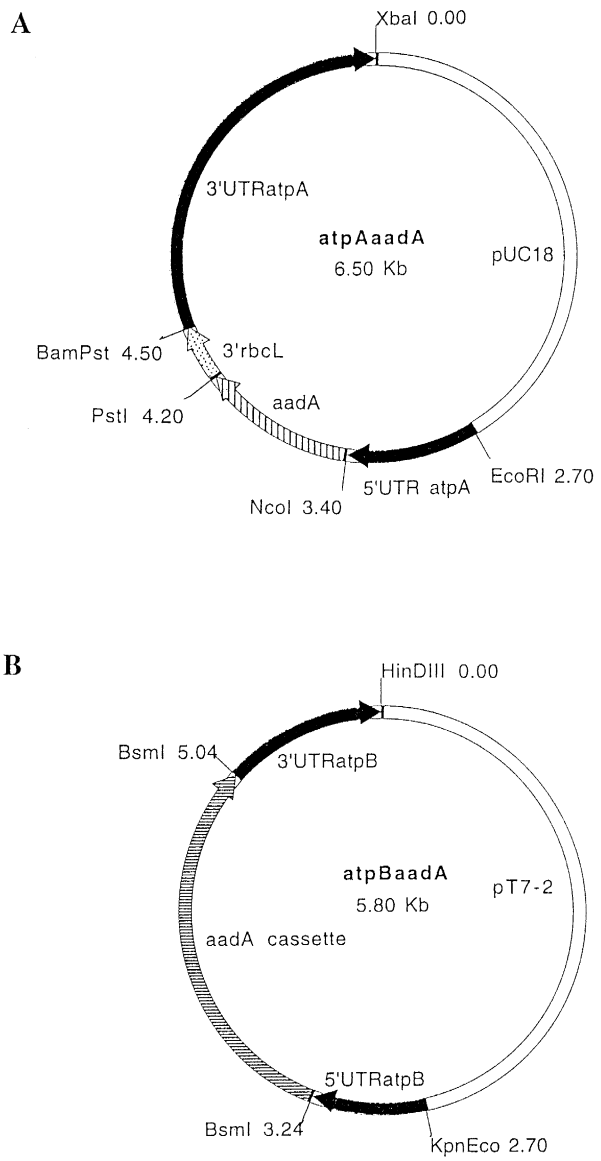

Fig. 1. Constructs used for deletion of *atpA* and *atpB* in cw15. A: Construct used to obtain the *atpA* deletion mutant: the pUC-atpX-AAD [5] plasmid was supplemented with a 2 kbp fragment covering the 3′ untranslated region of the *atpA* gene and the resulting construct was used to transform cw15. B: Construct used to obtain the *atpB* deletion mutant: the *atpB* open reading frame was excised from plasmid atpBpT7 [22] with *Bsm*I and the *aadA* expression cassette, excised with *Sma*I and *Eco*RV from pUC-atpX-AAD, was blunt end ligated into the remaining plasmid containing the 5′ and 3′ untranslated domains of *atpB*.

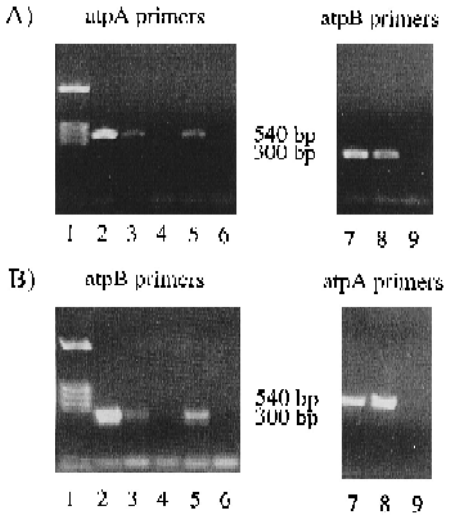

Fig. 2. PCR analysis of the deletion mutants. A: *atp*A deletion mutant (cw15Δatp A): The following template DNA's were amplified using *atp*A specific primers (2–6) or *atp*B specific primers (7–9): (2) 100 ng cw15 DNA; (3) 1 ng cw15 DNA; (4) 100 ng cw15Δatp A DNA; (5) 100 ng cw15Δatp A DNA + 1 ng cw15 DNA; (6) control without DNA; (7) 100 ng cw15 DNA; (8) 100 ng cw15Δatp A DNA; (9) control without DNA; B: *atp*B deletion mutant (cw15Δatp B): The following template DNA's were amplified using *atpB* specific primers (2–6) or *atpA* specific primers (7–9): (2) 100 ng cw15 DNA; (3) 1 ng cw15 DNA; (4) 100 ng cw15Δatp B DNA; (5) 100 ng cw15Δatp B DNA + 1 ng cw15 DNA; (6) control without DNA; (7) 100 ng cw15 DNA; (8) 100 ng cw15Δatp B DNA; (9) control without DNA. Lane 1 shows pBS-SK + plasmid DNA digested with *Hin*fI.

pUC-atpX-AAD with *Sma*I and *Eco*RV, was ligated into this plasmid fragment. *C. reinhardtii* cw15 was transformed with the resulting plasmid (Fig. 1B) by vortexing in the presence of glass beads [7]. Transformants were selected on HSHA/spec media under dim light, until clones unable to restore photoautotrophic growth were obtained.

Total DNA was isolated from cw15Δatp A, from cw15Δatp B and from cw15. The absence of all *atpA* or *atpB* gene copies in the mutant strains was demonstrated by PCR. PCR amplification of 100 ng of DNA derived from cw15Δatp A and cw15Δatp B, using *atpA* or *atpB* specific primers, respectively, did not yield any PCR products (Fig. 2A, lane 4 and 2B, lane 4). Control amplifications under the same conditions, of 100 and 1 ng of cw15 DNA and 1 ng of cw15 DNA mixed with 99 ng of each deletion mutant DNA, or with *atpB* specific primers for amplification of the cw15Δatp A DNA and *atpA* specific primers

for amplification of cw15ΔatpB DNA, yielded PCR products of the expected sizes (300 bp for *atpB* and 540 bp for *atpA*) (Fig. 2). These experiments showed that none of the DNA preparations contained contaminations that inhibit PCR amplification and confirmed that no copies of the *atpA* or *atpB* genes, respectively, are present in the deletion mutants.

For Southern blot analysis of cw15ΔatpA, total DNA from cw15ΔatpA and from cw15 was digested with *Eco*RI, subjected to agarose gel electrophoresis and transferred to nitrocellulose. The nitrocellulose was probed with probes specific for the fragment of the *atpA* gene missing in the deletion mutants or for the *aadA* gene. Correct insertion of the *aadA* cassette in place of the deleted part of the *atpA* gene leads to elimination of the *Eco*RI site in the *atpA* gene [9]. As expected, with cw15 DNA a 3.5 kb fragment (*Eco*RI fragment 22) hybridized to the *atpA* specific probe, while with the cw15ΔatpA DNA no signal was obtained. With the *aadA* specific probe no signal was obtained with cw15 DNA, while with the deletion mutant DNA a 9.5 kb band was observed as a result of the joined *Eco*RI fragments 15 plus 22, with a 600 bp addition due to the size difference between the *aadA* cassette and the deleted *atpA* fragment (not shown, [23]). For Southern blot analysis of cw15ΔatpB, DNA from cw15 and from cw15ΔatpB was digested with *Bam*HI and *Xho*I. Hybridization with an *atpB* specific probe did not yield any signal in cw15ΔatpB DNA, while in cw15 DNA a 7.6 kb band was detected. In the cw15ΔatpB DNA the predicted 4.7 kb fragment (resulting from insertion of a new *Xho*I site within the *aadA* cassette) hybridized with the *aadA* probe (not shown [23]).

Several walled *C. reinhardtii atpB* deletion mutants have previously been identified [1], characterized and used for transformation [3], for studying regulation of gene expression [24] and for mutagenesis of the tentoxin interaction site in the ATP synthase [25]. Mutants that do not synthesize subunits β, ϵ, I and IV were previously described [26,27]. These mutants were either completely or partially deficient in all ATP synthase subunits, even though all subunit genes except one were transcribed and translated as demonstrated by pulse labeling experiments.

Since a *C. reinhardtii* mutant deleted in *atpA* has not yet been described, we analyzed the *atpA* or *atpB* deletion mutants for the presence of ATP synthase subunits by SDS-PAGE and immunostaining of Western blots. The absence of partially assembled subcomplexes was checked by sucrose gradient fractionation of solubilized thylakoid membranes. We were not able to isolate any subunit-deficient CF_0CF_1 subcomplex from the deletion mutant thylakoids employing the procedure established for isolation of *C. reinhardtii* cw15 CF_0CF_1 [11]. The polypeptide patterns obtained from thylakoid membranes indicated that α and β subunits are absent in both deletion mutants and clearly demonstrated the absence of the proteolipid (subunit III) in the deletion mutant thylakoids, which is the only prominent thylakoid protein with an apparent molecular weight of 8 kDa detected in *C. reinhardtii* cw15 membranes. The complete lack of CF_0 subunit III coincides with the fact that these thylakoids showed a decreased permeability for protons and maintained a proton gradient that was significantly higher than that in wild-type thylakoids (see Section 3). CF_0CF_1 itself is known to contribute significantly to the leakiness of the thylakoid membrane, in particular in the absence of nucleotides ('proton slip') [28]. The polypeptide patterns of wild-type and deletion mutant thylakoids were also analyzed by immunostaining of Western blots with an antiserum raised against purified CF_1. Subunits γ and ϵ, that could be clearly identified in the cw15 thylakoids, were not detectable in both deletion mutants (not shown). Our results confirm the observation that lack of one ATP synthase subunit appears to result in the immediate degradation of the unassembled subunits.

3.2. Transformation of cell wall deficient mutants by particle gun bombardment

The above experiments proved that the two mutants were deleted in all copies of the *atpA* or *atpB* genes, and that the *aadA* cassette was incorporated in the expected way in the chloroplast genome, in place of the corresponding open reading frames. As these mutants were designed as recipients for transformation with mutated *atpA* or *atpB* genes, we attempted to transform them with the corresponding subcloned wild-type genes. Transformation of the cell wall deficient deletion mutants proved to be rather difficult. Low frequency transformation ($\sim 2/10^8$ photoautotrophic cells) was observed after vortexing of the

cw15ΔatpA deletion mutant with plasmid atpApBS, containing the *atpA* gene flanked by 1200 bp of 5′ and 1800 bp of 3′ flanking regions subcloned into pBS. Initial attempts to transform the mutants by particle gun transformation were equally inefficient, probably due to the sensitivity of cell wall deficient cells to hydrodynamic shock and high vacuum. Varying parameters such as: vacuum, particle speed and target distance did not result in an improved transformation efficiency. Only when the cells were spread onto the target plates resuspended in soft agar, under strictly defined conditions, the transformation efficiency was significantly improved. Spreading $\sim 2.5 \times 10^7$ cells, resuspended in 0.25% agar, and subsequent drying of the plates for 2–4 h at room temperature, yielded more than 50 photoautotrophically growing cells per plate upon bombardment of the plate in the second holder position at a vacuum of 26 psi and a rupture pressure of 800 psi (~ 2 transformants per 10^6 plated cells). The cw15ΔatpB deletion mutant could be successfully complemented as well, by transformation with plasmid p17, which contains the subcloned *Bam*HI 10 fragment with the whole *atpB* gene, and by a series of mutated *atpB* genes that were able to restore photoautotrophic growth. No spontaneous revertants were ever observed in control experiments, with either cw15ΔatpA or cw15ΔatpB, when the mutant cells were spread onto minimal medium and exposed to selection for autotrophic growth or were subjected to bombardment with tungsten particles not coated with DNA and selected for photoautotrophic growth.

The transformation efficiency obtained with cw15ΔatpA and cw15ΔatpB was sufficiently high to select for kanamycin resistance allowing us to use a kanamycin phosphotransferase expression cassette as a selectable marker. Since both cw15ΔatpA and cw15ΔatpB are resistant to spectinomycin, selection for an additional resistance marker is required for transformation with genes, that do not restore photoautotrophic growth. Meanwhile we have succeeded in transforming mutated *atpB* genes, not leading to assembly of an active enzyme, into ΔatpB, by linking them to this kanamycin expression cassette (unpublished results). With these techniques available we can now easily produce ATP synthases with mutated α or β subunits and correlate the effects of the mutations introduced with the resulting enzymatic and biochemical properties.

Table 1
Protein/chlorophyll and chlorophyll *a*/chlorophyll *b* ratios of thylakoids isolated from cw15 cells which were grown either mixotrophically or heterotrophically and of thylakoids isolated from the heterotrophically grown deletion mutants cw15ΔatpA and cw15ΔatpB

	mg protein/mg chlorophyll	chl *a*/chl *b*
cw15, mixotrophic	11.2 (±2.3)	2.52 (±0.17)
cw15, heterotrophic	11.4 (±2.2)	2.52 (±0.15)
cw15ΔatpA, heterotrophic	18.0 (±5.4)	2.34 (±0.26)
cw15ΔatpB, heterotrophic	13.2 (±3.3)	2.30 (±0.16)

Mean values of 6 independent experiments and standard deviations.

3.3. Photosynthetic properties of cw15 and deletion mutant thylakoids

Photophosphorylation by *C. reinhardtii* thylakoids has previously been characterized [12,29]. It appears that the thylakoids in these preparations were impaired during the drastic treatment required to break the cell wall of the wild-type alga since phosphorylation activity decreased rather rapidly upon illumination. We managed to minimize membrane damage by using the cell wall deficient *C. reinhardtii* strain cw15 and succeeded in preparing well coupled and stable thylakoids by breaking the cells at low pressure in the Yeda press [15]. We successfully used the same method to prepare active thylakoids from cw15ΔatpA and cw15ΔatpB. In order to establish the analytical basis for the characterization of mutated ATP synthase and of thylakoid membranes lacking the ATP synthase complex, we determined the chlorophyll/protein and chlorophyll *a*/*b* ratios, the photosynthetic electron transport, proton gradient and ATP formation with these thylakoid preparations.

Table 1 shows the chlorophyll and the protein contents of thylakoids isolated from cells harvested in the log phase. The *C. reinhardtii* cw15 cells were grown either mixotrophically or heterotrophically with acetate as carbon and energy source. The *atpA* and *atpB* deletion mutants were grown heterotrophically in the presence of spectinomycin. The membrane protein/chlorophyll ratio as well as the chlorophyll

Table 2
Rates of photosynthetic electron transport of thylakoids from *cw15* cells (mixotrophically and heterotrophically grown) and the deletion mutants cw15ΔatpA and cw15ΔatpB (heterotrophically grown) as measured by O_2-consumption with methylviologen/O_2 as electron acceptor system

	No additions	Nigericin	P_i	ADP	P_i + ADP
	μmol e^-/(mg chl · h)				
cw15, mixotrophic	231.6	377.2	267.6	243.6	302.4
cw15, heterotrophic	219.6	347.6	249.2	222.0	268.4
ΔatpA, heterotrophic	334.4	705.2	396.4	341.2	358.8
ΔatpB, heterotrophic	273.6	424.0	260.4	257.6	258.4
	μmol e^-/(mg protein · h)				
cw15, mixotrophic	20.8	33.6	24.0	21.6	27.2
cw15, heterotrophic	19.2	30.4	22.0	19.2	23.6
ΔatpA, heterotrophic	18.4	39.2	22.0	18.8	20.0
ΔatpB, heterotrophic	20.8	32.0	19.6	19.6	19.6

The electron transport rates were either calculated relative to the chlorophyll contents or to the protein contents of the thylakoids. The concentrations of additions were as follows: 1 μM nigericin, 5 mM phosphate, 1 mM ADP. The numbers are mean values of four independent experiments.

a/*b* ratio were not significantly different in the mixotrophically or heterotrophically grown cw15 cells. A significantly higher protein/chlorophyll ratio was found in the cw15ΔatpA mutant, a fact that has to be considered when comparing thylakoid activities which are usually related to the chlorophyll content.

Rates of photosynthetic electron transport with H_2O as electron donor and methylviologen/O_2 as the electron acceptor, were monitored by the consumption of O_2. The medium contained either no additions, or phosphate alone, or ADP alone (basal electron transport), or ADP and phosphate together (coupled electron transport) or nigericin (uncoupled electron transport) (Table 2). While the control thylakoids showed the expected acceleration of electron transport in the presence of ADP and phosphate, no such stimulation was observed in thylakoids from cw15ΔatpA or cw15ΔatpB. On the other hand, electron transport rates in the mutant as in the cw15 thylakoids were enhanced by the uncoupler nigericin. These results show that both deletion mutants have a fully intact electron transport chain but lack a functional ATP synthase. Obviously, the deficiency is not

Table 3
PSI-dependent electron transport from DCPIP/ascorbate to methylviologen/O_2 of thylakoids isolated from cw15 cells grown under mixotrophic or heterotrophic conditions and from the deletion mutants cw15ΔatpA and cw15ΔatpB grown under heterotrophic condition

	μmol e^-/mg chlorophyll	μmol e^-/mg protein
cw15, mixotrophic	908.2	81.1
cw15, heterotrophic	939.4	82.4
ΔatpA, heterotrophic	1224.0	68.0
ΔatpB, heterotrophic	1042.8	79.0

Electron transport rates were calculated relative to the chlorophyll or protein contents of the thylakoids. The numbers are mean values of three experiments.

Table 4
Photophosphorylation of thylakoids isolated from cw15, cw15ΔatpA and cw15ΔatpB assayed under demodulated (oxidized) or thiol-modulated (reduced) conditions, respectively

	μmol ATP/ (mg chl·h)
cw15, mixotrophic, oxidized	65.5
cw15, mixotrophic, reduced	231.3
cw15, heterotrophic, reduced	141.6
ΔatpA, heterotrophic, reduced	3.5
ΔatpB, heterotrophic, reduced	4.6

Thiol modulation was carried out by preillumination for 3 min in the presence of 10 mM DTT. Thylakoids at a final concentration of 25 μg chl/ml were suspended in a final volume of 2.5 ml in a medium containing: 25 mM tricine buffer (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 50 μM PMS, 10 mM DTT (except the oxidized enzyme), 5 μM 9-aminoacridine, 50 nM valinomycin, ^{32}P-labeled inorganic phosphate, 1 mM ADP and an ADP regenerating system (10 mM glucose and 12 U/ml salt-free hexokinase). The proton gradient formation was monitored by quenching of 9-aminoacridine fluorescence measured as described in Ref. [19].

accompanied by an increased leakiness of the thylakoids for protons. In Table 2 the rates of electron transport were related either to chlorophyll or membrane protein content of the different thylakoid preparations. The basal and the uncoupled electron transport rates of the cw15ΔatpA thylakoids, were higher than those of the control thylakoids when related to chlorophyll. However, the activities related to protein were similar in all four thylakoid preparations. These results show that electron transport from water to methylviologen is limited by an electron transfer step rather than by light absorption, since the cw15ΔatpA thylakoids show a significant rate increase of uncoupled electron transport at equal chlorophyll concentrations, corresponding to the resulting increase in protein. On the other hand, comparison of PSI-dependent electron transport from $DCPIPH_2$ to methylviologen (Table 3), which is much faster than the electron transport from water to methylviologen, suggests that this electron transport span may be limited by light absorption.

Based on the increment of the basal electron transport rate observed after the addition of ADP and phosphate (Table 2) and assuming a P/e_{2-} ratio close to 1, we can predict a phosphosphorylation rate of about 25–35 μmol ATP/mg chlorophyll per h in the non-cyclic system (~1/5 of the rate obtained with spinach thylakoids). In cyclic photophosphorylation with PMS as electron mediator, higher rates were obtained, which are nevertheless significantly lower than those obtained under similar conditions with spinach chloroplasts. In the two deletion mutants virtually no ATP was formed (Table 4). The thylakoids prepared from the cell wall deficient strains maintained a proton gradient which allowed assessment of ΔpH formation dependent on light intensity and availability of substrates for photophosphorylation. Time courses of light-induced proton gradient formation, determined by fluorescence quenching of 9-aminoacridine, are shown in Fig. 3. In the absence of ADP and phosphate, a steady-state fluorescence quench of 80% was observed with wild-type thylakoids which corresponds to a ΔpH of 3.4. With thylakoids from both deletion mutants, a 94% fluorescence quench corresponding to a ΔpH of 3.95 was attained. This result suggests that thylakoid membranes from the mutants are less permeable to protons. Addition of ADP and phosphate caused a drop of ΔpH in the wild-type but not in the deletion mutant membranes (Fig. 3), confirming that in the deletion mutants the transmembrane proton gradient cannot be used for ATP formation.

3.4. Regulation of C. reinhardtii ATP synthase activity in membrana

Preillumination of thylakoids in the presence of DTT results in the reduction of a disulfide bond in a regulatory segment of the γ subunit of CF_1 ('thiol modulation'). In higher plant thylakoids, the reduced ATP synthase has a lower ΔpH barrier for activation than the oxidized enzyme [30]. At the same proton

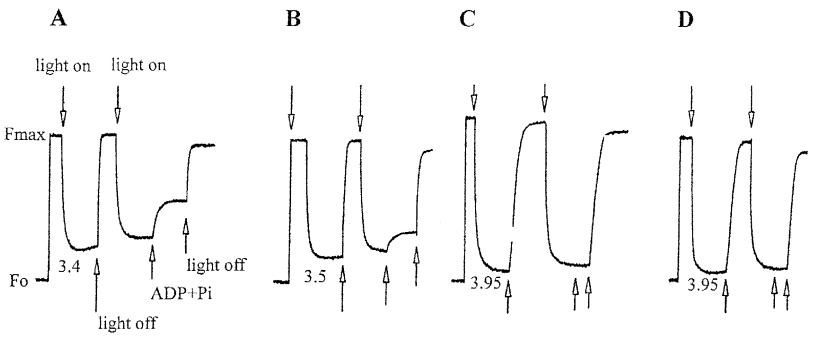

Fig. 3. Light-induced proton gradient formation of thylakoids, isolated from cw15 and from cw15ΔatpA and cw15ΔatpB, as monitored by 9-aminoacridine fluorescence quenching. A: cw15, grown at 900 lux in high salt medium plus 0.2% acetate (mixotrophic). B: cw15, grown in dim light in high salt medium plus 0.4% acetate (heterotrophic). C: cw15ΔatpA, grown in dim light in high salt medium plus 0.4% acetate and 150 μg spectinomycin/ml (heterotrophic). D: cw15ΔatpB, grown in dim light in high salt medium plus 0.4% acetate and 150 μg spectinomycin/ml (heterotrophic). For assay conditions see Section 2. ADP and phosphate were added at concentrations of 1 mM and 5 mM, respectively. The numbers indicate the calculated ΔpH values.

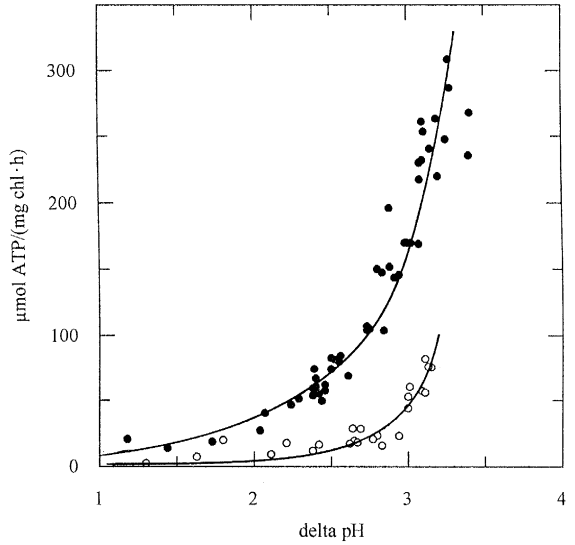

Fig. 4. PMS-cyclic photophosphorylation as function of ΔpH of cw15 thylakoids containing unmodulated (open symbols) or thiol modulated (filled symbols) CF_0CF_1, respectively. Thiol modulation was carried out by preillumination for 3 min in the presence of 10 mM DTT. Thylakoids at a final concentration of 25 μg chl/ml were suspended in a final volume of 2.5 ml in a medium containing: 25 mM tricine buffer (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 50 μM PMS, 10 mM DTT (except for the oxidized enzyme), 5 μM 9-aminoacridine, 50 nM valinomycin, ^{32}P-labeled inorganic phosphate, 1 mM ADP and an ADP regenerating system (10 mM glucose and 12 U/ml salt-free hexokinase). The proton gradient formation was monitored by quenching of 9-aminoacridine fluorescence measured as described in Ref. [19].

gradient, the rate of ATP formation by *C. reinhardtii* cw15 thylakoids was accelerated 3.5-fold by preillumination and DTT treatment (Table 4), indicating that the *C. reinhardtii* ATP synthase is also regulated by thiol modulation. Fig. 4 shows that the phosphorylation/ΔpH dependency is shifted to lower ΔpH values when the enzyme is in its reduced state. Indeed, the segment in the sequence responsible for thiol modulation, comprising two cysteine residues, is present in the *C. reinhardtii* γ subunit [31,32]. This regulatory mechanism is found in higher plant and green algal chloroplasts but is lacking in most cyanobacteria [33] or diatom chloroplasts [34]. We have observed that CF_1 isolated from *C. reinhardtii* cw15, as the higher plant CF_1, has a very low latent ATPase activity ($\sim 1\ \mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$). The rate of hydrolysis is increased to about 40–60 $\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ when assayed in the presence of 20% ethanol. These results suggest that previous findings reporting high ATPase activity without the need of activation [1,12], may have resulted from the harsh conditions used to break the walled *C. reinhardtii* cells. In variance to higher plants, the activity of the light-triggered ATP hydrolyzing activity was very low in *C. reinhardtii* thylakoids. We suggest that CF_0CF_1 is rapidly deactivated in this organism even in its thiol-modulated state.

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