Algae cells with deletion of the segment D210-R226 in γ subunit from chloroplast ATP synthase have lower transmembrane proton gradient and grow slowly

S. PONOMARENKO

Institut für Biochemie der Pflanzen, Heinrich Heine Universität Düsseldorf, Germany

Abstract

The γ-subunits of chloroplast ATP synthases are about 30 amino acids longer than the bacterial or mitochondrial homologous proteins. This additional sequence is located in the mean part of the polypeptide chain and includes in green algae and higher plants two cysteines (Cys198 and Cys204 in *Chlamydomonas reinhardtii*) responsible for thiol regulation. In order to investigate its functional significance, a segment ranging from Asp-D210 to Arg-226 in the γ-subunit of chloroplast ATP synthase from *C. reinhardtii* was deleted. This deletion mutant called T2 grows photoautotrophically, but slowly than the parental strain. The chloroplast ATP synthase complex with the mutated γ is assembled, membrane bound, and as CF_0CF_1 displays normal ATPase activity, but photophosphorylation is inhibited by about 20 %. This inhibition is referred to lower light-induced transmembrane proton gradient. Reduction of the proton gradient is apparently caused by a disturbed functional connection between CF_1 and CF_0 effecting a partially leaky ATP synthase complex.

Additional key words: *Chlamydomonas*; deletion mutant; proteins.

Introduction

The green alga *Chlamydomonas reinhardtii* is a suitable organism for the investigation of photosynthesis. *Chlamydomonas* can grow either photoautotrophically on CO_2 or heterotrophically on acetate as carbon source, and can be transformed in the chloroplast as well as in the nuclear genome (Strotmann *et al*. 1998). These features are used to create photosynthetic mutants, which help to examine the structure and function of the photosynthetic apparatus (Harris 1989). As photosynthesis of *C. reinhardtii* resembles that of higher plant cells (Strotmann *et al*. 1998), this alga can be regarded as a photosynthetic model organism.

The chloroplast ATP synthase catalyzes photosynthetic phosphorylation and is thus one of the essential enzyme complexes of photosynthetic membranes. Homologous enzymes are involved in oxidative phosphorylation of mitochondria and heterotrophic bacteria. All ATP synthases consist of a membrane-integral sector F_0 and an extrinsic sector F_1. The catalytic entity F_1 consists of five different subunits of the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. The alternately arranged α and β subunits form a hexagonal ring with a central hole (Abrahams *et al*. 1994, Groth and Pohl 2001, Yoshida *et al*. 2001). The tertiary (3D) structure determined by X-ray crystallography of the $\alpha_3\beta_3$ ring of the chloroplast F_1 (CF_1) (Groth and Pohl 2001) is essentially the same as that of mitochondrial F_1 (MF_1) (Abrahams *et al*. 1994). The coiled α-helical N- and C-terminal ends of the γ subunit structure form an axis located within the central cavity of $\alpha_3\beta_3$. The 3D structure of γ'ε dimer has been determined at high resolution (Rodgers and Wilce 2000), but the mid part of the γ subunit can be only proposed (Hisabori *et al*. 2003). This sequence segment is different in oxidative and photosynthetic ATP synthases.

Received 9 December 2004, *accepted* 22 December 2005.
Fax: (49) 211-8113706, e-mail: ponomarenko@t-online.de

Abbreviations: *AtpC*, nuclear gene coding expression of the CF_0CF_1 subunit γ; *BRP*, bleomycin resistance gene; CF_0CF_1, ATP synthase from chloroplast; Chl, chlorophyll; PEG, polyethylene glycol; TAP, Tris-acetate-phosphate medium; TP, TAP medium without acetate.

Acknowledgements: This work was supported by Deutsche Forschungsgemeinschaft (project Str 103/21-1) and Fond der Chemischen Industrie. Cordial thanks to Dr. E.H. Harris (The Chlamydomonas Genetics Center, DU, NC, USA) for the strains *–γ CC3022*, *nit-305*, and some plasmids, to Dr. S. Purton (University College London) for plasmid pSP124S with *ble* gene, to Dr. D. Drapier (Institut de Biologie Physico-Chimique, CNRS) for the *C. reinhardtii* chloroplast ATP synthase subunit γ antibodies, to Prof. Dr. P. Kroth (Konstanz University, Germany) for helpful discussion, and to all colleagues from Institute of Plant Biochemistry of the Heinrich Heine University Düsseldorf for support and good help.

In the γ-subunit of chloroplast F_1 of green algae and higher plants an insertion of about 30 additional amino acids is present being about 200 amino acids distant from the N-terminus and about 100 amino acids from the C-terminal end. The significance of this sequence motif is not clear except for the initial part (198C–204C in *C. reinhardtii*). These two cysteines can reversibly form a disulfide bridge. Reduction of the disulfide group is effected by thioredoxin *f* which is reduced by the photosynthetic electron transport chain, and provides the so-called thiol modulation (Ross *et al.* 1995, Strotmann *et al.* 1998, Hisabori *et al.* 2003). It is specific for chloroplast enzymes of higher plants and green algae and is lacking in other ATP synthases (Strotmann *et al.* 1998). However, the ability of thiol modulation can be transferred to cyanobacterial (Werner-Grüne *et al.* 1994) or bacterial (Hisabori *et al.* 1997) F_1 by introduction of the respective sequence by genetic engineering.

The segment 205V-231I following the dithiol/disulfide group is present in all photosynthetic ATP synthases and shows high similarity in chloroplasts and cyanobacteria. Not much is known about the significance of this sequence. I have deleted by genetic engineering the 17 amino acids (D210-R226) of γ subunit from *C. reinhardtii* CF_1 and studied the effects on function of the enzyme and some relevant cell parameters.

Materials and methods

Cell strains and cultivation: The *C. reinhardtii* cell wall deficient strain *cw15* mt^- served as a wild type stock in this study. Two strains *nit1-305* (Kindle *et al.* 1989) and *CC3022* (Smart and Selman 1991) derived from *cw15* were gifts of Dr. E. Harris (The Chlamydomonas Genetics Center, Duke University, Durham, NC, USA). The *nit1-305* strain obtained by Lefebvre completely lacks nitrate reductase (Kindle *et al.* 1989). The acetate auxotrophic strain *CC3022* (the progenitor strain of which was *nit1-305*) is a mutant lacking γ-subunit of chloroplast ATP synthase. It contains a modification in the structure of the *AtpC* gene resulting in the complete lack of any *AtpC* RNA transcripts. In this strain no CF_1 is assembled (Smart and Selman 1991).

C. reinhardtii cells were usually cultivated on TAP (Tris-acetate-phosphate) with 1.5 % agar plates or as liquid cultures in TAP medium on a rotary shaker containing 0.2 % acetate (Harris 1989) at 22 °C in a light/dark cycle of 14/10 h. Irradiance ("white light") was as a rule about 45 μmol m^{-2} s^{-1}. Alternatively, the cells were grown in continuous light. Cells were harvested at the mid-logarithmic phase and collected by centrifugation for 10 min at 3 000×*g*.

Preparation of DNA: Genomic DNA was isolated from a nuclei-enriched fraction. To this end, the *Chlamydomonas* cells were washed with medium *Iso-1* (25 mM *Tricine*/NaOH, pH 8.0, 50 mM NaCl, 300 mM sucrose, and 1 mM $MgCl_2$) and re-suspended in the same medium. After breaking of the cells by a *Yeda-Press* (twice at 5.5×10^5 N m^{-2}) and dilution with *Iso-1*, a fraction enriched in nuclei was obtained by centrifugation for 5 min at 1 000×*g*. DNA was isolated and purified by the corresponding *Qiagen Kits*: total genomic DNA by *DNeasy Plant Kit*; plasmid DNA by alkaline method *QIAprep Spin Miniprep* or *HiSpeed Plasmid Midi Kit*. DNA fragments were extracted from agarose gels using the *QIAquick Spin Kit* or *MiniElute Gel Extraction Kit* for very low quantity of DNA.

Cloning procedures were carried out after Sambrook *et al.* (1989) using commercially available restriction endonucleases and DNA-modifying enzymes according to the suppliers' instructions. The complete genomic gene *AtpC* was amplified from the *C. reinhardtii* nuclear DNA as a template using *Long Template Kit* (*Roche*). Another one, *Fidelity Template Kit* (*Roche*), was used for amplification of shorter DNA fragments. PCR procedures were done as a program *54* for template DNA of about 1 kb and as *Touchdown* PCR (Program *1*) for long template accordingly to the guideline of the kits for PCR amplification. PCR was carried out in 25 or 50 mm^3 *Boehringer* reaction buffer with DNA polymerase, which also contained 20–60 ng of DNA, 2 mM total $MgCl_2$, 250 nmol of each primer, and 250 μM dNTPs. The PCR product was purified by agarose gel electrophoresis followed by extraction from gel. The eluted DNA was used for ligation at 5–8 °C over night.

The PCR products were ligated to *pGEM-T* or *pGEM-T Easy Vector* (*Promega*) using *Promega* cloning kits and transformed by the heat shock method (Sambrook *et al.* 1989) in *E. coli* complement cells *JM109* (*Promega*) or *XL1 blue* (*Stratagene*). The DNA of *AtpC* genes from *cw15*, *nit1-305*, and mutant *T2* was sequenced by *SEQLAB* or *Genotype* (Germany). Stock cell cultures of cloning products were saved at –75 °C.

Site-directed mutagenesis: The deletion mutant (ΔγD210-R226) was engendered by oligonucleotide-directed mutagenesis. Oligonucleotides were synthesized by *MWG Biotech-AG* (Germany); the primers were usually 24–31 nucleotides long (Fig. 1). Introduction of the mutation into the genomic *AtpC* gene was confirmed by PCR, restriction analysis, and DNA sequencing using specific primers (Fig. 1). The DNA fragments were cloned into suitable cloning vectors containing extended 5'- and 3'-flanking regions and antibiotic resistance cassette as markers for successful transformation. The resulting plasmid was transformed into *E. coli XL1-Blue* cells.

Transformation: For nuclear transformation the transfection unit was linearized by *KpnI* endonuclease (Fig. 2). Transfection was achieved by vortexing in the presence of glass beads and PEG or by electroporation (*Bio-Rad*). The transfection procedure was done after Kindle and Lawrence (1998). 150–200 cm^3 culture grew to mid-log phase ($1–2\times10^6$ cells per cm^3) in TAP medium under continuous bright radiation (~90 μmol m^{-2} s^{-1}). Cells precipitated at 5 000 rpm at 4 °C for 10 min and were re-suspended in fresh TAP by gentle pipetting to a concentration of $2–10^8$ cells per cm^3.

To 300 mm^3 of the cell suspension transferred to sterile screw-top 15 cm^3 tubes containing ~0.3 g sterile glass beads (diameter 0.4 mm) 1–4 μg of linearised transfection vector plus herring sperm DNA as a carrier were added. Moreover, a DNA-free control was set up. The cell/glass beads/DNA/PEG suspension was vortexed for 15 s at maximal speed. The cells were transferred to a 50 cm^3 sterile screw-top tube containing 10 cm^3 of TAP medium, and grew for about 18 h at lower irradiance by shaking at 100 rpm to allow for recovery and expression of the *BRP* gene. After centrifugation for 10 min at 4 000 rpm the cells were gently re-suspended in 0.5 cm^3 TAP medium and then plated on 3.5 cm^3 of the TAP + 0.5 % agar (the temperature of the molten agar was below 42 °C and contained no antibiotic) onto TAP 2 % agar supplemented with zeocin (10 μg per cm^3). Plate controls were without antibiotic.

After allowing the agar to set, the plates were inverted and left in the light (~45 μmol m^{-2} s^{-1}) at ~22 °C. The transfection efficiency was $1–4\times10^{-6}$. Colonies of mutant cells were visible after 10–12 d.

Selective growth: To select autotrophic and antibiotic-resistant growing cells, each colony was incubated 5 d in soluble TP (TAP without acetate) + 10 μg per cm^3 zeocin into 6-well plates by constant shaking, and then spread on plates with TP medium containing 10 μg zeocin per cm^3. The photosynthetically active and zeocin-resistant GBT2 clone (named T2) was chosen for growth experiments and further analysis.

Thylakoid membrane isolation: Active thylakoid vesicles were prepared as described above. The cultures were grown to a cell density of $6–8\times10^6$ cells per cm^3. Then the cells were harvested by centrifugation, washed, and re-suspended in medium *Iso-1* to reach chlorophyll (Chl) concentration of 1 mg cm^3. The cells were broken by passage through a *Yeda-Press*, the thylakoids were precipitated and washed in 2 mM *Tricine*/NaOH, pH 8.0, 50 mM NaCl, and 1 mM $MgCl_2$, and re-suspended in a small volume of the medium *Iso-1*.

CF1 isolation: Chloroplast ATPase was extracted from thylakoid membranes by the method of Younis *et al.* (1977). Soluble protein was purified using gel-filtration in *Sephacryl S-300-HR* (*Sigma*), by *Pharmacia HiLoad System* (*Pharmacia*), in buffer: 20 mM Tris, pH 7.5, 2 mM EDTA, 1 mM ATP, and 100 mM $(NH_4)_2SO_4$. Protein was precipitated in $(NH_4)_2SO_4$ and the column *Bio-Spin* (*Bio-Rad*) was used to remove ammonium sulfate.

ATPase activity: 10–20 μg of thylakoid membranes were pre-incubated in 25 mM *Tricine*/NaOH, pH 8.0, with 5 mM $MgCl_2$ and 25 % EtOH at 37 °C, then the reaction was started by the addition of 5 mM ATP. The reaction was stopped by the addition of 3 % trichloroacetic acid (end concentration), and the released inorganic phosphate was determined photometrically as phosphomolybdate complex as described by Fiedler *et al.* (1997).

Rate of photophosphorylation of thylakoids from *C. reinhardtii* cells was measured as described by Fiedler *et al.* (1997). The reaction medium contained in a final volume of 2.5 cm^3: 25 mM *Tricine*, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 50 μM phenazine methosulfate, and thylakoid vesicles to give a final concentration of 25 μg Chl per cm^3. After a 1 min-pre-incubation, the reaction was initiated by addition of 1 mM ADP and 5 mM P_i, and terminated at 15, 30, 60, and 120 s by adding 2.5 % trichloroacetic acid (end concentration). The supernatant was neutralized (pH 7.7) with 0.25 M Tris/acetate (pH 9.5) and the amount of synthesized ATP was determined with the ATP *Bioluminescence Assay Kit CLSII* (*Roche*).

Transmembrane proton gradient was measured by monitoring the fluorescence quench of 9-aminoacridine which was calibrated as described by Fiedler *et al.* (1997).

Gel electrophoresis and blotting: Proteins were separated by SDS-PAGE electrophoresis and silver stained or transferred to nitrocellulose (0.2 μm *Schleicher&Schuell*) and immuno-stained using specific rabbit antiserum for *C. reinhardtii* γ-subunit CF_1 (gift from Dr. D. Drapier) and BM Biolumescent Blotting Substrate (*POD*) (*Roche*)

Protein and Chl contents were determined as described by Fiedler *et al.* (1997).

Bioinformatic methods: The following software was used: for alignment of sequences *Clustal-X*, for sequence check *Chromas-2*, for PCR primer check and for restriction sites' finding *Winclone*.

Results

Generation of the mutant Δγ210D-226R: Three of the nine subunits of the chloroplast ATP synthase, namely the CF_1 subunits γ, δ, and CF_0-subunit II (= b_2 for bacteria) are encoded in the nuclear genome. Nuclear transformants arise from random integration of the donor DNA into chromosomal DNA (Kindle *et al.* 1989). Due to the ability of *Chlamydomonas* cells to grow heterotrophically (Harris 1989), it is possible to delete even essential polypeptides involved in photosynthesis. Thus inactivation of the essential *AtpC* gene coding for γ-subunit of the chloroplast ATP synthase (as in strain *CC3022*) is not lethal for *Chlamydomonas*. Successful complementation of the *AtpC*-defective *Chlamydomonas* strain with *AtpC* gene has been demonstrated by Ross *et al.* (1995).

The utilisation of cell strains with auxotrophic mutations as selection markers is a suitable method of transformation with low frequency of reversion (Harris 1989, Kindle and Lawrence 1998). Here nitrate reductase deficient strain *nit1-305* was employed which depends on the supply of ammonia as nitrogen source and is derived from the cell-wall deficient strain *cw15*. Comparison of two nuclear genes *AtpC* from strains *nit1-305* and *cw15* showed that they have identical nucleotide sequences. Both genes fit to complete structure for genomic 3.6 kb DNA of the CF_1 γ-subunit from the *C. reinhardtii* cells (Kindle and Lawrence 1998) were generated by PCR using downstream (SalI-pr1) and upstream (γ-rev) primers (Fig. 1). It means that CF_0CF_1 from *nit1-305* is equal to *wt*.

(*1*) forward primer **SalI-pr1**: 5'- CATG*GTCGAC*GGACTTCAGGCGGACGGCGTG
(*2*) reverse primer **γ-rev**: 5'- CAT*CTTAAG*AGCCATAATGCCTTACATGCATCGCC
(*3*) forward primer **gNco**: 5'-CCCCATGGGCGAGCTGTGCGACGTGGACGGCAAGTGCGTGG
ACGCCGCTGAC**GAGAAGACCACCATCGAGACCGAGGCC**
(*4*) forward primer **gNcWT**: 5'-ATCTAGACCCTGCTGCCCATGACCC*CCATGG*GCG

Fig. 1. Primers used for DNA amplification by PCR for DNA fragments cloning and analysis. *In italics* restriction sites are shown.

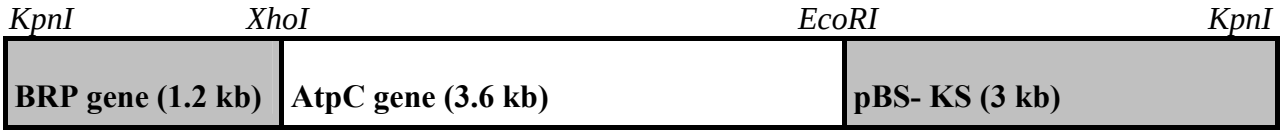

Fig. 2. Transformation unit. Scheme of the 7.8 kb plasmid digested by *KpnI* and used for transfection of CC3022 cells. The DNA fragment contains *BRP* gene, *gAtpC* gene and vector pBS-KS II +. *In italics* restriction sites are shown..

Preparation of the γΔ210D-226R deletion mutant named T2 required the ordinary molecular genetic techniques described above: isolation, purification, and cloning of the *AtpC* gene, generation of the mutation by PCR in the *AtpC* gene, construction of the transformation vector including a drug resistance gene (Fig. 2), and its transfer to the receptor strain CC3022. In order to use resistance against zeocin (= bleomycin) as a selection marker, the mutated *AtpC* gene was fused with gene of bleomycin resistance protein (*BRP*). *BRP* binds bleomycin stoichiometrically thereby prevents damage of nuclear DNA (Stevens *et al.* 1996). The plasmid with the wild type *gAtpC* in *pBS-KS+II* (*Stratagene*) vector was named pA. The deleted 51 bases in *AtpC* gene coding for 17 amino acid residues of the γ-subunit are indicated in bold letters in Fig. 3. Corresponding vector was named pΔA1. Wild type *gAtpC* has three *BglII* restriction sites but one of them the *BglII*-1977 was eliminated in the deletion mutant T2 (Fig. 3), and endonuclease *BglII* cut the vector pΔA1 only twice (Fig. 4). The deletion

201 210
GacGgcAagTgcGtgGacGccGctGac**GacG*agAtcT*tcAagCtgAccAccAagGgcGgc**
D G K C V D A A D **D E I F K L T T K G G**

221
GagTtcGccGtgGagCgcGagAagAccAccAtcGagAccGagGccCtgGacCccTcgCtc
E F A V E R E K T T I E T E A L D P S L

Fig. 3. The D201-L240 fragment of amino acids and nucleotide sequence of *C. reinhardtii* ATP synthase subunit gamma. Restriction site 1977***agAtcT*** for BglII endonuclease. Deleted 51 nucleotides and 17 amino acids are shown *in bold letters*.

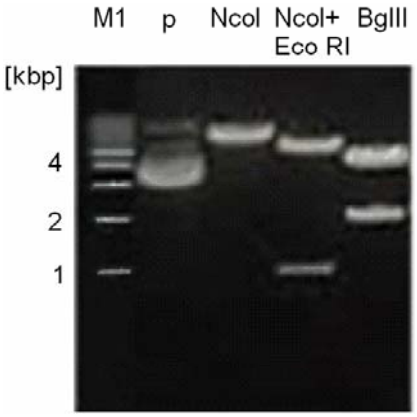

Fig. 4. Restriction analysis of the plasmid pΔA1. M1-DNA marker, p-purified plasmid pΔA1, NcoI, NcoI+EcoRI, BglII – plasmid pΔA1 restricted by these endonucleases.

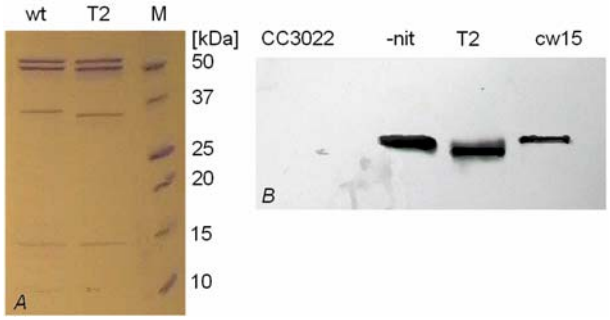

Fig. 5. *A*: SDS-PAGE of *C. reinhardtii* CF_1 isolated from *nit1-305* (wt) and T2 after silver staining, 4 μg of protein in each slot. M – 10 μg of *All Blue prestained Precision Plus Protein* (*BIO-RAD*). *B*: Western blot of subunit γ from strains: *CC3022*, *-nit*, T2 (cell proteins corresponding 10 mg Chl in each slot), and *cw15* (5 mg Chl) after 4–20 % gradient SDS-PAGE.

γΔD210-R226 was checked by restriction analysis followed by sequencing using primers: down gNcWT and reverse γ-rev (Fig. 1) for DNA amplification. Two methods of introduction of DNA into *C. reinhardtii* cells were used: electroporation or vortexing in the presence of glass beads and PEG. The latter technique gave higher transfection yields. Plating on starch of the transformation mix medium (Shimogawara *et al.* 1998) increased the efficiency ($1–4\times10^{-6}$) over plating on 0.5 % agar media ($1–2\times10^{-7}$). Anyhow, expression of the *BRP* does not affect assembly of the γ-polypeptide in chloroplast ATP synthase (Kindle and Lawrence 1998).

Selection for the successful transfection was achieved under autotrophic growth conditions in a well plate containing liquid TP medium + zeocin. As the next step the cells were plated on solid medium of otherwise the same composition. The mutant cells grew in medium TP with 10 μg per cm^3 zeocin, *i.e.* they are also autotrophic organisms. The genomic DNA of the deletion mutant T2 was isolated and purified and the mutation was verified by restriction and sequence analysis.

Properties of the mutant cells: Growth rates under photoautotrophic and photoheterotrophic conditions, respectively, of strains T2 and *nit1-305* were compared. In Table 1 cell duplication times are presented. With acetate the γD210-R226 deletion mutant grew as well as the parental strain *nit1-305*. However, at continuous irradiation T2 cells grew slowly under photoautotrophic conditions compared with *nit1-305* (Table 1) or *cw15* (Fiedler *et al.* 1997). The growth rates of the mutant cells in medium with 10 μg zeocin per cm^3 or without antibiotic were the same.

Table 1. Some phenotypic properties of *C. reinhardtii* strains *nit-305* and deletion mutant T2. *Columns 2 and 3*: The cell duplication time [d] was determined under conditions in liquid TP medium at continuous irradiation (autotrophic growth) with "white light" of 90 μmol(photon) m^{-2} s^{-1} at 22 °C. The amounts of chlorophyll (Chl) and total protein were determined as in Fiedler *et al.* (1997). *Column 4*: ATPase activity [mmol(ATP) kg^{-1}(Chl) s^{-1}] of *C. reinhardtii* membrane bound chloroplast ATPase in the presence of 25 % EtOH (V_{Et}). ATPase activity was measured by photometric determination of released inorganic phosphate (P_i). 10–20 μg of chlorophyll of thylakoid membranes were incubated for the indicated times in 25 mM *Tricine*/NaOH, pH 8.0, with 5 mM $MgCl_2$, 5 mM ATP, and 25 % EtOH. *Column 5*: Protein/Chl ratio in the thylakoid membranes.

Strain	24 h light	14/10 h light/dark	V_{Et}	Protein/Chl
nit-305	1.00	1.2	83.3	10.1
T2	1.25	1.1	80.0	10.3
CC3022	-	-	3.3	nd

Properties of the ATP synthase: The CF_1 sector of the chloroplast ATP synthase complex was isolated by chloroform extraction and analyzed by SDS-PAGE (Fig. 5). While subunits α, β, γ, and ε were clearly visible, subunit δ was absent in agreement with known results about F_1 chloroform extraction (Younis *et al.* 1977). The identity of γ-subunit was proven by a specific antibody against γ-subunit of the chloroplast ATP synthase of *C. reinhardtii* (Fig. 5). As expected, the migration of the mutated γ-subunit in CF_1 in the SDS-PAGE was slightly faster than that of the wild type indicating its lower molecular mass. There was, however, no significant difference in immuno-staining intensity of the γ-bands indicating the same expression and assembly rates. In *CC3022* not only γ-subunit was absent, but all other CF_1 subunits were lacking. This finding is in agreement with previous observations (Smart and Selman 1991).

Table 2. Transmembrane proton gradient (ΔpH) and rate of photophosphorylation (V) of thylakoids from *C. reinhardtii* strains *nit-305* and deletion mutant T2. The ΔpH generated by thylakoids by monitoring the fluorescence quench of 9-amino-acridine. The reaction medium contained in a final volume of 2.5 cm^3: 25 mM Tricine, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 50 μM phenazine methosulfate, 50 nM valinomycin, 10 mM glucose, 12 units per cm^3 hexokinase, 5 μM 9-aminoacridine, and thylakoid vesicles to give a final concentration of 25 μg(Chl) per cm^3. ATP synthase activity was investigated under the same conditions. After a 1-min-pre-incubation, the reaction was initiated by adding 1 mM ADP and 5 mM P_i, and terminated at 15, 30, 60, and 120 s by adding 2.5 % trichloro-acetic acid. The solution was neutralized to pH 7.7 with 0.25 M Tris/acetate (pH 9.5) and the amount of synthesized ATP was determined with ATP Bioluminescence Assay Kit *CLSII* (*Roche*).

Strain	ΔpH	[mmol(ATP) kg^{-1}(Chl) s^{-1}]
nit-305	3.16	983
T2	2.72	767

Activities of T2 thylakoids: Chloroplast ATPase of plants and *C. reinhardtii* (Kneusel *et al.* 1982) is much higher in the presence of solvents in contrast to other ATPases. The ATPase rates of the thylakoid membrane-bound ATP synthase measured in the presence of ethanol were equal in T2 and *nit1-305*. As expected, the thylakoids isolated from CC3022 showed virtually no ATP hydrolyzing activity. The rate of ATP formation was by around 20 % lower by T2 than *nit1-305* cells (Table 2). Data in Table 2 show the influence of γ 210D-226R deletion on light-induced proton gradient. Photophosphorylation depends on scale of trans-thylakoid ΔpH. The H+ gradient was measured by the 9-aminoacridine fluorescence quenching technique. At the same irradiance thylakoid vesicles isolated from deletion mutant T2 have a significantly lower ΔpH than from *nit1-305* (Table 2) or *cw15* (Fiedler *et al.* 1997).

Hence the inhibition of photophosphorylation is due to a reduced driving force rather than a derangement of the catalytic device of the ATP synthase.

Discussion

Deletions in chloroplast γ-subunit have been reported before. Thus truncation at the C-terminal end did not affect ATPase activity (Sokolov *et al.* 1999) and consequently ATP-driven rotation (Noji *et al.* 1997, Hisabori *et al.* 1999) of γ in the $\alpha_3\beta_3$ head unless more than 12 C-terminal amino acids were cut off (Müller *et al.* 2002).

Functional significance of the chloroplast γ205V-231T motif was investigated using different approaches. The three clustered acidic amino acids in positions 209–211 (Fig. 3) are involved in enzyme regulation because replacement by three alanines effects an inverse regulation of reconstituted soluble F_1 by reduction of the disulfide group (Ueoka-Nakanishi *et al.* 2004). I proved that the deleted fragment γ210D-226R participates in ATP synthase regulation (Table 2).

Excision of γ216L-231K (215L-230T for *C. reinhardtii*) by trypsinolysis of spinach CF_1 decreased the affinity of the ε-deficient CF_1 for the added pure ε-subunit. Furthermore proteolytic cleavage suggested that the segment γ216L-231K is essential for ATP synthase activation by irradiation which depends from γ/ε interaction (Hightower and McCarty 1996, Hisabori *et al.* 2003). The γ/ε inter-subunit affinity in foot part (Andrews *et al.* 2001) of the catalytic sector F_1 connecting to F_0 can also be reduced by the γ210D-226R deletion.

Using bacterial F_1 reconstruction shows that experimental change of γ-subunit length alters its ATPase regulation and protein conformation (Hisabori *et al.* 1998). The conformation alteration in γ central part can change its evolutionary settled close-fitting interaction with both subunits ε and c. The discussed deletion of the motif γ210D-226R is a direct investigation of shortening of γ-subunit in its foot part of chloroplast ATP synthase. The deleted sequence is a piece of the γ-moiety that forms connection to subunit c (subunit III for chloroplast CF_0CF_1) of F_0 sector (Watts *et al.* 1995). Most likely the shorter γ-polypeptide altering γ-ε-III interaction causes some unproductive proton efflux. During ATPase reaction, the subunit γ rotates in unidirectional manner in chloroplast catalytic sector $(\alpha\beta)_3$ (Hisabori *et al.* 1999). According to the current rotation hypothesis a tight connection between γ and the proteolipid ring (c_{12}) is a prerequisite of efficient energy transduction in ATP synthases (Tsunoda *et al.* 2001). Impairment of the F_1/F_0 connection, *e.g.* by weakening of γ-ε-III subunit affinity can explain a partial intrinsic uncoupling which is the apparent reason for the observed decrease of the trans-thylakoidal proton gradient (Table 2).

Photophosphorylation of *C. reinhardtii* thylakoids depends on the magnitude of the transmembrane ΔpH generated by photosynthetic electron transport (Fiedler *et al.* 1997). As in mutant thylakoids the irradiation-induced proton gradient is low (Table 2), we may conclude that inhibition of photophosphorylation is referred to a decreased driving force. Apparently, the observed reduction of photophosphorylation by about 20 % (Table 2) is not due to inhibition of the CF_0CF_1 catalytic capability (Table 1). By the way, the chloroplast polypeptide γ lacking γ210D-226R fragment is similar in the length to homolog protein of mitochondrial ATP synthase which usually generates the lower transmembrane proton gradient than CF_0CF_1.

I show here using genetically engineered deletion mutant of *C. reinhardtii* that the motif γ210D-226R (Fig. 3) is functionally important part of the CF_0CF_1 subunit γ for regulation of photophosphorylation. The mutant *C. reinhardtii* cells with modified γ-subunit from

CF_0CF_1 are able to grow photoautotrophically, but more slowly. The motif elongating the middle part of subunit γ of photosynthethic ATP synthases is essential for γ-ε-III interaction, also for transmembrane proton generation, and can participate in regulation of photophosphorylation in chloroplasts.

References

Abrahams, J.P., Leslie, A.G.W., Lutter, R., Walker, J.E.: Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria. – Nature **370**: 621-628, 1994.

Andrews, S.H., Peskova, Y.B., Polar, M.K., Herlihy, V.B., Nakamoto, R.K.: Conformation of the γ subunit at the γ-ε-c interface in the complete *Escherichia coli* F1-ATPase complex by site-directed spin labeling. – Biochemistry **40**: 10664-10670, 2001.

Fiedler, H.R., Schlesinger, J., Strotmann, H., Shavit, N., Leu, S.: Characterization of atpA and atpB deletion mutants produced in *Chlamydomonas reinhardtii* cw15: electron transport and photophosphorylation activities of isolated thylakoids. – Biochim. biophys. Acta **1319**: 109-118, 1997.

Groth, G., Pohl, E.: The structure of the chloroplast F1-ATPase at 3.2 Å resolution. – J. biol. Chem. **276**: 1345-1352, 2001.

Harris, E.: The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. – Academic Press, San Diego – New York – Berkeley – Boston – London – Sydney – Tokyo – Toronto 1989.

Hightower, K.E., McCarty, R.E.: Proteolytic cleavage within a regulatory region of the γ subunit of chloroplast coupling factor 1. – Biochemistry **35**: 4846-4851, 1996.

Hisabori, T., Kato, Y., Motohashi, K., Kroth-Pancic, P., Strotmann, H., Amano, T.: The regulatory functions of the gamma and epsilon subunits from chloroplast CF1 are transferred to the core complex, alpha3beta3, from thermophilic bacterial F1. – Eur. J. Biochem. **247**: 1158-1165, 1997.

Hisabori, T., Kondoh, A., Yoshida, M.: The gamma subunit in chloroplast F(1)-ATPase can rotate in a unidirectional and counter-clockwise manner. – FEBS Lett. **463**: 35-38, 1999.

Hisabori, T., Motohashi, K., Kroth, P., Strotmann, H., Amano, T.: The formation or the reduction of a disulfide bridge on the gamma subunit of chloroplast ATP synthase affects the inhibitory effect of the epsilon subunit. – J. biol. Chem. **273**: 15901-15905, 1998.

Hisabori, T., Ueoka-Nakanishi, H., Konno, H., Koyama, F.: Molecular evolution of the modulator of chloroplast ATP synthase: origin of the conformational change dependent regulation. Minireview. – FEBS Lett. **545**: 71-75, 2003.

Kindle, K.L., Lawrence, S.D.: Transit peptide mutations that impair in vitro and in vivo chloroplast protein import do not affect accumulation of the gamma-subunit of chloroplast ATPase. – Plant Physiol. **116**: 1179-1190, 1998.

Kindle, K.L., Schnell, R.A., Fernandez, E., Lefebvre, P.A.: Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. – J. Cell Biol. **109**: 2589-2601, 1989.

Kneusel, R.E., Merchant, S., Selman, B.R.: Properties of the solvent-stimulated ATPase activity of chloroplast coupling factor 1 from *Chlamydomonas reinhardii*. – Biochim. biophys. Acta **681**: 337-344, 1982.

Müller, M., Pänke, O., Junge, W., Engelbrecht, S.: F1-ATPase, the C-terminal end of subunit γ is not required for ATP hydrolysis-driven rotation. – J. biol. Chem. **277**: 23308-23313, 2002.

Noji, H., Yasuda, R., Yoshida, M., Kinosita, K., Jr.: Direct observation of the rotation of F1-ATPase. – Nature **386**: 299-302, 1997.

Rodgers, A.J.W., Wilce, C.J.: Structure of the γ'–ε complex of ATP synthase. – Nature struct. Biol. **7**: 1051-1054, 2000.

Ross, S.A., Zhang, M.X., Selman, B.R.: Role of the *Chlamydomonas reinhardtii* coupling factor 1 gamma-subunit cysteine bridge in the regulation of ATP synthase. – J. biol. Chem. **270**: 9813-9818, 1995.

Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular Cloning: a Laboratory Manual. 2nd Ed. – Cold Spring Harbour Press, Cold Spring Harbour 1989.

Shimogawara, K., Fujiwara, S., Grossman, A., Usuda, H.: High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. – Genetics **148**: 1821-1828, 1998.

Smart, E.J., Selman, B.R.: Isolation and characterization of a *Chlamydomonas reinhardtii* mutant lacking the γ-subunit of chloroplast coupling factor 1 (CF_1) – Mol. cell. Biol. **11**: 5053-5058, 1991.

Sokolov, M., Lu, L., Tucker, W., Gao, F., Gegenheimer, P.A., Richter, M.L.: The 20 C-terminal amino acid residues of the chloroplast ATP synthase gamma subunit are not essential for activity. – J. biol. Chem. **274**: 13824-13829, 1999.

Stevens, D.R., Rochaix, J.D., Purton, S.: The bacterial phleomycin resistance gene *ble* as a dominant selectable marker in *Chlamydomonas*. – Mol. gen. Genet. **251**: 23-30, 1996.

Strotmann, H., Shavit, N., Leu, S.: Assembly and function of the chloroplast ATP synthase. – In: Rochaix, J.-D., Goldschmidt-Clermont, M., Merchant, S. (ed.): The Molecular Biology of Chloroplasts and Mitochondria in *Chlamydomonas*. Pp. 477-500. Kluwer Academic Publ., Dordrecht – Boston – London 1998.

Tsunoda, S.P., Aggeler, R., Yoshida., M, Capaldi, R.A.: Rotation of the c subunit oligomer in fully functional F1F0 ATP synthase. – Proc. nat. Acad. Sci. USA **98**: 898-902, 2001.

Ueoka-Nakanishi, H., Nakanishi, Y., Konno, H., Motohashi, K., Bald, D., Hisabori, T.: Inverse regulation of rotation of F1-ATPase by the mutation at the regulatory region on the γ subunit of chloroplast ATP synthase. – J. biol. Chem. **279**: 16272-16277, 2004.

Watts, S.D., Zhang, Y., Fillingame, R.H., Capaldi, R.A.: The gamma subunit in the *Escherichia coli* ATP synthase complex (ECF1F0) extends through the stalk and contacts the c subunits of the F0 part. – FEBS Lett. **368**: 235-238, 1995.

Werner-Grüne, S., Gunkel, D., Schumann, J., Strotmann, H.: Insertion of a "chloroplast-like" regulatory segment responsible for thiol modulation into γ-subunit of F_0F_1-ATPase of the cyanobacterium *Synechocystis* 6803 by mutagenesis of *atpC*. – Mol. gen. Genet. **244**: 144-150, 1994.

Yoshida, M., Muneyuki, E., Hisabori, T.: ATP synthase – a marvellous rotary engine of the cell. – Nature Rev. **2**: 669-677, 2001.

Younis, H.M., Winget, G.D., Racker, E.: Requirement of the δ subunit of chloroplast coupling factor 1 for photophosphorylation. – J. biol. Chem. **252**: 1814-1818, 1977.