The *C. reinhardtii* CF_1 with the mutation βT168S has high ATPase activity

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Abstract We have generated the mutation T168S in the β subunit of the chloroplast ATP synthase complex of *Chlamydomonas reinhardtii* by site directed mutagenesis and chloroplast transformation. CF_1 and the $\alpha_3\beta_3\gamma$ complex of this mutant strain were isolated and their enzymatic activities were characterized and compared to those of the corresponding wild type complexes. Without activation the mutant CF_1 exhibits MgATPase activity with at least 10 times higher rates than the wild type enzyme. The MgATPase activity could be stimulated to some extent by methanol, but less by ethanol and octylglucoside. The $\alpha_3\beta_3\gamma$ complex had an even higher MgATPase activity, which was only slightly enhanced by ethanol or methanol. The ATPase activities of the mutant complexes, like those of the wild type complexes, displayed a sharp concentration optimum for Mg^{2+}. Free ADP inhibited neither the mutant nor the wild type ATPase significantly. Azide, which strongly inhibited the ATPase activity of the wild type enzyme, inhibited the mutant enzyme only at an about 30 times higher concentration suggesting that the mutation T168S prevents trapping of a tightly bound MgADP by a catalytic site that regulates chloroplast ATPase activity. The mutant cells grew photoautotrophically at a growth rate of about 50%. Similar to the wild type the cells survived on minimal medium in the dark. Under heterotrophic conditions with acetate as energy and carbon source the mutant cells grew much faster than the wild type cells, but the chlorophyll content per cell decreased dramatically.

Key words: βT168S mutation; CF_1 ATPase; Regulation; Azide inhibition; *Chlamydomonas reinhardtii*

1. Introduction

The F_0F_1-type ATP synthases of bacteria, mitochondria and chloroplasts [1] are composed of two sectors, a membrane embedded F_0 which is responsible for transmembrane proton translocation and an extrinsic F_1 which carries the catalytic centers. The chloroplast F_1 (CF_1) [2–4] consists of five different subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. In contrast to bacterial F_1, isolated CF_1 is a latent enzyme with low ATPase activity which can be enhanced by treatment with proteases, heat and DTT [2–6], alcohols [7] or octylglucoside [8]. In membrana the ATP synthase (CF_0CF_1) is inactive in the dark adapted chloroplast, and its activation requires a transmembrane ΔpH. By reduction of a disulfide bond in the γ subunit the threshold ΔpH for activation is decreased [6,9]. The oxidized enzyme is immediately deactivated with relaxation of the proton gradient while the activity of the reduced enzyme persists upon membrane deenergization. Deactivation of the reduced enzyme in the deenergized membrane is caused by trapping of ADP by one of the catalytic sites which results in a 'tightly bound' ADP [6,10]. A similar, but much less pronounced effect is observed in the mitochondrial and bacterial enzyme [11,12]. The more stringent regulation of the chloroplast enzymes might be related to the fact that the photosynthetic membranes are subject to large variations in ΔpH due to changes in light intensity and to the diurnal light-dark cycle. Thus the possible meaning of the different control devices is to prevent degradation of the cellular ATP pools under conditions which would energetically favor ATP hydrolysis [6,9].

A PS3 $\alpha_3\beta_3\gamma$ complex with the mutation βT165S was recently reported to lack the ability to incorporate inhibitory MgADP into TF_1 [13]. T165 is located in the so called P-loop sequence that interacts with the phosphate groups of the nucleotide substrate [14]. Because of the mentioned significance of tight nucleotide binding for the regulation of the chloroplast enzyme, it was important to study a corresponding mutation in a chloroplast enzyme. The *Chlamydomonas reinhardtii* ATP synthase, which is similar to the one of higher plants with regard to its catalytic and regulatory properties [6,15–17], is now accessible to genetic studies by means of site directed mutagenesis and chloroplast transformation [6,15,16]. We have produced the corresponding βT168S mutation in *atpB* of *C. reinhardtii* and transformed the mutated gene into the *atpB* deleted strain FUD50 [18]. Here we report on the enzymatic characteristics of the CF_1 and $\alpha_3\beta_3\gamma$ complex from the resulting mutant and the phenotypic behavior of the mutant cells.

2. Materials and methods

The *atpB* deletion mutant FUD50 [18] was cultivated in Sager-Granick medium [19] containing 0.2% acetate, while transformant strains derived from this mutant were cultivated in Sager-Granick minimal medium. Cell density was determined using a hemocytometer, and chlorophyll concentrations were determined according to Arnon [20].

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). Transformation into *Escherichia coli* 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 done using the T7 DNA Sequencing kit (Pharmacia) and denatured plasmid DNA as template. The *atpB* gene containing the T168S mutation was produced by site directed mutagenesis using a PCR based mutagenesis method [21] with the muta-

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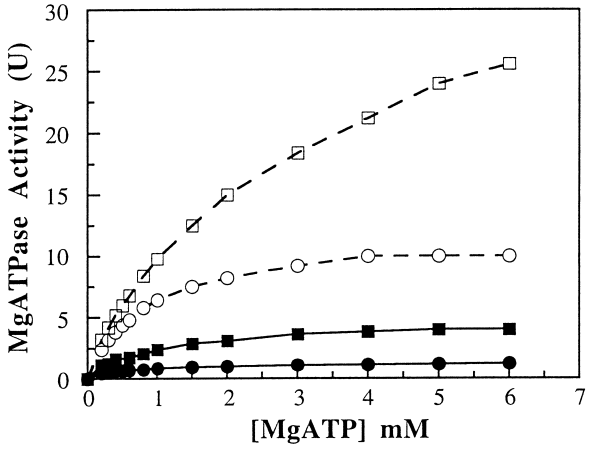

Fig. 1. MgATPase activities of wt CF_1 (●), wt $\alpha_3\beta_3\gamma$ complex (■), T168S mutant CF_1 (○) and T168S mutant $\alpha_3\beta_3\gamma$ complex (□) in dependence on the MgATP concentration. Reaction mixes were prepared with the indicated concentration of ATP (+2/3 the concentration of $MgCl_2$), and the reactions were started by the addition of 5 μg of βT168S mutant enzymes or of 10 μg of the wild type enzymes. Reactions were stopped after 5 min of incubation at 37°C and phosphate released was determined.

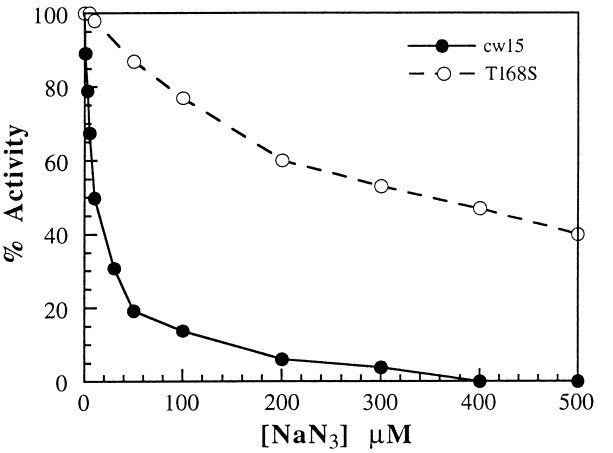

Fig. 2. NaN_3 inhibition of the MgATPase activity of WT and T168S mutant CF_1. Reaction mixes were prepared with the indicated concentrations of NaN_3, and the reactions were started by the addition of 5 μg of βT168S mutant enzymes or 10 μg of the wild type enzymes. Reactions were stopped after 5 min of incubation at 37°C and phosphate released was determined.

genic primers T168S-forward: 5′ GTA GGC AAA TCA GTT TTA A 3′ and T168S-reverse: 5′ TTA AAA CTG ATT TGC CTA C 3′.

The *atpB* fragments containing the mutant sites were cloned back into pBSatpB [15]. Presence of the correct mutation in the *atpB* gene was confirmed by DNA sequencing.

Chloroplast transformation was done with the Biolistic PDS1000-He Particle Delivery System of BioRad. After incubation overnight at low light intensity, cells were transferred to full light intensity (500–1000 lux) in CO_2 enriched atmosphere for selection of transformants. *C. reinhardtii* total DNA was isolated as described [15].

C. reinhardtii wild type and mutant CF_1 and $\alpha_3\beta_3\gamma$ complexes were purified as recently described [17]. ATPase activity was measured as described [15] with the additions indicated in the legends of figures and tables. Protein concentrations were determined using the BioRad protein assay. The content of bound nucleotide in isolated CF_1 and $\alpha_3\beta_3\gamma$ complexes was determined by the luciferase/luciferin assay as recently described [17].

3. Results and discussion

3.1. ATPase activity of isolated mutant CF_1

CF_1 and the $\alpha_3\beta_3\gamma$ complex were isolated from wild type cells and from the mutant strain FUD-βT168S and the ATPase activities of these preparations were analyzed. In contrast to the wild type CF_1 and its $\alpha_3\beta_3\gamma$ complex, the respective mutant complexes had significant MgATPase activity without activators present. The activities were about 10 times higher than those of the wild type complexes (Fig. 1, Table 1). The $\alpha_3\beta_3\gamma$ complexes of both the wild type and the mutant had about three times higher activities than the corresponding CF_1 preparations. This confirms the slight inhibitory role of the ε subunit on the MgATPase activity of CF_1 [17,22]. The isolated CF_1 and $\alpha_3\beta_3\gamma$ complexes of the mutant and of the wild type contained each 1 mol tightly bound ADP per mol (Table 2), indicating that this bound ADP by itself is not the reason for latency of the wild type enzyme.

Like the wild type enzyme, the FUD-βT168S enzyme was not significantly inhibited by ADP, displayed the same sensitivity to excess of free Mg^{2+} ions and showed optimal activity at 1 Mg^{2+}/1.5 ATP. In contrast, a correspondingly mutated $\alpha_3\beta_3\gamma$ complex produced by reconstitution from TF_1 subunits was no longer inhibited by excess Mg^{2+} [13]. Indeed, the threonine in question was identified as a Mg^{2+} binding site in the crystal structure of mitochondrial F_1 [14].

The mutated CF_1 and $\alpha_3\beta_3\gamma$ complex of *C. reinhardtii* (as the corresponding TF_1 $\alpha_3\beta_3\gamma$ complex) were much less sensitive to inhibition by NaN_3 than the wild type enzymes (Fig. 2). Azide inhibits octylglucoside activated or methanol stimulated MgATPase but not the CaATPase of spinach CF_1. The DTT activated CaATPase which is per se insensitive to azide, too, becomes susceptible to azide by preincubation with Mg^{2+} or MgADP [23]. The MgATPase of *E. coli* F_1 is strongly inhibited by azide under multisite conditions but not at all affected under uni-site conditions [24]. It was proposed that NaN_3 inhibits mitochondrial F_1-ATPase activity by stabiliz-

Table 1
ATPase activities of mutant and wild type ATPase complexes

	K_m	−DTT	+DTT	Ethanol	Methanol	Octyl-glucoside
CF_1-βT168S	0.76 mM	10.0	6.6	20.0	34.5	14.0
CF_1-cw15	K_m1 = 0.22 mM K_m2 = 0.59 mM	1.0	2.1	40.0	49.0	45.0
$\alpha_3\beta_3\gamma$-βT168S	1.48 mM	25.0	48.0	35.0	51.0	20.0
$\alpha_3\beta_3\gamma$-cw15	K_m1 = 0.25 mM K_m2 = 1.07 mM	×3.8	6.9	54.0	62.0	61.0

Catalytic properties of wt CF_1, wt $\alpha_3\beta_3\gamma$ complex, βT168S mutant CF_1 and βT168S mutant $\alpha_3\beta_3\gamma$ complex. Reaction mixes were prepared with 6 mM ATP (+4 mM $MgCl_2$), and the reactions were started by the addition of 5 μg of βT168S mutant enzymes or of 10 μg of the wild type enzymes for reactions in the absence of activators. In the presence of activators all reactions were performed with 5 μg/ml of enzyme present. Reactions were stopped after 5 min of incubation at 37°C (or 2 min of incubation in the presence of activators) and phosphate released was determined.

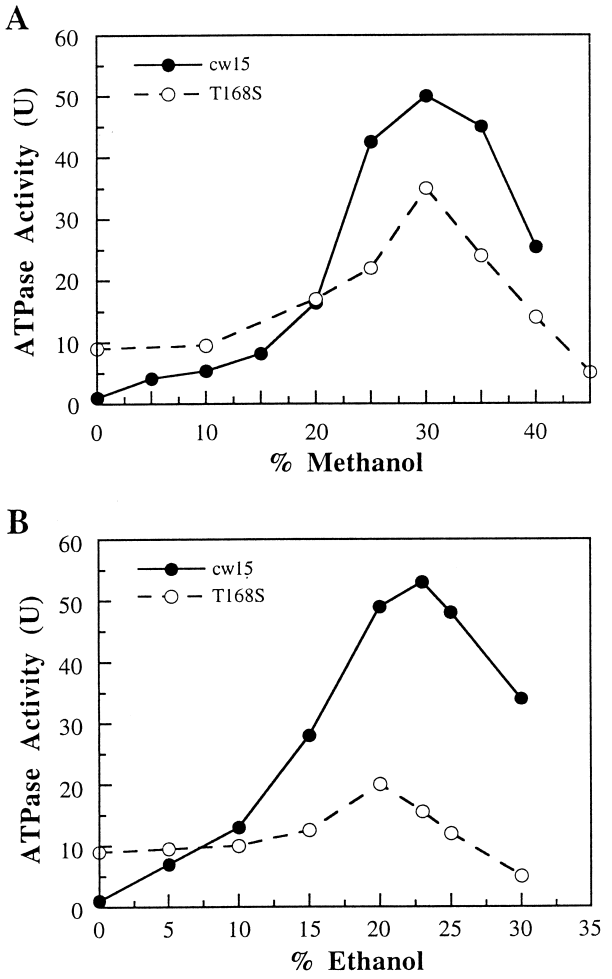

Fig. 3. Activation of the βT168S CF_1 and wild type CF_1 by methanol (A) and ethanol (B). Reaction mixes containing 6 mM ATP, 4 mM $MgCl_2$ and the indicated concentrations of ethanol or methanol were prepared. The reactions were started by the addition of 5 μg of enzyme, and phosphate released was determined after 2 min incubation at 37°C.

ing the inactive enzyme conformation with MgADP entrapped in one of the catalytic sites [13]. This interpretation which also fits the result observed with CF_1, suggests that trapping of ADP via Mg^{2+} to T168 in one of the three catalytic sites controls multisite activity, and that this control is abolished by replacement of T168 for S. It appears that the change from T to S changes the nature of the catalytic site so that trapping of ADP is no longer possible. If this is correct it has to be concluded that (1) the tightly bound ADP found in the isolated CF_1 of the wild type as well as the mutant is not inhibitory by itself and (2) that the threonine in position 168 of β subunit is not essential for binding of substrate MgATP in catalysis.

Table 2
Content of tightly bound nucleotide in mutant and wild type ATPase complexes

	ADP (mol/mol)	ATP (mol/mol)
CF_1-βT168S	1.04	0.13
CF_1-cw15	1.03	0.17
$\alpha_3\beta_3\gamma$-βT168S	0.95	0.12
$\alpha_3\beta_3\gamma$-cw15	1.19	0.11

Content of bound nucleotide in the purified CF_1 and $\alpha_3\beta_3\gamma$ complexes isolated from cw15 and from the FUD-βT168S mutant strain. The amount of nucleotide was determined by the luciferin/luciferase assay in supernatants derived from enzymes precipitated with perchloric acid and was calculated as mol of nucleotide bound per mol of enzyme.

3.2. Kinetic properties of the mutant enzymes

Rates of ATP hydrolysis of the wild type and mutant CF_1 and $\alpha_3\beta_3\gamma$ complexes were analyzed as function of substrate concentration. The wild type enzymes display non-linearity in Lineweaver-Burk plots from which two K_ms can be estimated. It is possible that the complex kinetics indicates transition between two different states of cooperativity. In the mutant enzymes only one K_m is observed which roughly corresponds to the higher of the two K_ms found in the wild type enzyme (Table 1). This result is in agreement with the conclusion drawn above, that control of multisite catalysis is abolished by replacement of T168 for S.

3.3. Activation of wild type and mutant enzymes

The effects of DTT, ethanol, methanol and octylglucoside on the βT168S enzymes differ from those on the wild type complexes (Table 2). DTT, which stimulates the MgATPase activity of wild type *C. reinhardtii* CF_1 by a factor of about 2.5, inhibits the activity of the mutant CF_1. On the other hand, the activity of the mutant $\alpha_3\beta_3\gamma$ complex like the wild type complex was stimulated by DTT. Methanol and ethanol stimulate the mutant enzymes, but the maximal activities obtained with the mutant complexes are lower than those of the wild type. Octylglucoside only poorly stimulates the activity of the mutated CF_1 and slightly inhibits the activity of the mutant $\alpha_3\beta_3\gamma$ complex while both wild type enzymes are strongly activated by the detergent. This confirms a previous observation that the pathways of activation by octylglucoside and alcohols are different [15]. It appears that the FUD-βT168S enzyme is locked in a partially activated state, and that further activation can only be obtained near the optimal methanol or ethanol concentration.

The ATPase activities of CF_1 and $\alpha_3\beta_3\gamma$ complex from

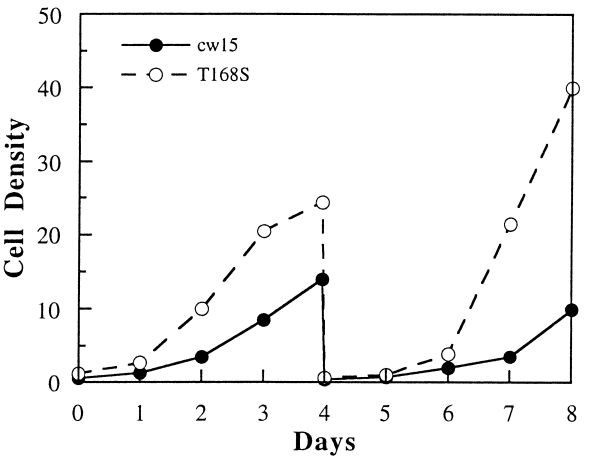

Fig. 4. Growth of wild type and FUD-βT168S in acetate containing media in the dark. Cells grown in the dark for 4 days were diluted to 10^5 cells/ml into acetate containing medium, and cell densities (10^6 cells/ml) in the culture were determined every 24 h. After another 4 days the cultures were diluted again and cell densities were determined for another 4 days. Cell densities during the two last 4 day periods are shown.

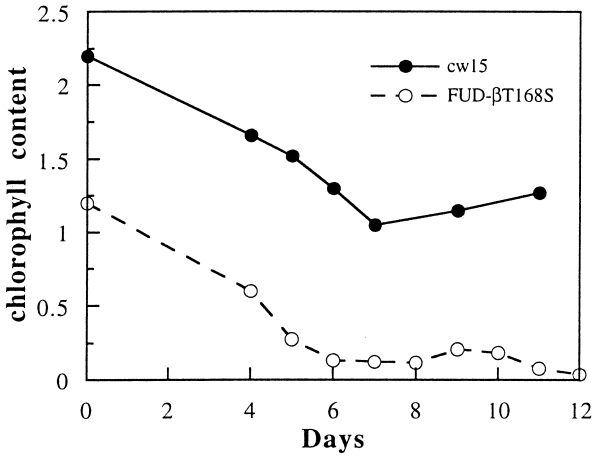

Fig. 5. Chlorophyll content of cw15 and FUD-βT168S cells during extended growth on acetate in the dark. The chlorophyll content in the cultures (given as μg chlorophyll/10^6 cells) was calculated from the cell density and chlorophyll concentration of the cultures and is plotted over the 12 day growth period described in Fig. 4, that included two dilution steps. Day 0 represents the chlorophyll content in the photoautotrophic cultures used to inoculate the dark grown cultures.

FUD-βT168S and from the wild type cells are differently affected by increasing concentrations of alcohols, especially ethanol (Fig. 3). The alcohol concentration optima indicate superposition of an activating effect with an inhibitory effect at higher alcohol concentrations. With the mutated enzyme, however, the inhibitory effect seems to be prevailing which causes a lower activity at a lower optimal ethanol concentration and a stronger inhibition at superoptimal alcohol concentrations. Interestingly, the same type of effect was found in a βE72K mutant [15], indicating that the activation mechanism by ethanol comprises not yet understood long range conformational interactions and involves large parts of the enzyme complex.

3.4. Phenotypic characterization of FUD-βT168S

The mutant *C. reinhardtii* strain bearing the βT168S mutation grew photoautotrophically at about half the growth rate of wild type cells. When transferred to the dark on HS minimal medium plates, the mutant cells survived equally well as the wild type cells. It will have to be investigated how the constitutive activity of CF_1 in FUD-βT168S is controlled so that the chloroplast ATP synthase does not cause depletion of the cellular ATP in the dark.

Surprisingly in the presence of acetate the mutant cells grew more rapidly in the dark than the wild type cells (Fig. 4), but the chlorophyll content per cell decreased dramatically in the mutant cells to about 2% of the content in the light (Fig. 5). In contrast the wild type cells maintained the chlorophyll content at around 1.1 mg/10^6 cells, corresponding to about 50% of the chlorophyll content in the light (Fig. 5). It will have to be investigated in which way these effects are related to the βT168S mutation in the chloroplast ATP synthase.

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