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Expression by *Chlamydomonas reinhardtii* of a chloroplast ATP synthase with polyhistidine-tagged beta subunits

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Abstract

The green alga *Chlamydomonas reinhardtii* is a model organism for the study of photosynthesis. The chloroplast ATP synthase is responsible for the synthesis of ATP during photosynthesis. Using genetic engineering and biolistic transformation, a string of eight histidine residues has been inserted into the amino-terminal end of the β subunit of this enzyme in *C. reinhardtii*. The incorporation of these amino acids did not impact the function of the ATP synthase either in vivo or in vitro and the resulting strain of *C. reinhardtii* showed normal growth. The addition of these amino acids can be seen through altered gel mobility of the β subunit and the binding of a polyhistidine-specific dye to the subunit. The purified his-tagged CF1 has normal Mg^{2+}-ATPase activity, which can be stimulated by alcohol and detergents and the enzyme remains active while bound to a nickel-coated surface. Potential uses for this tagged enzyme as a biochemical tool are discussed.

Keywords: *Chlamydomonas reinhardtii*; ATP synthase; CF1; Polyhistidine; His-tag

1. Introduction

Chloroplast ATP synthase belongs to the family of F-type ATPases, characterized by their ability to couple the translocation of protons to the synthesis and hydrolysis of ATP. This coupling of proton movement to ATP synthesis was first suggested by Mitchell in his chemiosmotic hypothesis [1]. In chloroplasts, the ATP synthase couples proton movement – down the electrochemical gradient established by light-dependent electron-transport – to ATP synthesis. This enzyme is composed of two distinct domains and is sometimes referred to as CF1CFo. The CF1 domain is the hydrophilic headgroup, whereas CFo is composed of transmembrane polypeptides and includes a ring of peptides within the thylakoid membrane. CF1 is composed of five different polypeptide chains in the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ and contains six nucleotide binding sites, three of which act cooperatively during catalytic activity [2]. The movement of these domains is coupled to the movement of the peptide ring of CFo so that transportation of protons across the thylakoid by CFo can drive the synthesis of ATP in CF1. The structure of CF1 is known from electron microscopy [3], partial crystal structures [4,5], and inference to the crystal structure of the mitochondrial ATPase [6]. The core catalytic unit of CF1 is a ring of alternating α and β subunits, which contain the nucleotide binding sites, around a central γ subunit.

Much of the recent work involving the ATP synthase has involved single-molecule studies of the enzyme while it is bound to an immobile surface [7]. This binding is possible because some enzymes have been engineered with additional regions of polyhistidines (his-tag) at the amino-terminal ends of the α or β subunits, which allow attachment to nickel-coated surfaces without interfering with the catalytic domains of the enzyme. Studies involving these bound, his-tagged enzymes have shown the ability of the central γ stalk to rotate within the heterohexameric ring formed by the α and β subunits [7–9].

For the chloroplast ATP synthase, no native enzyme has been available with an incorporated histidine tag. Single molecule studies have been attempted using untagged

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enzyme [10], genetically manipulated cyanobacterial enzyme [11] and hybrid enzymes reconstituted from plant and bacterial sources [9]. But without comparison to a native in vivo assembled enzyme, interpretation of these results is difficult.

Further work in defining the activity of the chloroplast ATP synthase would benefit from an enzyme that can be easily manipulated for single-molecule studies. Placing a polyhistidine region at the amino-terminus of the β subunit of CF1 and expressing this altered β subunit within enzymes which are assembled in vivo will provide a important tool for the study of ATP synthase.

The unicellular alga *Chlamydomonas reinhardtii* is an excellent model organism for the genetic manipulation of photosynthetic enzymes [12]. The techniques to alter photosynthetic genes, especially genes present in the chloroplastic genome, have been well established and extensively used [13]. Polyhistidines have been incorporated into proteins of both photosystem I [14] and photosystem II [15] as well as the oxygen evolving complex [16] of *C. reinhardtii*. In all cases, the tagged proteins assembled normally and were active.

In this paper, we will demonstrate that his-tagged ATP synthase can be expressed in *C. reinhardtii*, that the strain containing this altered ATP synthase behaves normally, that CF1 isolated from this strain is fully active, and that the isolated CF1 is active when bound to a nickel-coated surface.

2. Materials and methods

2.1. Cell culture

C. reinhardtii strain CC-1287 (FUD50) was obtained from the Chlamydomonas Culture Collection and maintained on sterile TAP agar [17] in the dark. After transformation, photosynthetic strains were maintained under low light on sterile TAP agar or grown at 25 °C in 1-l glass flasks using TAP media, bubbled with sterile air and stirred while continuously illuminated with cool-white fluorescent bulbs at an intensity of 150 $\mu E/m^2/s$. Chlorophyll concentrations were determined by the method of Arnon [18]. DNA extraction from small cultures of cells was performed using existing techniques [19].

2.2. Plasmid construction

Escherichia coli cells were grown either on LB agar or in LB media at 37 °C. Plasmid isolation was performed using QIAPrep DNA isolation (Qiagen) and digestion of plasmid DNA followed the supplier's protocols (New England Biolabs). DNA was separated using agarose gel electrophoresis and excision of DNA from agarose gels used QIAquick Gel Extraction columns (Qiagen). Mutation of plasmid DNA was performed by megaprimer mutagenesis [20]. Primers were obtained from Invitrogen. PCR amplification was performed in a thermal cycler (MJResearch) according to manufacturer's instructions.

2.3. Biolistic transformation

A 100-ml culture of FUD50 was grown in the dark in TAP media until it reached a cell density of approximately 3.0×10^6 cells/ml. The cells were then pelleted by centrifugation at $2000\times g$ for 5 min at 20 °C. The cells were resuspended in 4 ml of TAP media and 1 ml was spread onto each of four nylon membranes placed atop TAP agar plates. The cells were allowed to dry onto the membranes for 30 min prior to transformation. Plasmids were coated onto 0.6 μm gold particles and loaded into a PDS-1000He biolistic transformation apparatus according to manufacturer's protocol (Bio-Rad). Transformations were performed by placing the petri dish in the second highest position and using 1100-psi rupture disks. After transformation, the plates were kept in the dark overnight, then placed under 50 $\mu E/m^2/s$ light for approximately 48 h. The nylon membranes were then transferred from the TAP plates and layered onto plates with minimal media [17]. These were then placed under 100 $\mu E/m^2/s$ light and monitored until colonies formed (approximately 7–10 days). Resulting colonies were transferred to TAP media and grown under low light.

2.4. CF1 purification

CF1 was isolated from *C. reinhardtii* using modifications to existing techniques [21,22]. One liter cultures of rapidly growing cells were harvested by centrifugation at $1000\times g$ for 5 min and the cell pellets were resuspended in ice cold TNM buffer (10 mM Tricine–NaOH (pH 8.0), 10 mM NaCl and 5 mM $MgCl_2$) to a concentration of 0.2 mg/ml chlorophyll. The cell suspension was passed through an Emulsiflex cell disruptor (Avastin) at 5000 psi and centrifuged at $30{,}000\times g$ for 10 min at 4 °C. The pellet was resuspended in ice cold TNM buffer to a concentration of 0.1 mg/ml chlorophyll and then centrifuged at $30{,}000\times g$ for 10 min at 4 °C. The pellet was resuspended in the above buffer, centrifuged, and then washed for a third time. The resulting washed thylakoid membranes were resuspended in 0.75 mM EDTA (pH 8.0) at room temperature to a concentration of 0.05 mg/ml chlorophyll and stirred for 30 min. The thylakoid membranes were sedimented by centrifugation at $30{,}000\times g$ for 10 min and discarded. All subsequent steps were carried out at room temperature. The supernatant, containing released CF1, was supplemented with Tricine–NaOH (pH 8.0) to 20 mM and ammonium sulfate to 10 mM. DEAE-Sephadex, pre-equilibrated with 20 mM Tricine–NaOH (pH 8.0), was added and the mixture stirred for 30 min at room temperature. The solution was then poured across a small funnel lined with Miracloth to retain the DEAE-Sephadex, that was then loaded onto a small chromatography column. After washing the column with 3 column volumes of 20 mM Tricine–NaOH (pH 8.0), 20 mM ammonium sulfate, and 0.5 mM ATP, protein was eluted from the column using 20 mM Tricine–NaOH (pH 8.0), 300 mM ammonium sulfate and 0.5 mM ATP. The first 50 ml of elutant was retained and loaded into a 100,000 MWCO centrifugal concentrator (Millipore) and concentrated to a volume of less than 1 ml. ATP was added to 1 mM and ammonium sulfate added to 50% saturation, and the resulting suspension was stored at 4 °C.

2.5. Activity assays

ATP was isolated from rapidly growing cells in TAP media. Cells were centrifuged at $1000\times g$ for 2 min and quickly resuspend to approximately 0.1 mg/ml chlorophyll and aliquoted into 1 ml fractions on ice. Twenty μl of 50% TCA were added and the suspension vigorously vortexed for 1 min. The suspension was then flash frozen in liquid nitrogen for 5 min then transferred to wet ice for 5 min. The suspension was centrifuged at $14{,}000\times g$ for 5 min and the supernatant transferred to a fresh tube and stored at −80 °C until used. For measurement, the tube was thawed on ice and 10 μl of the solution was added to 90 μl of 0.1 M Tris–acetate (pH 7.75) and the ATP concentration was determined by the luciferin–luciferase method (Roche).

Photosynthetic oxygen evolution and dark respiration of cell cultures were determined using rapidly growing cells diluted with TAP to 2 μM chlorophyll concentration. Samples were placed in an oxygen electrode (Qubit Systems) held at a constant 20 °C and supplemented with 50 mM sodium bicarbonate. The sample chamber was kept in the dark for 5 min then exposed to 1200 $\mu E/m^2/s$ light through a yellow filter for 2 min followed by an unilluminated period to determine respiration.

CF1, stored as an ammonium sulfate precipitate, was centrifuged for 10 min at room temperature at $14{,}000\times g$ to pellet the protein. The pellet was dissolved in 20 mM Tricine–NaOH (pH 8.0) and passed through two Sephadex G-50 centrifuge columns to remove residual ammonium sulfate [23]. Protein concentration was then determined using the method of Bradford [24]. ATP hydrolysis rates of washed membranes or purified CF1 were measured at 37 °C in 20 mM Tricine–NaOH (pH 8.0), 5 mM ATP and 2 mM $MgCl_2$ with the addition of additives as noted. Phosphate released was determined colorimetrically [25].

3. Results

3.1. Transformation of *C. reinhardtii* with altered β subunit gene

The *Bam*HI DNA fragment containing the *atpB* gene (*Bam*HI 10) from the *C. reinhardtii* chloroplast genome was obtained from the Chlamydomonas Genetics Center as part of the P-17 plasmid. The *Bam*HI 10 fragment was excised from P-17 and ligated into a pUC-18 plasmid, predigested with *Bam*HI, creating the plasmid pUCatpB(wt). The pUCatpB(wt) plasmid was then mutated to incorporate an unique *Xho*I restriction site within the *atpB* gene (Fig. 1) by altering the sequence CTATTGAAA (bases 161178–161169 using published [26] numbering) to CTCTCGAGA. The mutation was performed by mega-primer mutagenesis using primers A and B (Table 1) for the first PCR amplification, and using primer C to elongate the amplified fragment beyond the mutation site. The fragment was digested with the restriction enzymes *Sty*I and *Cla*I, each of which has unique restriction sites within the pUCatpB(wt) plasmid. The DNA fragment was then isolated by gel extraction and ligated into pUCatpB(wt) that had been likewise digested and gel purified, creating the new plasmid pUCatpB(*Xho*I). The polyhistidine tag was incorporated into the pUCatpB(*Xho*I) plasmid by first annealing primers D and E as listed in Table 1. These primers are complementary and create a small DNA fragment with either end being degenerate *Xho*I cut sites (see Fig. 1). This fragment was ligated into a pUCatpB(*Xho*I) plasmid predigested with *Xho*I. The resulting ligation mixture was digested with *Xho*I thereby linearizing all plasmids without the new fragment. Since the new fragment could ligate into the plasmid in either orientation, colonies of *E. coli* following transformation with this ligation mixture were screened for correct insertion. Candidate colonies were screened by PCR amplification using primers D and F from Table 1. This primer combination amplified only those plasmids containing inserted DNA in the correct orientation. Plasmid DNA from positive colonies was designated pUCatpB(His) and confirmed by DNA sequencing.

Both the pUCatpB(wt) and the pUCatpB(His) plasmids were used to transform the FUD50 strain [26] of *C. reinhardtii*.

Table 1
List of primers

Primer	Sequence (5′ to 3′)
A	CTA TGA GTG ATT CTC TCG AGA CAA AAA ACA TG
B	GCA GGA GCA GGG TCA GTA AGG
C	CGT CGT CGT GAA TTC CAA CAA G
D	TCG AAC ACC ATC ACC ATC ACC ATC ACC ATA
E	TCG ATA TGG TGA TGG TGA TGG TGA TGG TGT
F	CCA TAA TTA AAA CTG TTT TGC CTA CAC

FUD50 (CC-1287) contains an *atpB* gene with a large deletion, so that no functional β subunit can be produced, causing an assembly failure of the chloroplast ATP synthase and a thylakoid membrane devoid of the enzyme. Due to the lack of ATP synthase on the thylakoid membrane, this strain is not capable of photoautotrophic growth [27]. The plasmids were introduced into the chloroplasts of the FUD50 cells using biolistic transformation [28]. After transformation, the cells were transferred to acetate deficient media and selection was based upon photoautotrophic growth. Colonies capable of growth on minimal media were grown in TAP media and whole cell DNA extracted for PCR screening. The DNA was screened for the presence a segment of the *atpB* gene missing in the FUD50 strain. Colonies were also screened for the inserted segment. Fig. 2 shows representative PCR amplification of DNA from colonies following transformation with either pUCatpB(wt) or pUCatpB(His). PCR fragments were also confirmed by DNA sequencing. Positive transformants of the pUCatpB(wt) plasmid were designated as the atpB(wt) strain, while transformants of the pUCatpB(His) plasmid were designated as atpB(His).

3.2. Characterization of new strains

The transformation of the FUD50 strain with the pUCatpB (wt) plasmid resulted in wildtype growth of the new strain and in all assays no difference was seen between this strain and previous results conducted on wildtype *C. reinhardtii*. The wildtype strain was used as a control when analyzing the effects of the polyhistidine insert on the physiology of the new strain of algae.

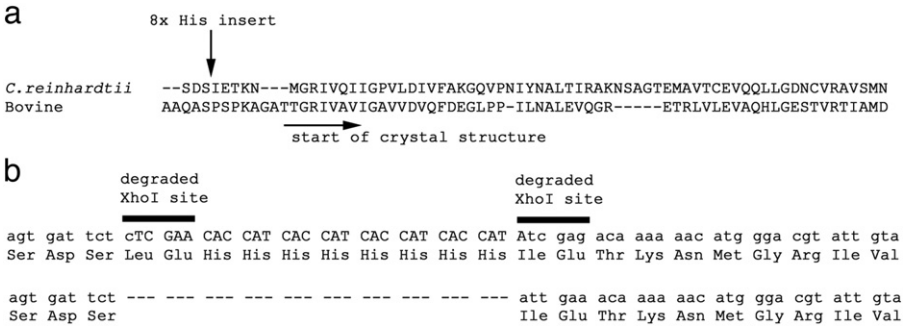

Fig. 1. Insertion site within β subunit. (a) Peptide alignment of the β subunits of the chloroplast ATP synthase from *C. reinhardtii* and the mitochondrial ATP synthase from *Bos taurus*. Alignment was performed using CLUSTALW. Horizontal arrow marks beginning of secondary structure in the mitochondrial subunit [6]. Vertical arrow shows the point of insertion for the histidine tag into the chloroplast subunit. (b) Codon sequence of the gene *atpB* from *C. reinhardtii*. Upper line shows sequence of atpB(His) while lower line shows sequence of atpB(wt). Dark lines show degraded *Xho*I sites after insertion of new DNA.

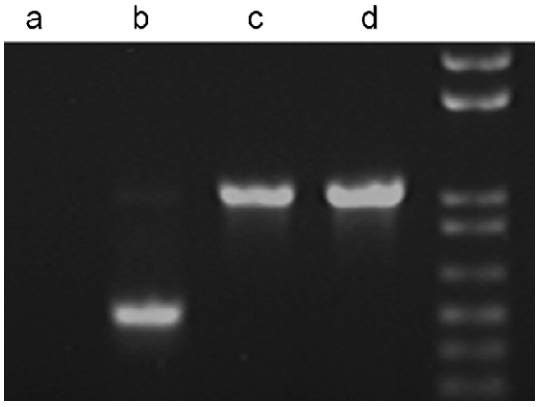

Fig. 2. PCR amplification of gene fragments from DNA extracts. Whole cell DNA extracted from actively growing cells. Samples shown in lanes a and c used DNA from the strain atpB(wt) while lanes b and d used DNA from the atpB (His) strain. Samples shown in lanes a and b used primers D and F as listed in Table 1 while samples in lanes c and d used primers C and F as listed in Table 1. The far right lane is 1.0 kb DNA ladder (Invitrogen). Both of these primer sets were also used with samples containing DNA extracted from wildtype cells (CC124) and FUD50 strain. Samples from CC124 behaved identically to the atpB(wt) strain while FUD50 gave no amplification to either primer set(not shown).

The presence of the polyhistidine insert within the ATP synthase of the atpB(His) strain did not affect growth of the cells on agar media. Both the atpB(wt) and the atpB(His) strains grew well on TAP agar in the light and in the dark, as well as on minimal media when grown in the light (data not shown). The FUD50 background strain cannot grow in the light on agar with either TAP or minimal media. In liquid cultures the atpB(His) strain grew at the same rate as the atpB(wt) strain (Fig. 3) when grown side-by-side with the same light source and in the same media. Analysis of the biochemical properties of the two strains (Table 2) shows normal chlorophyll *a* to chlorophyll *b* ratios

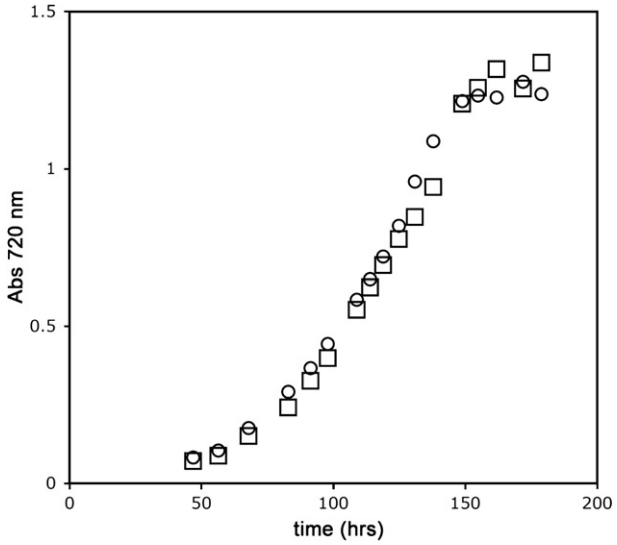

Fig. 3. Photosynthetic growth of algal strains in TAP media. Cells grown in stirred, 1-l bottles of sterile TAP media bubbled with air and illuminated as described under Materials and methods. Bottles were inoculated at time zero with 1.0×10^7 cells. Circles shows readings from the atpB(wt) strain, squares show readings from the atpB(His) strain.

Table 2
Biochemical properties of algal strains

	atpB-wt	atpB-His
Chl a/Chl b	2.8±0.1	2.7±0.1
Respiration	13.3±0.7	13.0±1.7
Oxygen evolution	38.3±2.1	36.5±3.7
ATP	112±16	104±5

Respiration and oxygen evolution rates are given in mmol O_2 mol chl^{-1} s^{-1}. Whole cell ATP is measured as nmol ATP mg chl^{-1}.

and content, demonstrating that the polyhistidine tag has not affected the assembly of the thylakoid membranes of the atpB(His) strain. Both respiration and oxygen evolution rates are equivalent in the two strains. Whole cell ATP content was also the same in both strains, further proof that the atpB(His) strain is capable of the same metabolic activity as wildtype *C. reinhardtii*.

Although photophosphorylation rates have been difficult to measure in *C. reinhardtii* thylakoids due to difficulty in isolating well-coupled membranes, ATP hydrolysis can be easily measured. Under normal conditions, ATP hydrolysis rates of thylakoids are very low in the dark. This activity can be stimulated by additives like detergents such as β-octyl-D-glucopyranoside, also referred to as octylglucoside, or alcohols such as ethanol. Table 3 shows that when octylglucoside is absent from the reaction mixture, activity of the membranes is low for both the atpB(wt) and atpB(His) strains. The addition of octylglucoside causes a large increase in the Mg^{2+}-ATPase activity of membranes from both strains. Given the similarity between these two strains, we believe that the ATP synthase of the atpB(His) strain is expressed normally on the thylakoid membranes of these cells and that the expressed protein is capable of normal activity.

3.3. Comparison of isolated CF1 from C. reinhardtii

CF1 was isolated from thylakoid membranes from both the atpB(wt) and the atpB(His) strains. Fig. 4a shows the electrophoretic separation of the individual subunits of wildtype and his-tagged CF1. CF1 from the atpB(wt) strain shows normal distribution of the subunits from the ATP synthase and the migration of the subunits from the atpB(His) strain seems to

Table 3
Mg^{2+}-ATPase activity of isolated membranes and purified protein

	Additions	CF1(wt)	CF1(His)
Washed membranes	None	72	65
	+ Octylglucoside	510	479
Purified CF1	None	2	1
	+ Octylglucoside	31	29
	+ Ethanol	26	25

Mg^{2+}-ATPase activity of washed membranes measured using 5 μg of chlorophyll at 37 °C for 10 min (without additive) or 5 min (with additive) and given as μmol P_i mg chl^{-1} h^{-1}. Mg^{2+}-ATPase activity of isolated CF1 measured using 5 μg protein at 37 °C for 10 min (without additive) or 2 min (with additive) and given as μmol P_i mg $protein^{-1}$ min^{-1}. Octylglucoside is added to the reaction mixture at 1.2% (w/v) and ethanol is added to the reaction mixture at 25% (v/v).

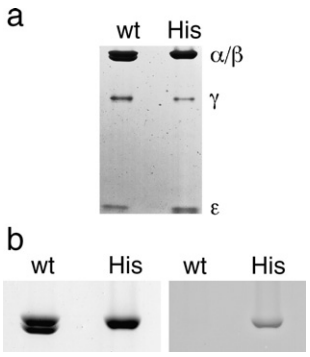

Fig. 4. Staining of purified CF1 separated by SDS-PAGE. (a) Purified CF1 was run onto an 12% SDS-PAGE gel (2 μg/lane) and stained by Coomassie Brilliant Blue. (b) Enlarged section of 12% SDS-PAGE gel. Left image shows gel stained with coomassie, and the right image is the same gel stained with InVision gel stain prior to coomassie stain. Two images aligned using his-tagged molecular weight markers (Invitrogen). InVision stain was detected in a Typhoon imager (Amersham) using 560 nm laser excitation.

be equivalent to wildtype. Both CF1 preparations do not show a band corresponding to the δ subunit of CF1; however this subunit has been difficult to visualize by other groups as well [22], and may have been lost or degraded during the purification procedure. Loss of the δ subunit does not affect the Mg^{2+}-ATPase activity of isolated CF1. Fig. 4b shows the α and β subunits electrophoresed under conditions that maximize separation of the two subunits. In the coomassie stained gel, it is apparent that the β subunit of the atpB(His) CF1 moves more slowly than the corresponding β subunit from the atpB(wt) strain. The presence of eight additional histidines, plus the additional two amino acids found on the polyhistidine insert (see Fig. 1) would add approximately 1.8 kDa to the molecular weight of the β subunit. Since the α subunit is approximately 55 kDa and the wildtype β is approximately 53 kDa [29], the additional amino acids found in the CF1 from the atpB(His) strain would account for the roughly equivalent mobility of both the α and β subunits in this strain. The presence of the his-tag was confirmed by using the gel stain InVision (Invitrogen) which is specific to polyhistidine containing proteins. When a SDS-PAGE gel containing CF1 from both atpB(wt) and atpB (His) strains was stained with InVision, only the band corresponding to the β subunit of the atpB(His) CF1 was stained (Fig. 4b).

Isolated CF1 from both strains show low Mg^{2+}-ATPase activity. The Mg^{2+}-ATPase activity of both CF1 were stimulated by the addition of octylglucoside and ethanol (Table 3). Rates of ATP hydrolysis by our CF1 preparations are lower than those reported by earlier groups [21,22]. The his-tagged and the non-tagged CF1, however, had equivalent rates of ATP hydrolysis.

CF1 from the atpB(His) strain was also active when bound to a surface. CF1 from both strains, in 20 mM Tricine–NaOH (pH 8.0), was exposed to the surface of a 96-well plate coated with nickel. The enzyme, in increasing concentrations, was placed in the wells of the plate and left at room temperature overnight without shaking. The wells were emptied and washed to remove unbound enzyme. The wells were then filled with a solution containing Mg^{2+}-ATP and octylglucoside in order to stimulate hydrolysis activity by CF1 bound to the well. The amount of P_i was detected colorimetrically. The results are seen in Fig. 5. Only the wells exposed to CF1(His) tested positive for octylglucoside-dependent Mg^{2+}-ATPase activity and the amount of P_i released was dependent upon the initial loading of CF1. Thus, purified CF1 from the atpB(His) strain, unlike native CF1, binds to a nickel-coated surface and remains active while bound.

4. Discussion

The incorporation of a his-tag into the chloroplast ATP synthase allows for the quick and efficient manipulation of the enzyme. The location of the tag was chosen in order to minimize the impact of the additional amino acids on the structure and function of the enzyme and was chosen by comparing the *C. reinhardtii* β subunit to the crystal structure of the bovine mitochondrial β subunit. The bovine subunit contains a short segment of twelve amino acids at the amino-terminal (termed residues −4 to 8) that form an disordered region before the beginning of the first β-sheet domain of the structure [6]. Aligning the amino acid sequence of the two β subunits (Fig. 1a) reveals an equivalent segment of eight amino acids in the algal β subunit. Adding the his-tag to an unstructured region at the amino-terminus of the peptide increases the chances that the tag will be solvent-exposed. This is also the region where a his-tag was incorporated into the thermophilic ATPase [7].

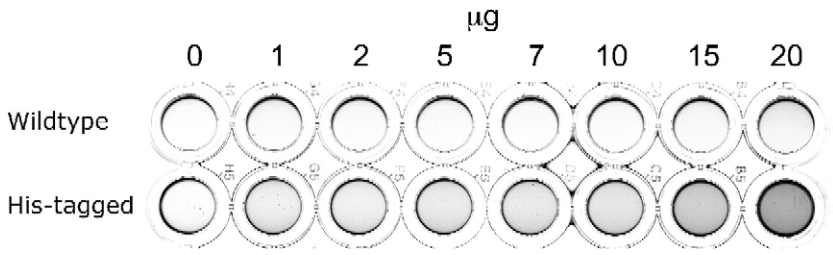

Fig. 5. ATPase activity of CF1 attached to nickel-coated wells. Isolated CF1 from strains atpB(wt) and atpB(His) was added to the wells of a nickel-coated 96-well plate (Sigma-Aldrich) and allowed to bind overnight at room temperature. The wells were then washed to remove unbound protein. ATPase activity present in the wells was determined using Mg^{2+}-ATPase reaction mix (100 μl) as described in Materials and methods with 1.2% (w/v) octylglucoside added. The reaction was stopped by adding 100 μl of 0.5 N TCA and P_i visualized using 5% (w/v) $FeSO_4 \cdot 7H_20$, 1.6% ammonium molybdate in 1.0 N H_2SO_4 (100 μl).

An initial application involving his-tagged ATP synthase will be single-molecule studies of the enzyme labeled with either gold beads or actin filaments. Such studies have been performed with other forms of the chloroplast ATP synthase [9–11], but use of an in vivo assembled, his-tagged CF1 will be novel and potentially informative. These studies will aim to define some of the unique attributes of CF1, especially in regard to its regulation [30,31].

Additional changes to the *atpB* gene can be incorporated into the pUCatpB(His) plasmid allowing single-molecule experimentation on CF1 containing additional β subunit mutations. Several alterations to the β subunit have already been accomplished in *C. reinhardtii*, all have resulted in functional, assembled CF1 [22,32,33]. The technique of co-transformation [34] can also be used to create mutations in other CF1 subunits while still producing his-tagged CF1. Current work in our laboratory is focused on using the co-transformational method to produce strains of *C. reinhardtii* containing both the polyhistidine tag incorporated into the pUCatpB(His) plasmid and alterations to the ε subunit made to the pUCatpE plasmid [19].

The capacity of *C. reinhardtii* for genetic manipulation allows for a unique opportunity in the study of ATP synthase: the ability to create and assemble in vivo chloroplast ATP synthase containing a histidine-tag necessary for immobilizing the enzyme on specific surfaces. This work will allow native-assembled eukaryotic ATP synthase enzymes to be used in single molecule studies.

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