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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].