Final Technical Report

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Title: "Molecular genetic analysis of biophotolytic hydrogen production in green algae."

The principal objective of this project was to identify genes necessary for biophotolytic hydrogen production in green algae, using *Chlamydomonas reinhardtii* as an experimental organism. The main strategy was to isolate mutants that are selectively deficient in hydrogen production and to genetically map, physically isolate, and ultimately sequence the affected genes.

The first step in the execution of this strategy was the generation of a population of mutant strains that could be screened for hydrogen evolution deficiency. We generated a population of 4500 insertional mutant strains, each with an independent insertion of the bleomycin-resistance (BLE) marker at an unselected site in the genome. One and one half percent of these strains are deficient in some aspect of photosynthesis and cannot grow without a fixed carbon source (acetate). Another 0.5% are deficient in aerobic respiration and cannot grow in the absence of light. These mutant proportions are in line with expectation for roughly random insertion of the BLE marker.

The second step in our mutant isolation strategy was to screen the mutagenized strains for photosynthetically competent strains that are deficient in light-driven hydrogen evolution (*hev*). Screening was conducted by analyzing strains individually for photoinduced hydrogen production transients in a closed chamber with a Clark-type Pt-Ag/AgCl electrode system biased for hydrogen detection. We isolated more than a dozen *hev* strains from this mutant collection and were in a position to clone them at the termination of the project. An example hydrogen evolution transient for one of these mutants is shown in Figure 1a. We were surprised to find an additional set of mutants with elevated net hydrogen evolution (see Figure 1b – note the change in scale from that in Figure 1a). The net photoevolution of hydrogen by wild type cells is rapidly superseded by a net hydrogen uptake process, which is absent in this class of mutants. This hydrogen uptake is not directly light-dependent, but shows characteristics of the oxyhydrogen reaction, metabolically combining the photosystem-produced O_2 with the accumulated H_2 to regenerate water. We classify this interesting category of mutants as hydrogen uptake-deficient (*hup*). It is clear from the properties of these mutants that metabolic uptake of hydrogen by the cells plays a much more significant role in determining net hydrogen evolution than has been previously recognized. Efforts to achieve the technologic objective of oxygen-insensitive hydrogen evolution by the algae must take this into account. Our findings also demonstrate that suppression of the oxyhydrogen reaction can be achieved by genetic modification of the algae. Most of the *hup* mutants are also deficient in autotrophic growth in the dark due to a deficiency in mitochondrial oxidative electron transport. The example mutant shown in Figure 1b is deficient in mitochondrial cytochrome b. Thus, cytochrome oxidase and mitochondrial electron transport play a critical role in the oxygen uptake segment of the oxyhydrogen reaction.

DOE Patent Clearance Granted

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We also isolated and sequenced the gene encoding the hydrogenase enzyme from *Chlamydomonas*. The single subunit catalytic activity purified and N-terminal sequenced by Happe and Naber (Eur J Biochem, 214:475-481, 1993) from *Chlamydomonas* contained iron but lacked nickel, placing it in the category of Fe-hydrogenases. We employed RT-PCR to identify a cDNA clone from *C. reinhardtii* encoding a protein with strong homology to the Fe-hydrogenase of *Clostridium pasteurianum*. The sequence is published in GenBank as accession AF289201. The clone encodes a 56 amino acid N-terminal extension consistent with the expected localization of the enzyme in the chloroplast. The 441 amino acid mature protein sequence is completely lacking the N-terminal region of the *Clostridium* enzyme that binds several iron-sulfur clusters involved in electron transfer to the active site. The mature *Chlamydomonas* sequence is highly homologous to the C-terminal catalytic domain of the *C. pasteurianum* enzyme, suggesting that this region of the 1FeH crystal structure for that enzyme can be used for modeling the expected structure of the *Chlamydomonas* enzyme. The *Chlamydomonas* sequence contains two insertions relative to the *Clostridium* sequence, both of which map to surface loops in the structure. Anaerobic induction of hydrogenase activity in *Chlamydomonas* is enhanced by the addition of nickel salts to the culture. We found that nickel also stimulates induction of the Hyd1 mRNA, providing a sufficient explanation for the nickel effect without requiring the conclusion that there is a Ni-Fe hydrogenase in addition to the Fe hydrogenase that has been characterized. Our genetic analysis places the Hyd1 gene as a single locus on linkage group III of *C. reinhardtii*, 17.5 cM from the centromere.

Figure 1. Light-driven H₂ evolution transients. Cells grown aerobically on TAP medium were resuspended in fresh medium at a concentration of 20 μM chl and adapted to anaerobiosis by bubbling with N₂ in darkness for two hours. They were placed in a closed, stirred, thermostatted (20°C) chamber equipped with a Clark-type Pt-Ag/AgCl electrode system polarized for H₂ detection and exposed to light at time zero. The curves labeled "wild type" show typical H₂ production transients for *C. reinhardtii*, with a rapid net evolution phase followed by an active net H₂ uptake phase within a few seconds. Mutant G27, upper panel, is deficient in light-driven hydrogen evolution (*hev*). Mutant dum1, lower panel, is deficient in hydrogen uptake (*hup*).