Thermotolerant Photodependent Hydrogen Production by Platinized Photosystem I Reaction Centers and Recombinant Cytochrome c_{553} from *Thermosynechococcus elongatus*

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Abstract

Photosystem I (PSI) from plants, algae, and cyanobacteria has been shown to mediate hydrogen evolution both *in vivo* and *in vitro*. We observed that metallic platinum could be photo-precipitated at the reducing end of PSI according to the reaction, $[PtCl_6]^{-2} + 4e^- + h\nu \Rightarrow Pt\Downarrow + 6Cl^-$. This platinum nanocluster can then receive the photo-generated electrons from PSI and function as a nanocatalyst for the evolution of molecular hydrogen *in vitro*. In this biomimetic reaction, sodium ascorbate is present as a sacrifical source of reducing equivalents with cyt c_{553} acting as a relay between ascorbate and the lumenal side of PSI. To enable a more thermostable and kinetically optimized system for hydrogen evolution in this biomimetic photosynthetic reaction, we have begun characterizing the hydrogen evolution capabilities of trimeric PSI isolated from the thermophilic cyanobacterium, *Thermosynechococcus elongatus (T. elongatus)*. This organism utilizes only c-type cytochrome as the primary electron donor to P_{700}. An expression system for *T. elongatus* cyt c_{553} in *E. coli* that involves the concurrent over-expression of the entire *E. coli* Type I heme insertion pathway (*ccm*A-H) plus ALA supplementation of minimal, synthetic media have been developed and optimized. CD spectroscopy has shown that cyt c_{553} is thermally stable up to a temperature of $>75^0$C. Also CD spectroscopy of PSI from *T. elogatus* suggests that most of the chlorophyll molecules maintain their structure until ~67° and Chl a, the key pigment that serves as an electron donor called the special pair, is still intact up to 95°C. We have demonstrated that a Cyt-PSI-Pt system based on this cytochrome is capable of evolving hydrogen in a cyt c_{553}- and light-dependent fashion. Hydrogen evolution increases with temperature up to 55°C. In addition, stability studies have shown the hydrogen evolution to be stable for >80 days. We have also demonstrated through a sequence-of-addition reaction that although sodium ascorbate can directly reduce P_{700} during photo-dependent hydrogen production, these experiments unequivocally show that cyt c_{553} acts as a catalyst in this reaction.

Introduction

Provision of an abundant, clean and secure renewable energy source is one of the key energy challenges facing mankind. Molecular hydrogen is a renewable fuel that can be used with hydrogen fuel cells to provide a clean and efficient source of energy. If an environmentally and economically sustainable method of producing hydrogen can be found, it could become the primary energy carrier of the future. It has been known since the turn of the century that microorganisms have the capability to produce hydrogen. Recently there has been

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considerable interest in using green algae to produce hydrogen as a fuel since, in principle they can employ the highly efficient process of photosynthesis to produce H_2 from two simple yet renewable resources, sunlight and water. Previous work has identified two independent pathways of H_2 production. Greenbaum and co-workers demonstrated the existence of a low-level but continuous electron transport pathway based on oxygenic photosynthesis (1-3). This pathway is distinct from a fermentative pathway of electron transport for H_2 production reported by Gaffron, Gibbs, and co-workers (4-8). However, it is now clear that both of these electron-transport processes have limitations in both sustainability and yield, *in vivo*. Although the incompatibility of simultaneous photoproduction of O_2 and H_2 remained problematic for many years (9), in the late 1990's a simple and elegant solution was reported (10). By using a nutrient-modulated two-stage growth cycle the processes of oxygen evolution and hydrogen production may be separated temporally and sustained for hours (11).

An alternative approach to *in vivo* photosynthetic hydrogen production has been proposed that employs biochemically-isolated PSI reaction centers that have kinetic and structural properties that are unsurpassed by synthetic systems. In PSI, photon absorption initiates an electron transfer sequence that generates a 1 V potential over a distance of 6 nm (12), a reaction that is completed within 150 ns (13). The quantum yield of the photochemical reaction is close to 100% (14, 15). By coupling the low-potential electron-emergent end of PSI complexes to either platinum nanoparticles (16, 17) or covalently linked hydrogenase (18, 19), the photochemically produced electrons can catalyze the reduction of protons to hydrogen *in vitro.* The fact that PSI has been shown to be active in both the solid-state (20) and in solution (this work) for many months suggests that we will be able to produce a highly stable, cell-free hydrogen-evolving system that may prove to be a feasible, solar-driven energy solution (21). Furthermore, future improvements may allow direct electron extraction from water using a coupled PSII to PSI system capable of recapitulating the photosynthetic electron transport chain in a new hybrid nanoparticle similar to what has been done in solution (22).

This work is directed toward demonstrating that a cell-free system derived from the thermophilic cyanobacterium, *Thermosynechococcoccus elongatus*, can provide a stable, thermotolerant, hydrogen-evolving nanoparticle. Using two biological components, PSI isolated from cultivated *T. elongatus* cells and a recombinant form of *T. elongatus* cyt c_{553} expressed in *E. coli* and platinum metal, we are able to demonstrate sustainable hydrogen evolution in an entirely resolved system. Moreover, we demonstrate both the thermostablity of the individual components as well as an enhanced hydrogen evolution rate for temperatures up to 60°C and also the temporal stability of this system by its ability to evolve hydrogen for > 80 days. These properties, as well as the renewable nature of most of the key components, suggest that this mode of photosynthetic hydrogen evolution may be a sustainable source of hydrogen.

Results and Discussion

Photosystem I (PSI) was isolated from the thermophilic cyanobacteria *Thermosynechococcus elongatus.* Tris-Tricine SDS-PAGE revealed the presence of a small amount of phycobiliproteins, anion exchange chromatography was later used to ensure consistent purity and to concentrate the final PSI trimers. Unlike plants, this cyanobacterium only encodes a c-type cytochrome (cyt c_{553}) that functions as the primary donor to P_{700}. The electron donor to PSI is a cyt c_{553}. Thus to maximize the rate of hydrogen evolution of P_{700} it

was necessary to have this cytochrome available in large quantities. Using gene-specific primers we have cloned the gene (petJ) for cyt $c_{553,}$ Using an optimized expression system, large amounts (>10 mg/l) of the mature length holoenzyme were purified via immobilized metal affinity chromatography (IMAC) followed by anion exchange chromatography.

Circular Dichroism spectroscopy was used to observe the thermostability of both PSI and cyt c by monitoring the non-covalent pigment organization of PSI and the protein primary structure of cyt c_{553}. These experiments indicated that T_m for the coordination of the bulk chlorophyll is ~55°C for PCC 6803 and >67°C for *T. elongatus* (data not shown).

Initial experiments were carried out to investigate the parameters under which *T. elongatus* PSI can support photo-dependent hydrogen evolution and also to study the stability of the system. A sequence-of-addition reaction was carried out to demonstrate the effect of each reagent on the rate of photo-dependent hydrogen evolution (Fig. 1A). This was accomplished by adding PSI and platinum hexachloroplatinate to an MES pH 6.4 buffer solution. Sodium ascorbate (the sacrificial electron donor) was then added, followed by cyt c_{553}, and providing light (2 hours on, 2 hours off).

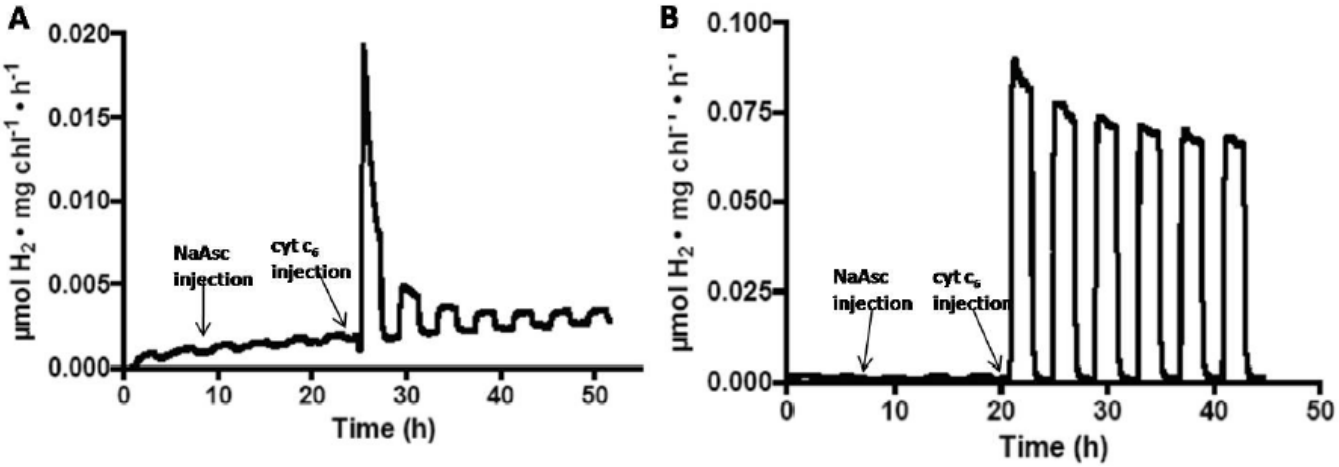

Figure 1 A. Sequence of addition on fresh PSI preparation. PSI at a Chl concentration of 80 µg/ml and 0.5 mM $[PtCl_6]^{2-}$ were added at time t = 0 at a temperature of 25°C. 1 mM NaAsc was injected through a side port into the photo-reactor at t = 7.18 h in the dark as indicated with an arrow. 9.4 µM cyt c_{553} was then added into the system at time t = 24.58 h in the dark as indicated with an arrow. **B.** Sequence of addition on platinized PSI complex. Cyt c_{553}-free platinized PSI was initially added at time t = 0.1 mM NaAsc was added at time t = 7.5 h and 9.4

The platinum nanoclusters were self-assembled as the platinum ions were reduced to platinum metal in progressively larger nanospheres. After platinization, the PSI-Pt congugates were isolated and purified and then resuspended in buffer. The presence and structure of Pt in the nanoparticles were confirmed by energy-dispersive X-ray spectroscopy and TEM imaging of the particles (data not shown). Hydrogen evolution activity on the re-isolated platinized PSI (Fig. 1B) demonstrates that the platinum catalyst was truly intergrated into the PSI nanoparticle. Also, the hydrogen evolving ability of these platinized PSI nanoparticle still required cyt c_{553} as an electron donor, to facilitate rapid re-reduction of P_{700}.

μM cyt c_{553} was then added at time t = 20.09 h. The activity of hydrogen evolution was continuously monitored at 25^0C.

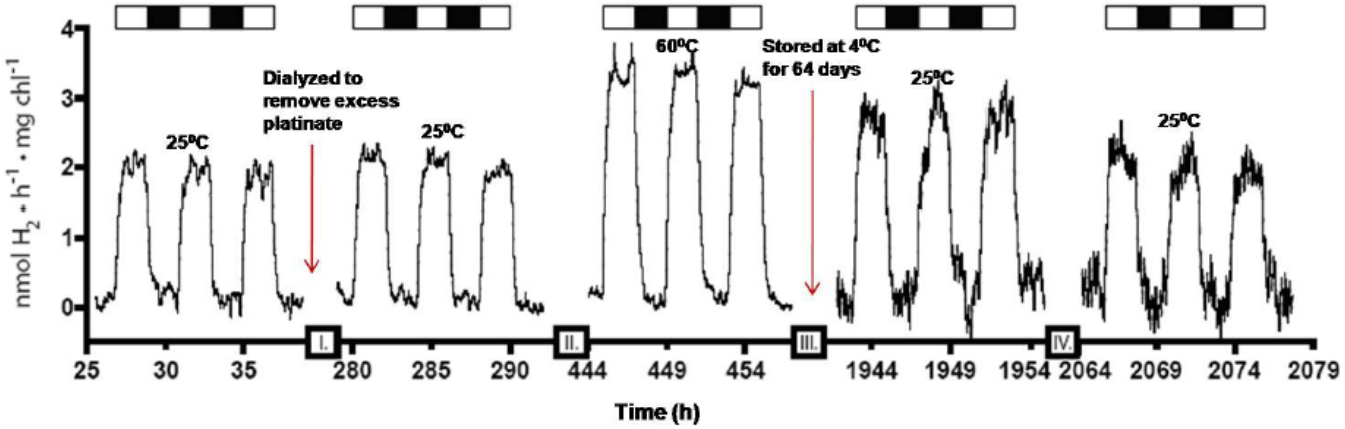

Figure 2 Temporal stability of H_2 evolution. PSI (80 μg chl/ml), 9.4 μM cyt c_{553}, 0.5 mM $Na_2[PtCl_6]$, 20 mM MES buffer pH 6.4, and 20 mM NaAsc, was used for this experiment.

To evaluate the sustainability of this cell-free hydrogen evolution system we ran an extended experiment to test one lot of platinized PSI for over a three month period. The results of this are shown in Fig. 2. After 170 hours of photo-dependent hydrogen evolution at 25°C, the temperature was increased to 60°C and the H_2 evolution rate increased to approximately double the rate at 25°C. At 180 hours of continuous operation, the reagent mixture was stored for 64 days at 4°C, later warmed to 25°C, and re-exposed to 4 hour light-dark cycles as before. The rate previous to storage was almost completely recovered. These data show that our PSI preparations maintain operational stability for extended time periods.

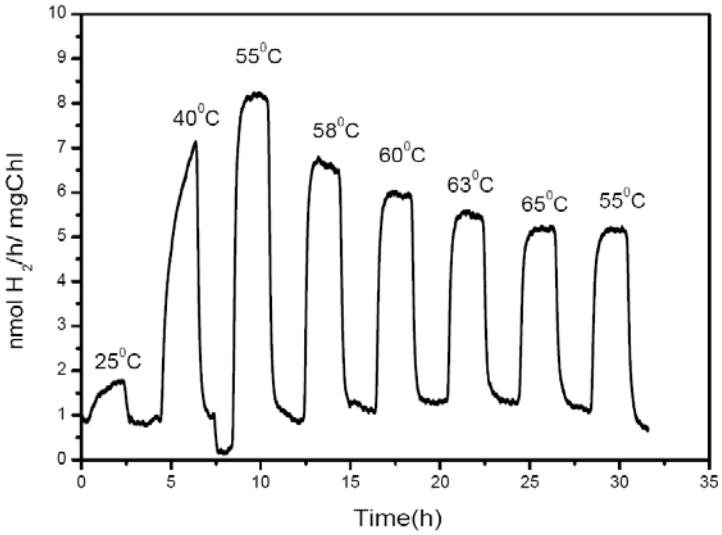

Figure 3 Temperature-activity measurements using the dialyzed platinized PSI preparation. An external water bath was used to incrementally increase the temperature during successive 2 hours light on/off cycles allowing the reaction to equilibrate during the dark cycles. Hydrogen evolution was continuously monitored at each temperature as indicated.

Study of temperature dependence on the rate of hydrogen photo-evolution was performed in order to optimize the rate of hydrogen evolution for the platinized PSI and cyt c_{553} system. Temperature-activity measurements were carried out using dialyzed platinized PSI preparation in the presence of MES buffer pH 6.4 and NaAsc (Fig. 3). The highest rate of hydrogen evolution was observed at 55°C with a yield of 14.8 nmole H_2/mg Chl. This indicates that we can expect ~15-fold increase in the sustained rate of hydrogen evolution as the temperature is increased from 25 - 55°C, which is the physiological growth temperature for *T. elongatus*.

Conclusion

In summary, we have characterized the hydrogen evolution capabilities of PSI from the thermophile, *Thermosynechococcus elongatus* by a "re-wiring of the electron transport pathway of PSI. Using a solution-based, self-assembled platinization of the PSI nanoparticles, we have demonstrated a NaAsc-Cyt-PSI-Pt-H_2 electron transport that yields light-dependent hydrogen. The system is thermostable with hydorgen evolution rates increasing up to 55^0C and stability studies have shown the hydrogen evolution to be stable for > 80 days. Through optimization we obtained a peak H_2 yield of ~ 5.5 µmol H_2/mg Chl/h (data not shown) close to those recently reported by researchers working with synthetically attached catalysts. Future work has to be done in characterizing and optimizing the platinization process and enhancing the rate of the cell-free hydrogen evolution by engineering the interaction between PSI and cyt c_{553}.

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