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Terry M. Bricker
Louisiana State University

Jason Morvant
Louisiana State University

Najy Masri
Louisiana State University

Holly M. Sutton
Louisiana State University

Laurie K. Frankel
Louisiana State University

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Rapid report

Isolation of a highly active Photosystem II preparation from *Synechocystis* 6803 using a histidine-tagged mutant of CP 47

Terry M. Bricker *, Jason Morvant, Najy Masri, Holly M. Sutton, Laurie K. Frankel

Department of Biological Sciences, Biochemistry and Molecular Biology Section, Louisiana State University, Baton Rouge, LA 70803, USA

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Abstract

Site-directed mutagenesis was used to produce a *Synechocystis* mutant containing a histidine tag at the C terminus of the CP 47 protein of Photosystem II. This mutant cell line, designated HT-3, exhibited slightly above normal rates of oxygen evolution and appeared to accumulate somewhat more Photosystem II reaction centers than a control strain. A rapidly isolatable (< 7 h) oxygen-evolving Photosystem II preparation was prepared from HT-3 using dodecyl-β-D-maltoside solubilization and Co^{2+} metal affinity chromatography. This histidine-tagged Photosystem II preparation stably evolved oxygen at a high rate (2440 μmol O_2 (mg chl)$^{-1}$ h^{-1}), exhibited an α-band absorption maximum at 674 nm, and was highly enriched in a number of Photosystem II components including cytochrome c_{550}. Fluorescence yield analysis using water or hydroxylamine as an electron donor to the Photosystem II preparation indicated that virtually all of the Photosystem II reaction centers were capable of evolving oxygen. Proteins associated with Photosystem II were highly enriched in this preparation. 3,3′,5,5′-Tetramethylbenzidine staining indicated that the histidine-tagged preparation was enriched in cytochromes c_{550} and b_{559} and depleted of cytochrome *f*. This result was confirmed by optical difference spectroscopy. This histidine-tagged Photosystem II preparation may be very useful for the isolation of Photosystem II preparations from mutants containing lesions in other Photosystem II proteins. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: CP 47; His-Tag mutant; Photosystem II; (*Synechocystis* 6803)

1. Introduction

Photosystem II (PS II) is a light-driven, water-plastoquinone oxidoreductase. In higher plants and cyanobacteria at least six intrinsic proteins appear to be required for oxygen evolution by the photosystem. These are CP 47, CP 43, the D1 protein, the D2 protein, and the α and β subunits of cytochrome b_{559}. Insertional inactivation or deletion of the genes for these components results in the complete loss of oxygen evolution activity. Additionally, a number of low molecular mass components appear to be associated with PS II [1,2], although the functions of these proteins remain obscure. While PS II complexes containing only these components can evolve oxygen, they do so at low rates (about 25–40% of

Abbreviations: chl, chlorophyll; DCBQ, 2,6-dichlorbenzoquinone; DCMU, dichloromethylurea; HA, hydroxylamine; His-tagged, (histidine)$_6$-tagged; MES, 2-[*N*-morpholino]ethanesulfonic acid; PCR, polymerase chain reaction; PEG, polyethylene glycol 10 000; PMSF, phenylmethylsulfonyl fluoride; PS I, Photosystem I; PS II, Photosystem II; TMBZ, 3,3′,5,5′-tetramethylbenzidine

* Corresponding author. Fax: (504) 3884638;
E-mail: btbric@lsuvm.sncc.lsu.edu

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control), are extremely susceptible to photoinactivation, and require high, non-physiological levels of calcium and chloride for maximal activity [3,4]. For optimal levels of oxygen evolution at physiological salt concentrations, three extrinsic proteins appear to be required. These have apparent molecular masses of 33, 24 and 17 kDa in higher plants and 33, 17 (cytochrome c_{550}) and 12 kDa in cyanobacteria [5].

The cyanobacterium *Synechocystis* 6803 offers a number of unique advantages for study of PS II. The organism can grow photoheterotrophically in the absence of PS II, allowing the study of PS II mutants which normally possess lethal phenotypes. It is naturally transformable and exhibits a high frequency of homologous recombination, which allows the facile introduction of mutations into the PS II genes of interest [6]. Finally, *Synechocystis* 6803 is the only organism capable of oxygenic photosynthesis for which the genomic DNA has been completely sequenced.

A necessary prerequisite for the study of structure and function of PS II is the ability to isolate PS II preparations which exhibit high rates of oxygen evolution, are stable, and can be rapidly isolated. While such preparations exist in higher plants [7–9], the isolation of cyanobacterial PS II preparations is significantly more difficult. Most cyanobacterial PS II preparations require ultracentrifugation, gel filtration and/or ion exchange chromatography [10–12]. These steps can often be time-consuming and often are technically difficult to perform.

The production of His-tagged mutations of a variety of proteins has proved to be an effective means of simplifying the purification of the target protein from complex protein environments. Membrane protein complexes which have been purified by this methodology include cytochrome oxidase [13,14], the bacterial photosynthetic reaction center [15] and PS II from the green alga *Chlamydomonas reinhardtii* [16]. In this communication, we report the introduction of a His-tag onto the C terminus of the CP 47 protein of PS II. We have characterized the resulting mutant and developed a procedure to isolate a highly active and stable PS II preparation from this mutant cyanobacterial strain.

2. Materials and methods

Control and mutant strains of *Synechocystis* 6803 were grown in liquid BG-11 media as previously described [17] at a light intensity of 70 μmol photons m^{-2} s^{-1} in 150 ml cultures at 30°C with air bubbling. For the analysis of oxygen evolution capacity, cells were grown photoautotrophically. For the analysis of the fluorescence properties, cells were grown photoheterotrophically in the presence of 5 mM glucose and 10 μM DCMU. For isolation of the His-tagged PS II complex, cells were grown in 15 l carboys either autotrophically or mixotrophically.

Restriction digests, cloning, growth and transformation of bacterial strains, and isolation of DNA were performed according to standard procedures [18]. *Synechocystis* transformation procedures [6], the characteristics of control strain K3 and the deletion strain Del-1, and construction and properties of the plasmid pTZ18K3 have been described elsewhere [17]. The desired mutation was introduced into pTZ18K3 by oligonucleotide-directed mutagenesis [19]. The mutagenic primer, 5′-TCCACCCGGAAAGAAGCCCACCACCACCACCACCACTAGGTGCTTCTTGCACAG-3′, was used to introduce six histidyl codons at the end of the *psbB* gene. This yielded the recombinant plasmid designated pTZ18His. This alteration was then confirmed by sequencing prior to transformation of the recipient *Synechocystis* strain Del-1. Putative transformants were isolated and streaked six times to allow sorting out of the multiple *Synechocystis* genomic copies. Genomic DNA was isolated from several of these putative transformants, the *psbB* gene was amplified by PCR, and the PCR products were sequenced directly on an Applied Biosystems Model 377 DNA sequencer.

For the isolation of the His-tagged PS II preparation, cells were harvested from a 30 l culture of HT-3 cells which had been grown either photoautotrophically or mixotrophically for 8–10 days with air bubbling. The cells were concentrated to about 1 l with a Pellicon tangential ultrafiltration device (Millipore) equipped with a 0.45 μm filter. All further operations were performed at 4°C. The cells were centrifuged in a Sorvall GSA rotor at 10 000 × g for 10 min, resuspended in 1.0 l of 50 mM Na-K phosphate buffer,

pH 7.4, centrifuged at $10\,000\times g$ for 10 min in a Sorvall GSA rotor, and resuspended at 300 μg/ml chl in Buffer A [12] (50 mM MES-NaOH, pH 6.0, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 25% glycerol) and incubated for 1 h. The cells were then centrifuged at $10\,000\times g$ for 10 min in a Sorvall GSA rotor and resuspended to 1.0 mg/ml chl in Buffer A. The cell suspension was brought to 1.0 mM PMSF, 1.0 mM benzamidine, 1 mM ε-amino caproic acid and 50 μg/ml DNAase. The cells were loaded into a pre-chilled Bead-Beater chamber (Bio-Spec Products) and glass beads (0.1 mm) were added to give a 1:1 ratio of glass beads to cell suspension. The Bead-Beater chamber was cooled with a water-ice jacket. The cells were then broken with 12 break cycles, each cycle consisting of 15 s of homogenization followed by 5 min of cooling. After breakage, the cell homogenate was decanted from the glass beads and the beads were washed once with Buffer A to recover additional homogenate. The cell homogenate was brought to 1% dodecyl maltoside by addition of a 10% freshly prepared dodecyl maltoside stock solution and was immediately centrifuged at $36\,000\times g$ for 10 min in a Sorvall SS-34 rotor to remove unbroken cells and residual glass beads. The solubilized cell homogenate was then loaded onto a 30 ml Co^{2+} metal affinity column (Clontech) which had been pre-equilibrated with Buffer A+0.04% dodecyl maltoside. The column was washed with 90 ml of Buffer A+0.04% dodecyl maltoside and the bound His-tagged PS II was eluted with Buffer A+0.04% dodecyl maltoside+50 mM histidine. We used histidine to elute the PS II complex from the metal affinity column rather than the more usual imidazole. Because of its much larger size (MW 209.6 vs. 68.08), and zwitterionic charge, we believe that histidine would be less likely to interact with PS II reaction centers (particularly, mutant reaction centers) than imidazole. The PS II fraction eluted as a tight, intensely green band. The eluted fractions were pooled and the PS II complex was precipitated by addition of an equal volume of 25% PEG-8000 in 50 mM MES-NaOH, pH 6.0 and incubation for 30 min. The precipitated PS II complex was harvested by centrifugation at $36\,000\times g$ in a Sorvall SS-34 rotor for 30 min. The precipitated PS II complex was resuspended in 1.0 ml of Buffer A+0.04% dodecyl maltoside and either used immediately or stored at −80°C until further use.

For cells, PS II activity was measured by oxygen polarography using a Hansatech oxygen electrode. Cells were assayed in BG-11 media with DCBQ added as an electron acceptor. Oxygen evolution was measured at a light intensity of 5000 μmol photons m^{-2} s^{-1} of copper sulfate-filtered white light at 20°C. Variable fluorescence yield measurements were performed on a Walz PAM 101 fluorimeter as previously described [20,21]. The chlorophyll concentration was 10 μg/ml. For the His-tagged PS II complex, oxygen evolution was measured with 250 μM DCBQ and 1 mM potassium ferricyanide as electron acceptors in a buffer consisting of 50 mM MES-NaOH, pH 6.5, 25 mM $CaCl_2$ and 10 mM NaCl. The chlorophyll concentration in these measurements was 2.5–5.0 μg/ml. Fluorescence assays were performed in the same media. Samples were brought to 250 μM DCBQ and 1 mM potassium ferricyanide and incubated for 30 s, then DCMU was added to a concentration of 40 μM and the sample incubated for another 30 s prior to the onset of pulsed illumination. In experiments where HA was used as an electron donor, the sample was brought to 40 mM HA after the DCMU addition and the samples were incubated for an additional 20 s prior to pulsed illumination.

Electrophoresis, 'Western' blotting, antibody probing, and TMBZ staining were performed as previously described [17,22]. Chl was extracted from *Synechocystis* cells with MeOH and its concentration was determined from its absorbance at 665 nm [23]. Visible spectra were recorded on a Varian 100 UV/VIS spectrophotometer. Optical difference spectra were also recorded on a Varian 100 UV/VIS spectrophotometer. His-tagged PS II samples (20 μg/ml chl) were reduced with a few grains of dithionite and oxidized with a few grains of either ascorbate or potassium ferricyanide.

3. Results and discussion

3.1. Construction and characterization of the HT-3 cell line

Transformation of the deletion strain Del-1 with the recombinant plasmid pTZ18His yielded hundreds of transformants. The *psbB* gene of several of these transformants was amplified by PCR and sequenced.

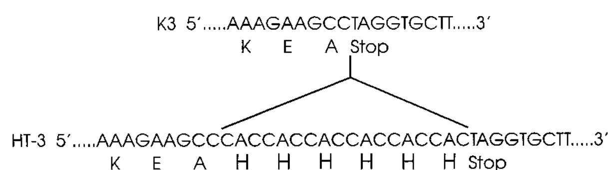

Fig. 1. Nucleotide and amino acid sequence in the vicinity of the introduced His-tag of CP 47. K3, control strain; HT-3, mutant strain carrying the introduced His-Tag at the end of the *psbB* gene. The amino acid sequences are shown below the respective nucleotide sequences. Amino acids are designated by their single letter code.

All of the transformants whose DNA was sequenced contained the desired mutation (data not shown). One transformant was chosen for further study and designated HT-3. The DNA sequences of a portion of the *psbB* genes from HT-3 and the control strain K3 [17] are shown in Fig. 1. Comparison of these DNA sequences indicated that six histidyl codons had been introduced at the end of the *psbB* gene in the strain HT-3.

Ideally, the characteristics of a mutant protein bearing a His-Tag should be identical to the characteristics of the wild type protein. Any major alteration in activity and/or structure in the mutant protein would make extrapolation of the properties of the mutant protein to those of the wild type protein difficult, if not impossible. The mutant HT-3 cell line exhibited PS II characteristics which were very similar to those observed in the control strain K3. Table 1 summarizes some of these characteristics. The oxygen evolution rate observed in HT-3 was marginally higher than that observed in K3. While this effect was small it was consistently observed. Interestingly, the variable fluorescence yield using either HA or water as a donor (in the presence of DCMU) was also higher in the mutant. Variable fluorescence yield can provide a semi-quantitative estimate of the number of PS II centers which can utilize either water or HA as an electron donor [21]. These results appear to indicate that the HT-3 strain accumulates somewhat more active PS II reaction centers than the control strain K3. We also examined the fluorescence rise time in the control strain K3 and the HT-3 mutant using both water and HA as an electron donor and in the presence of DCMU. When water is used as an electron donor, the fluorescence rise time is a measure of the ability of the oxygen-evolving complex to donate electrons to Q_A. When HA is used as an electron donor, the fluorescence rise time is a measure of the ability of PS II to transfer electrons from Z to Q_A. The fluorescence rise times of the HT-3 mutant, using either water or HA as an electron donor, were very similar to those of the control strain. Taken together, these results indicate that the PS II characteristics of K3 and HT-3 are quite similar. The oxygen evolution rates, fluorescence yield measurements, and fluorescence rise time meas-

Table 1
Characteristics of the control strain K3 and the CP 47 His-tagged mutant, HT-3

	Cell type	
	K3	HT-3
O_2 evolution rate[a]		
Cells	450	475
Variable fluorescence yield[b]		
$H_2O \rightarrow Q_A^-$	0.41	0.60
$HA \rightarrow Q_A^-$	0.48	0.84
$T_{1/2}$ fluorescence rise time (ms)		
$H_2O \rightarrow Q_A^-$	5	6
$HA \rightarrow Q_A^-$	12	14

[a] μmol O_2 (mg chl)$^{-1}$ h^{-1}.
[b] Variable fluorescence yield=$F_m - F_o$.

Table 2
Flow chart for isolation of the His-tagged oxygen-evolving PS II preparation

Step	% chl yield
1. Cell harvesting (1 h)	100
2. Cell breakage (2 h)	50[a]
3. Detergent extraction, column loading and washing (2 h)	48
4. PS II elution (0.5 h)	1–3
5. PEG precipitation (1 h)	1–3

Approximate time required for each step is shown in parentheses.
[a] Virtually all of this loss is due to the limited washing of the glass beads used in this procedure. Increasing the number of washes would increase the yield but lengthen the procedure.

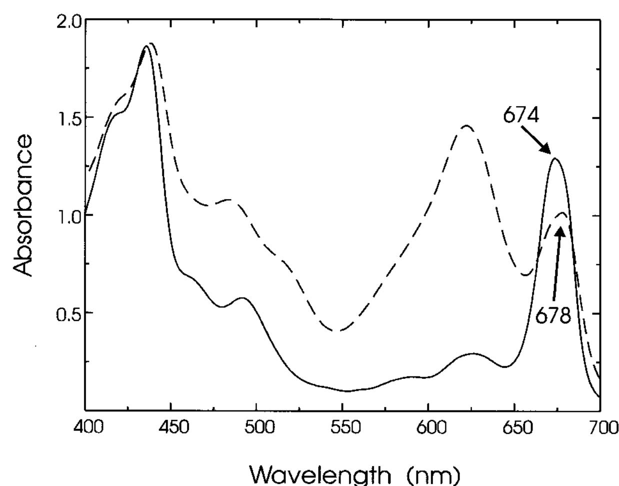

Fig. 2. Visible spectra of the His-tagged PS II preparation and the cell homogenate. Dashed line, cell homogenate; solid line, His-tagged PS II preparation. Absorption maxima of the α-bands are indicated with arrows.

urements indicate that the two strains accumulate active PS II centers and that these centers exhibit similar abilities to evolve oxygen.

3.2. Isolation and characterization of the His-tagged PS II preparation

The entire procedure from harvesting of the cells to freezing of the PS II preparation took less than 7 h (Table 2). The PS II preparation eluted from the Co^{2+} affinity column as a tight, intensely green band located at the void volume of the column. During ion exchange chromatography [11,12], PS II elutes as an extremely broad peak which is located between two PS I peaks. PS II-containing fractions must be identified by continuously monitoring the absorption maximum of the eluting material. This is unnecessary in our preparation. The visible spectra of the PS II fraction is shown in Fig. 2. The PS II fraction exhibited an absorption maximum at 674 nm as opposed to an absorption maximum of 678 nm for the cell detergent extract. Additionally, the PS II fraction was highly depleted of phycobiloproteins, which exhibit absorption maxima at 600–650 nm. The small peak at about 624 nm does not appear to be due to residual phycobiloproteins and is observed in both highly purified *Synechocystis* PS II preparations [11,12] and PS II preparations isolated from higher plants (which lack these components) [24,25]. The visible spectrum of the His-tagged PS II preparation appears to be identical to that obtained from wild type *Synechocystis* PS II preparations which are isolated by classical techniques [11,12].

The His-tagged PS II preparation exhibited an oxygen evolution rate of 2440 μmol O_2 (mg chl)$^{-1}$ h^{-1} which was comparable to that observed for wild type oxygen-evolving PS II preparations (Table 3) [11,12]. This oxygen-evolving activity was quite stable. After 48 h storage at 4°C the preparation retained more than 85% of its original activity (data not shown). Variable fluorescence was used to estimate the proportion of PS II centers present in the

Table 3
Some characteristics of wild type and His-tagged PS II oxygen-evolving preparations

	Cell type	
	Wild type	HT-3
O_2 evolution rate[a]	(2500)[b]	2440
Visible spectra		
λ maximum α band	(673.6 nm)[c]	674 nm
Cytochrome b_{559} content (per 60 chl)	(1.5–2.1)[d]	1.47
Variable fluorescence yield[e]		
$H_2O \rightarrow Q_A^-$	ND[f]	0.41
$HA \rightarrow Q_A^-$	ND	0.43

[a] μmol O_2 (mg chl)$^{-1}$ h^{-1}.
[b] Oxygen evolution rate observed in PS II preparations isolated from wild type *Synechocystis* 6803 [11,12].
[c] Tang and Diner [12].
[d] MacDonald et al. [30].
[e] Variable fluorescence yield=$F_m - F_o$.
[f] ND, not determined.

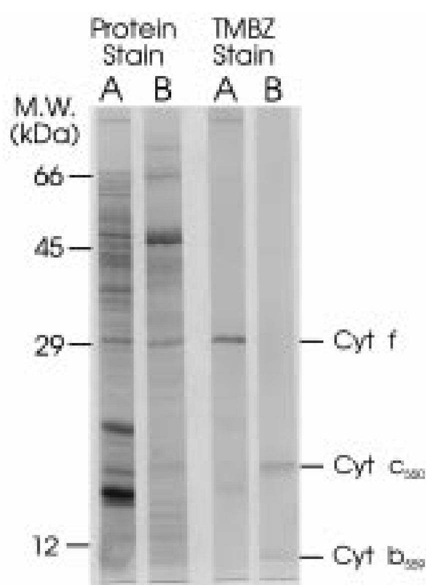

Fig. 3. Polyacrylamide gel electrophoresis of the *Synechocystis* cell homogenate and His-tagged PS II preparation. The results of Coomassie blue staining and TMBZ staining are shown. A, cell homogenate; B, His-tagged PS II. The locations of molecular weight standards are shown to the left. The identities of the TMBZ stained cytochrome bands are shown to the right.

His-tagged PS II preparation which were capable of evolving oxygen (Table 3). The variable fluorescence yield using water as an electron donor was 95% of that which was observed using HA as an electron donor. Given the semi-quantitative nature of these fluorescence yield measurements we conclude that most of the PS II centers present in the His-tagged preparation can evolve oxygen.

Polyacrylamide gel electrophoresis, TMBZ staining, and 'Western' blotting were used to examine the protein composition of the His-tagged PS II preparation. The Coomassie blue staining pattern demonstrated that the vast majority of the proteins present in the cell extract have been depleted in the PS II preparation (Fig. 3). Comparison of the polypeptide composition of the His-tagged preparation with PS II preparations obtained from wild type *Synechocystis* indicates that this preparation has fewer polypeptide components than the preparation described by Noren and Barry [11] but more components than that described by Tang and Diner [12]. Interestingly, the CP 47 protein exhibits significantly greater Coomassie blue staining than is normally observed. This is probably because of the introduction of the His-tag on the C terminus of this protein. Coomassie blue binds to positively charged amino acid residues and would be expected to bind quite strongly to a sequence of six histidines. A number of proteins were examined by immunological detection. CP 47, CP 43, D1, and the 33 kDa extrinsic protein were all enriched in the His-tagged PS II preparation (Fig. 4). RuBp carboxylase/oxygenase, PS I reaction center proteins, phycobiloproteins, and proteins associated with the cytochrome b_6/f complex were all highly depleted in this PS II preparation (data not shown).

We also examined the His-tagged preparation for the presence of TMBZ-stainable cytochromes. TMBZ staining exploits the inherent peroxidase activity of cytochromes after gel electrophoresis and is particularly useful for detection of *c*-type cytochromes [22,26] and under some conditions can also be used to identify *b*-type cytochromes [22,27]. Three cytochromes were observed (Fig. 3). The first, at an approximate molecular mass of 33 kDa, was present in the cell homogenate and was depleted in the PS II preparation. This cytochrome was tenta-

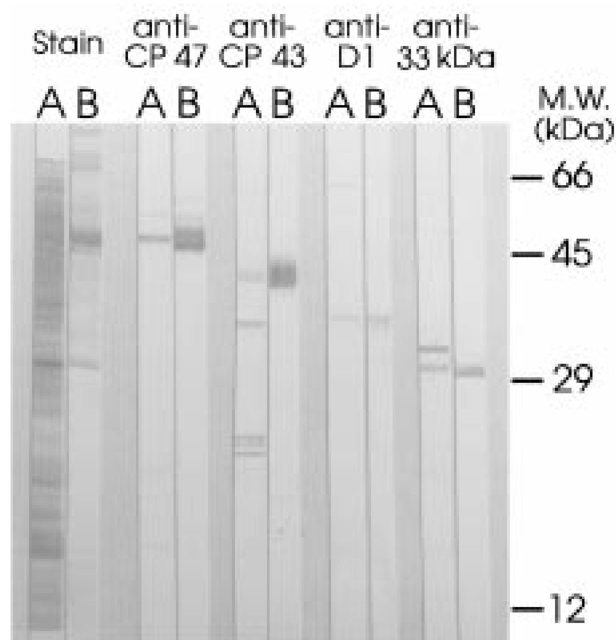

Fig. 4. 'Western' blot analysis of the *Synechocystis* cell homogenate and His-tagged PS II preparation. Coomassie blue stain and antibody probe are identified above. A, cell homogenate; B, His-tagged PS II. The locations of the molecular weight standards are shown to the right.

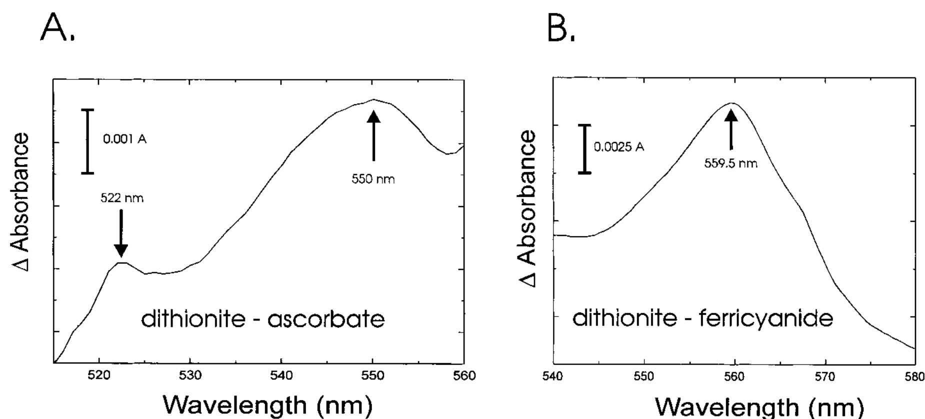

Fig. 5. Optical difference spectra of the His-tagged PS II preparation. (A) Dithionite-reduced minus ascorbate-oxidized difference spectrum. (B) Dithionite-reduced minus ferricyanide-oxidized difference spectrum. Absorption maxima are indicated by arrows. Please note different scales used in A and B.

tively identified as cytochrome *f* based on its apparent molecular weight. This identification was confirmed immunologically (data not shown). The second cytochrome exhibited an apparent molecular mass of about 17 kDa and was highly enriched in the His-tagged PS II preparation. We tentatively identified this component as cytochrome c_{550} based on its apparent molecular mass and its enrichment in the His-tagged PS II preparation. A third cytochrome was also enriched in the His-tagged PS II preparation. It exhibited an apparent molecular weight of about 9.5 kDa and was tentatively identified as cytochrome b_{559}.

To further clarify the identity of the cytochromes present in the His-tagged preparation, optical difference spectroscopy was performed (Fig. 4). The dithionite-reduced minus ascorbate-oxidized difference spectrum of the His-tagged PS II preparation is shown in Fig. 5A. Under these conditions, absorption maxima at 550 nm and 522 nm were observed. These absorption maxima are very similar to those observed from native cytochrome c_{550} [28,29]. No peak at 559 nm was observed. The dithionite-reduced minus ferricyanide-oxidized spectrum of the His-tagged PS II preparation is shown in Fig. 5B. Under these conditions an absorption maximum at 559.5 nm was observed with a slight broadening at 550 nm. This difference spectrum is very similar to that observed for the classically isolated *Synechocystis* PS II preparation previously described [11]. These results indicate that both cytochrome c_{550} and cytochrome b_{559} are present in the His-tagged PS II preparation. Additionally, the cytochrome b_{559} present in this preparation does not appear to be oxidized with ascorbate. The amount of cytochrome b_{559} present in this preparation was determined as previously described by MacDonald et al. [30] using deramped spectra and an extinction coefficient for cytochrome b_{559} of 20 mM^{-1} cm^{-1}. The His-tagged PS II preparation contained 1.47 cytochrome b_{559} per 60 chl. This confirms the value previously reported [30].

In this communication we have characterized a *Synechocystis* mutant which contains a His-tagged alteration at the C terminus of CP 47. This mutant exhibited PS II characteristics similar to a control cell line. We have also presented a method to isolate a His-tagged oxygen-evolving PS II preparation from this mutant cell line which exhibits high rates of oxygen-evolving activity. There are several advantages to using His-tag methodology to isolate PS II. First, this technique is very rapid, with the entire procedure being completed in less than 7 h. Isolation of PS II by classical methods from *Synechocystis* requires significantly more time (12–15 h). Most of the time savings in our procedure result from the use of metal-affinity chromatography to isolate the preparation. These columns can be washed and eluted rapidly. It should be noted that this PS II preparation was isolated using low pressure column chromatography; even less time would be required if medium or high pressure columns were to be used. Additional time savings were obtained by directly solubilizing the cell

homogenate with dodecyl maltoside. Other procedures for the isolation of PS II require the initial isolation of thylakoids from the homogenized cells. We have found this to be unnecessary. Second, because of the use of affinity chromatography, the binding and elution conditions can be varied. For instance, solubilization, binding and elution could be performed at significantly higher chloride and possibly calcium concentration than can be used in classical ion-exchange chromatography. This may be very useful for the isolation of PS II preparations from mutant cell lines which require high chloride concentrations to maintain active PS II reaction centers.

Previously, a His-tagged PS II isolation procedure had been described for *Chlamydomonas* [16]. In this preparation, the His-tag was incorporated onto the C terminus of the D2 protein. The mutant cell line grew somewhat slower than the control *Chlamydomonas* strain and exhibited a 16% decrease in oxygen-evolving capability. Thermoluminescence glow curves exhibited a lower yield of both the B- and Q-bands (17 and 13%, respectively). The His-tagged PS II preparation isolated from this mutant exhibited an oxygen evolution rate of 1030 μmol O_2 (mg chl)$^{-1}$ h^{-1}. It is possible that the incorporation of the His-tag onto the D2 protein induced a change in the configuration of the reaction center which led to these, albeit small, phenotypic changes. How these changes might be affected by the introduction of additional mutations into the His-tagged D2 protein background cannot be determined at this time.

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