Oxygen-Evolving Photosystem II Preparation from Wild Type and Photosystem II Mutants of *Synechocystis* Sp. PCC 6803†

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ABSTRACT: We present here a simple and rapid method which allows relatively large quantities of oxygen-evolving photosystem II- (PS-II-) enriched particles to be obtained from wild-type and mutants of the cyanobacterium *Synechocystis* 6803. This method is based on that of Burnap et al. [Burnap, R., Koike, H., Sotiropoulou, G., Sherman, L. A., & Inoue, Y. (1989) *Photosynth. Res. 22*, 123–130] but is modified so that the whole preparation, from cells to PS-II particles, is achieved in 10 h and involves only one purification step. The purified preparation exhibits a 5–6-fold increase of O_2-evolution activity on a chlorophyll basis over the thylakoids. The ratio of PS-I to PS-II is about 0.14:1 in the preparation. The secondary quinone electron acceptor, Q_B, is present in this preparation as demonstrated by thermoluminescence studies. These PS-II particles are well-suited to spectroscopic studies as demonstrated by the range of EPR signals arising from components of PS-II that are easily detectable. Among the EPR signals presented are those from a formal S_3-state, attributed to an oxidized amino acid interacting magnetically with the Mn complex in Ca^{2+}-deficient PS-II particles, and from S_2 modified by the replacement of Ca^{2+} by Sr^{2+}. Neither of these signals has been previously reported in cyanobacteria. Their detection under these conditions indicates a similar lesion caused by Ca^{2+} depletion in both plants and cyanobacteria. The protocol has also been applied to mutants which have site-specific changes in PS-II. Data are presented on mutants having changes on the electron donor (Y160F) and electron acceptor (G215W) side of the D_2 polypeptide.

Photosystem II is an enzyme which is able to reduce plastoquinone and oxidize water [reviewed in Babcock (1987); Rutherford, 1989; Debus, 1991]. The process is driven by light and results in the release of O_2. The PS-II reaction center contains two similar polypeptides, D_1 and D_2,[1] which form a heterodimeric complex [reviewed by Michel and Deisenhofer (1988)]. The D_1/D_2 complex carries the primary and secondary electron donors (P_{680} and Tyr_Z), the initial electron acceptor Ph, a pheophytin molecule, and the quinone electron acceptors, Q_A and Q_B, and it probably provides ligands for the Mn complex which accumulates the positives charges required to oxidize water.

Light induces a separation of charges between P_{680} and Ph. This charge separation is stabilized by electron transfer from Ph^- to Q_A and by the reduction of P_{680}^+ by Tyr_Z, the tyrosine 161 of the D_1 polypeptide (Debus et al., 1988b; Metz et al., 1989). Further stabilization of charges is obtained by reduction of Tyr_Z^+ by the Mn complex and by the oxidation of Q_A^- by the secondary quinone, Q_B. Q_B binds to a pocket in the D_1 polypeptide. In this site, Q_B^- is tightly bound and stable. After a second reduction and protonation, the resulting quinol (Q_BH_2) is released and is rapidly replaced by an oxidized quinone of the plastoquinone pool (Velthuys, 1981; Wraight, 1981). Four successive photoreactions, resulting in a storage of four positive charges, are required to oxidize two water molecules to one oxygen molecule (Joliot et al., 1969; Kok et al., 1970). Consequently, the oxygen-evolving complex cycles through five redox states, S_0–S_4 (Kok et al., 1970). The oxygen is released during the S_3 to S_0 transition in which S_4 is a transient state.

In addition to Tyr_Z, there is a second redox-active tyrosine in PS-II, Tyr_D, the tyrosine 160 of the D_2 polypeptide [Debus et al., 1988a; Vermaas et al., 1988; see also Barry and Babcock (1987)]. In the dark, Tyr_D^+ slowly oxidizes the S_0-state (Styring & Rutherford, 1987) and Tyr_D reduces the S_2- and S_3-states (Vass & Styring, 1991, and references cited therein). The physiological relevance of these processes is unknown.

Site-directed mutagenesis has begun to play an active role in the study of PS-II. It allowed the identification of the Tyr-160 in D_2 (Debus et al., 1988a; Vermaas et al., 1988) and the Tyr-161 in D_1 (Debus et al., 1988b; Metz et al., 1989) as Tyr_D and Tyr_Z, respectively. Several groups have also con-

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[1] Abbreviations: D_1 and D_2, polypeptides of the photosystem II (PS-II) reaction center; P_{680}, reaction center chlorophyll (Chl) of PS-II; P_{700} reaction center chlorophyll of photosystem I (PS-I); Q_A and Q_B, primary and secondary quinone electron acceptor; Ph, pheophytin, the intermediary electron acceptor between P_{680} and Q_A; Tyr_D, the tyrosine 160 of D_2, a side-path electron donor of PS-II; Tyr_Z, the tyrosine 161 of D_1, the electron donor to P_{680}; DCBQ, 2,5-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); PPBQ, phenyl-*p*-benzoquinone; DM, dodecyl maltoside; OG, octyl glucoside; EPR, electron paramagnetic resonance.

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structed mutations in D_1 and D_2 in order to study Q_A binding and to identify the Mn ligands [e.g., Diner et al. (1990) and Vermaas et al. (1990a,b)]. Most mutagenesis studies have involved the cyanobacterium *Synechocystis* 6803. This cyanobacterium is a facultative heterotroph, which can be propagated in the absence of functional PS-II when glucose is added to the media. Moreover, this cyanobacterium is transformable and incorporates DNA into its genome by homologous recombination (Grigorieva & Shestakov, 1982). Thus, *Synechocystis* 6803 is a suitable organism for the study of PS-II mutants.

The characterization of the site-directed mutants and the assignments of amino acid residues associated with redox-active components require spectroscopic analysis. Such analysis has been impeded by the lack of active PS-II preparations of *Synechocystis* 6803. Recently, two oxygen-evolving preparations from wild-type cells were reported (Burnap et al., 1989; Noren et al., 1991a). The preparation of Burnap et al. (1989) exhibited an oxygen-evolving activity on a chlorophyll basis which was twice that of the thylakoids although the PS-II was enriched by 10-fold. The particles of Noren et al. (1991a) retain more oxygen-evolving activity (5-fold over the thylakoids) and had very little contaminating PS-I (PS-I:PS-II approximately 0.07:1), but they used a relatively lengthy procedure, involving two different ion-exchange columns. A third preparation of PS-II particles was reported by Rögner et al. (1990); these particles were also of high purity but were inactive in oxygen evolution.

Here, we present a simple and rapid procedure to obtain a large quantity of oxygen-evolving PS-II-enriched particles of *Synechocystis* 6803. The procedure, which was based on the method of Burnap et al. (1989), takes only 10 h and involves a single purification step. The purified preparation exhibits a 5–6-fold increase of O_2-evolution activity over the thylakoids, and although the PS-I contamination (PS-I:PS-II 0.14:1) is higher than that recently reported by Noren et al. (1991a), it is nevertheless well-suited to spectroscopic studies such as low-temperature EPR. The preparation is shown to be applicable not only to wild-type cells but also to PS-II mutant cells. As test mutants we have used (1) a mutant lacking Tyr_D, i.e., the tyrosine 160 of the D_2 polypeptide was replaced by a phenylalanine (Vermaas et al., 1988), and (2) a mutant in which the Gly-215 of the D_2 polypeptide was replaced by a Trp (Gly-215 may be implicated in the binding of Q_A; Vermaas et al., 1990b).

Experimental Procedures

Growth Conditions. *Synechocystis* 6803 cells were grown in the mineral medium described by Herdman et al. (1973) with twice the concentration of nitrate. The medium was supplemented with 5 mM glucose for the growth of the mutants. The cells were grown in a rotary shaker at 34 °C in a CO_2-enriched atmosphere under illumination from fluorescent lights of about 70 $\mu E\ m^{-2}\ s^{-1}$.

Preparation of Thylakoid Membranes. Cells from a culture (about 6 L) reaching an OD of 1.5–2 at 580 nm (measured with a Shimadzu UV 160 spectrophotometer) were harvested by centrifugation at 9000 rpm for 15 min in a JA-10 Beckman rotor (≈9000*g*) at 4 °C. The pellets were resuspended in 20 mM MES–NaOH (pH 6.35)/5 mM $CaCl_2$/5 mM $MgCl_2$/25% glycerol (v/v) (buffer A) and then pelleted again by centrifugation. The cells were resuspended in 40–60 mL (final volume) of buffer A containing 50 μg/mL DNase, 1 mM caproic acid, 1 mM benzamidine, and 1 mM PMSF. The cells were added to a beadbeater chamber (100 mL) (Bio-spec Products, Oklahoma). The chamber was then filled with glass beads of 0.1-mm diameter and was incubated on ice for 60–90 min in the dark. The cells were broken in darkness with 8–10 cycles; a cycle consisted of a 20-s "on-phase" and a 5-min "off phase". The beadbeater chamber was cooled by an ice/water mixture in the outer jacket. The cellular material was separated from the beads by decantation, and the beads were washed 4–5 times with a total of about 200 mL of buffer A. Then, the cellular material was centrifuged for 5 min at 5000 rpm in a JA-20 Beckman rotor (≈2000*g*) to pellet unbroken cells and residual beads. For mutants, centrifugation at ≈ 1000*g* was used. The membranes fragments in the supernatant were pelleted by centrifugation at 48 000 rpm for 20 min in a 70 Ti Beckman rotor (179000*g*). The pellet was resuspended in buffer B, which was identical to buffer A except that it contained 20 mM $CaCl_2$ rather than 5 mM $CaCl_2$, and pelleted again once or twice. These additional washings remove the phycobiliproteins from the membranes. The pelleted thylakoids were resuspended in buffer B to give a chlorophyll concentration of about 1.2–1.5 mg/mL and were used immediately.

Preparation of the PS-II-Enriched Particles. Thylakoid membranes were solubilized with dodecyl maltoside (DM) and octyl glucoside (OG) (both from Sigma). A total of 150 μL of DM at 50 mg/mL was added to 5.55 mL of the sample (1.1 mg of Chl/mL). Then, after gentle mixing (about 30 s), 300 μL of OG at 100 mg/mL was added, and the solution was mixed again as before. The final volume was 6 mL, and the detergent to Chl ratio was 1.25 DM:5 OG:1 Chl (w/w). The mixture was then centrifuged at 48 000 rpm in a 50 Ti Beckman rotor (164000*g*) for 50 min at 4 °C. The supernatant, which was highly enriched in PS-II, was carefully removed. The PS-II-enriched fraction (approximately 5 mL) was precipitated by adding 2 mL of 25% poly(ethylene glycol) (PEG-6000), dissolved in buffer B, followed by centrifugation at 49 000 rpm for 20 min in a 50 Ti Beckman rotor (171000*g*). The pellet was resuspended in a minimal quantity of buffer B for immediate assay or frozen in liquid nitrogen. All steps of the preparation were done at 4 °C under dim light and when possible in darkness. The final preparation contained between 5 and 8% of the initial chlorophyll of the thylakoids.

PS-II enriched membranes from spinach were prepared as in Boussac and Rutherford (1988). Note that unlike the PS-II preparation from *Synechocystis*, PS-II-enriched membranes from spinach contain light-harvesting chlorophyll proteins.

Oxygen-Evolution Measurements. Oxygen evolution was measured at 25 °C by polarography using a Clark-type oxygen electrode with saturating white light. Oxygen evolution by thylakoids (6–10 μg of Chl/mL) or PS-II-enriched particles (1.5–3 μg of Chl/mL) was measured in buffer B in the presence of 1 mM DCBQ, an artificial electron acceptor. Oxygen evolution by whole cells (8–15 μg of Chl/mL; for the cells this was estimated from the OD at 580 nm) was measured in the growth medium in the absence or the presence of 2 mM DCBQ.

SDS–Polyacrylamide Gel Electrophoresis. The polypeptide composition of the PS-II particles and thylakoids was analyzed by SDS–PAGE after denaturation by incubation in 2% SDS sample buffer at 37 °C for 2 h using the method of Laemmli (1970). The resolving gel contained 4 M urea. The gel was stained with Coomassie blue.

Chlorophyll Assays. Chlorophyll *a* content was determined in methanol. The extinction coefficient at 665.5 nm (79.24 mL/mg of Chl cm^{-1}) was taken from Lichtenthaler (1987).

Thermoluminescence Measurements. Thermoluminescence was measured in a home-built apparatus as described by

Kirilovsky et al. (1991). For measurements of the $S_2Q_B^-$ band, the samples were incubated in the dark for 5 min and then a flash was given at −10 °C and the sample was frozen rapidly in liquid nitrogen. The thermoluminescence signal observed upon heating the sample was analyzed as described earlier (Kirilovsky et al., 1991).

Optical Assays. P_{700}^+ and P_{680}^+ were measured by flash-induced absorption changes at 820 nm in the microsecond time range. The measuring beam, provided by a tungsten–halogen lamp, was monochromated by interference filters between the light source and between the cuvette and the photodetector. The PS-II particles were used at 4 μg of Chl/mL. Excitation was provided by a (Nd–YAG)-pumped dye laser (broadband emission around 600 nm, pulse energy = 15 mJ, duration = 15 ns). The repetition rate was 1 Hz. All flashes were saturating. A silicium photodiode was used as a detector, and the output signal was filtered (10 Hz–1 MHz) and amplified before being recorded and digitized (5RTD710A from Tektronix). The extinction coefficients at 820 nm for P_{700}^+ and P_{680}^+ were taken as 6500 M^{-1} cm^{-1} and 7000 M^{-1} cm^{-1}, respectively (Mathis & Sêtif, 1981). P_{680}^+ was measured in the presence of ferricyanide, in Tris-washed samples. In these conditions, the donor side of PS-II is inhibited and the reduction of P_{680}^+ occurs in the microsecond time range (Conjeaud & Mathis, 1981).

EPR Measurements. For low-temperature EPR measurements, the samples were put into calibrated quartz tubes at about 1 mg of Chl/mL and dark-adapted for 30–60 min in ice. EPR spectra were recorded at helium temperatures with a Bruker ESR 200 X-band spectrometer equipped with an ER 035 M Bruker NMR gaussmeter and a 9350 B HP microwave frequency counter. The spectrometer was also equipped with an Oxford Instruments cryostat. Illumination of the samples was performed at different temperatures with the EPR tube submerged in one of the following baths: (1) in a stirred ice/water mixture for 0 °C illumination; (2) in a CO_2/ethanol bath for 198 K illumination; (3) in liquid nitrogen for 77 K illumination. In these three cases, a nonsilvered dewar was used. Illuminations at 77 K were usually followed by a dark incubation of several minutes at 198 K. This was done in order to allow P_{700}^+ to be reoxidized by an electron from the photoreduced electron acceptors in PS-I. For helium temperature illumination, the EPR sample was illuminated in the EPR cavity. For Sr^{2+} reconstitution, the PS-II particles were washed in buffer B with 50 μM EGTA instead of 20 mM Ca^{2+}. Then, the PS-II particles were resuspended in medium B with 20 mM $SrCl_2$ instead of $CaCl_2$. For Ca^{2+} depletion, the PS-II particles were washed and resuspended in buffer B with 500 μM EGTA instead of $CaCl_2$. For the NH_3 treatment, the PS-II particles were washed and resuspended in buffer B with 5 mM MES instead of 20 mM. Before freezing the sample, a small volume of a medium containing 1 M NH_4Cl and 1 M Hepes, pH 7.5, was added into the EPR tube to obtain a final concentration of 100 mM NH_4Cl and a final pH close to 7.5. The higher pH favors formation of NH_3, the reactive species. The short incubation at high pH alone has no marked effect on the EPR signal observed before annealing of the sample (not shown).

For measurements of the triplet state, the sample was prereduced essentially as described previously (van Mieghem et al., 1989) but with the minor changes described in the legend of Figure 4. Orientation of the PS-II particles was done by partial drying in darkness at 4 °C for approximately 24 h on Mylar strips in a 80% humidity/argon atmosphere. The samples used for the orientation experiment were prereduced by a treatment with dithionite and benzyl viologen and then washed and resuspended in 5 mM MOPS, pH 7.0/5 mM ascorbate/1 mM EDTA for drying [see van Mieghem et al. (1991)].

Fluorescence Spectra. Fluorescence emission spectra at 77 K were performed using a homemade fluorometer. For excitation, light from a tungsten–halogen lamp was filtered through broadband blue filters (390–480 nm) before reaching the sample. The emission was recorded through a motor-driven monochromator (Jobin-Yvon, M10, with slit equal to 1 nm), with a low-wavelength cut-off filter (640 nm). The fluorescence was detected with a red-extended (S20) photomultiplier.

Results and Discussion

Preparation of PS-II-Enriched Particles. The thylakoid preparation was similar to that described by Burnap et al. (1989) except that some changes were introduced to simplify and improve the method. During the course of the preparation, we used only two media which differed only in the $CaCl_2$ concentration. It was important to use 5 mM $CaCl_2$ until the unbroken cells were separated from the thylakoids. A higher concentration of Ca^{2+} caused the precipitation of both thylakoids and cells at a low speed of centrifugation. The thylakoids were resuspended in the presence of 20 mM $CaCl_2$ in order to stabilize the PS-II oxygen-evolution activity. The replacement of the 1 M sucrose, as used by Burnap et al. (1989), in the breaking buffer by 25% glycerol had no influence on the activity. The oxygen-evolving activity of the PS-II-enriched particles was increased by lowering the pH of the solution from 6.8, as used by Burnap et al. (1989), to 6.35.

The ratio of 1.25 mg of DM:5 mg of OG:1 mg of Chl corresponded to the best combination of the detergents to obtain the highest O_2-evolution activity and the best separation of PS-II from PS-I in wild-type cells. With wild-type cells, higher concentrations of any of the detergents resulted in a smaller PS-II to PS-I ratio and a lower PS-II activity. Note that the important factor seems to be the OG:Chl ratio. The OG concentration used is lower than that used by Burnap et al. (1989) (8 mg of OG:1 mg of Chl); however, it seems that this ratio must be optimized for specific cell culture conditions and for individual mutants (e.g., we used 1.25 DM:4 OG:1 Chl for the Y160F mutant; see below). Differences between detergent batches may also result in the optimal OG:Chl ratio being higher or lower than those given here.

After the membranes had been exposed to the detergents, dilution of the sample was avoided since sample dilution led to a marked loss of activity. After precipitation of the PS-II particles by PEG and their collection by centrifugation, each subsequent washing step resulted in a 10% loss in activity.

The whole procedure, from the cells to the PS-II-enriched particles, was done in 10 h. When the preparation was done over 2 days, a reduced activity was obtained. Freezing of the thylakoids (−80 °C in breaking buffer) or overnight storage of cells or the thylakoids at 0 °C in the dark resulted in only a 10% loss in activity measured in the thylakoids. However, the PS-II particles made from such thylakoids showed a disproportionate loss in activity; e.g., freezing of the thylakoids led to a 40% loss in activity measured in the PS-II particles.

Polypeptide Composition. Figure 1 shows the polypeptide composition of the PS-II particles isolated from cells of wild type (lane 2) and Y160F (lane 4). The two prominent bands at 54 and 52 kDa are probably the CF1 α and β subunits which are also contaminants in other cyanobacterial PS-II preparations [e.g., Bowes et al. (1983) and Noren et al. (1991a)]. The prominent band at approximately 60 kDa in the thylakoids polypeptide pattern (lane 5) corresponds to the

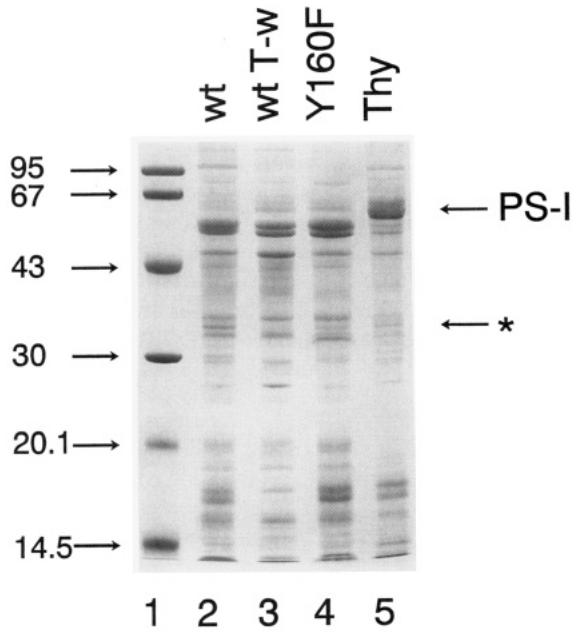

FIGURE 1: Polypeptide patterns on SDS–polyacrylamide electrophoresis gels of oxygen-evolving (lane 2) and Tris-washed PS-II particles (lane 3) from wild-type cells. Also shown are oxygen-evolving PS-II particles from the Y160F D_2 mutant (lane 4) and wild-type thylakoids (lane 5). Lane 1 shows a gel of standards with known molecular mass. The asterisk indicates the 33-kDa extrinsic polypeptide. Gel loadings were done on a chlorophyll basis: 1 μg of Chl/lane for PS-II particles, and 1.5 μg of Chl/lane for thylakoids.

PSI reaction center apoproteins. Only traces of this band were observed in the pattern of the PS-II particles of wild type (lane 2) although it is a little more marked in the Y160F mutant (lane 4). The polypeptide pattern in the particles of Y160F is generally very similar to that of the wild type; however, we note some slight differences around the 36–32-kDa region where the relative staining of the bands is different and an extra band at lower molecular mass is resolved. Tris washing of the PS-II membranes from plants removes the three extrinsic proteins of 17, 23, and 33 kDa [reviewed in Ghanotakis and Yocum (1990)]. Tris washing of the *Synechocystis* PS-II particles (Tris-HCl, 0.8 M, pH 8.5, 30 min, 4 °C) resulted in the removal of the 33-kDa extrinsic protein (lane 3). Cyanobacterial PS-II seems to lack the other two extrinsic proteins (Stewart et al., 1985). We noted that the 33-kDa protein was absent in our early preparations that did not show oxygen evolution. Three other polypeptides were removed by the Tris wash, having apparent molecular masses of 95, 30, and 17 kDa. These polypeptides may be components of phycobilisomes as shown by gel electrophoresis of isolated phycobilisomes [data not shown, but see Elmorjani et al. (1986)]. The gel pattern and the effect of Tris washing are similar to those found by Noren et al. (1991) for a similar PS-II preparation.

PS-II:PS-I Ratio. Illumination of thylakoids at 430 nm at 77 K gave rise to the fluorescence spectra shown in Figure 2A. The spectra exhibit a peak at 720 nm, attributed to PS-I, and two peaks at 696 nm and 685 nm, attributed to PS-II. The terminal energy acceptor of the phycobilisome also contributes to the emission at 685 nm [for a review, see Fork and Mohanty (1986)]. Comparison of spectra from wild-type (solid line) and from Y160F D_2 mutant (dotted line) thylakoids indicates that the mutant has a PS-II content which is about half that of the wild-type (Figure 2A).

The 77 K fluorescence spectrum of the PS-II-enriched particles from wild-type cells has major peaks at 685 nm and 696 nm and a minor peak at 720 nm (Figure 2B). Using the

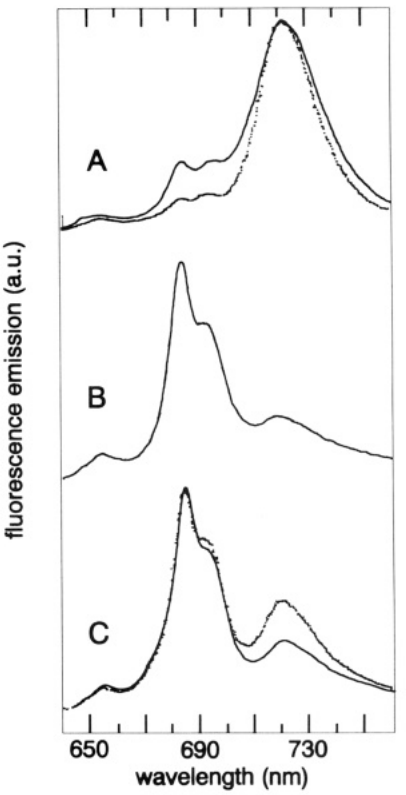

FIGURE 2: Fluorescence emission spectra at 77 K. Panel A: Thylakoids isolated from wild-type cells (solid line) and thylakoids isolated from Y160F mutant cells (dotted line). Panel B: PS-II-enriched particles from the wild-type cells. Panel C: PS-II-enriched particles from the Y160F mutant cells. For the wild-type (panel B), 5 of mg OG/1 mg of Chl has been used in the solubilization. For the mutant (panel C), two concentrations of OG were used in the solubilization: 5 mg of OG/1 mg of Chl (dotted line) and 4 mg of OG/1 mg of Chl (solid line).

same detergent to Chl ratio to isolate the PS-II particles from the Y160F mutant as for the wild-type resulted in a relative increase of the fluorescence at 720 mm (Figure 2C, dotted line). The decrease of the OG to Chl ratio from 5 to 4 (w/w) resulted in a better PS-II purity for the mutant (Figure 2C, solid line). Insufficient washing of the thylakoids to remove phycobilisomes resulted in an increase of the 685-nm peak relative to the 696-nm peak.

More precise estimates of the number of chlorophylls associated with PS-II were made by flash absorption–change spectrometry. With the same preparation as used in Figure 2B, there were 77 Chl/P_{680} and 570 Chl/P_{700}. Considering that in thylakoids there are approximately 120 Chl/P_{700} and 1000 Chl/P_{680} (Vermaas et al., 1990a,b), the ratio PS-I to PS-II diminished from 8.5:1 in the thylakoids to 0.14:1 in the particles. The enrichment of PS-II is consistent with the diminution of PS-I and its reaction center associated antenna chlorophylls. It is of note that the amount of PS-I contamination varied from preparation to preparation and determinations were also made on material having a PS-I:PS-II ratio of approximately 0.33:1.

Oxygen Evolution. The oxygen-evolution activity at 25 °C of wild-type cells of thylakoids and of PS-II-enriched particles is shown in Table I. DCMU (2×10^{-5} M) inhibited 85–90% of the activity in the particles. The PS-II preparation maintained the same activity after an overnight incubation in ice. However, freezing and thawing of the PS-II-enriched membranes caused the loss of about 30% of the total activity, and only 50% of the remaining activity was sensitive to 2×10^{-5} M DCMU (data not shown).

Table I: Oxygen-Evolving Activity in Cells, Thylakoids, and PS-II Particles

	oxygen evolution (μmol of O_2/(mg of Chl·h))	
	wild type	Y160F mutant
cells		
+DCBQ[a]	350	240
-DCBQ	110–160	100–140
thylakoids[b]	180–240	100–110
PS-II particles[b]	1000–1300	350–400

[a] 2 mM DCBQ. [b] 1 mM DCBQ.

The PS-II-enriched particles were active only in the presence of Ca^{2+} as previously observed by Burnap et al. (1989) and Noren et al. (1991a). The oxygen-evolution activity was totally inhibited after washing of the preparation with a solution lacking Ca^{2+}. Some of this activity recovered upon addition of Ca^{2+} or Sr^{2+}. The reconstituted activity with Sr^{2+} was only 40% of that seen when reconstitution was done with Ca^{2+} [data not shown, see Pistorius (1983)]. A similar situation occurs in plants (Ghanotakis et al., 1984); however, with the *Synechocystis* preparation, the washing procedures required for Ca^{2+} depletion resulted in a greater proportion of irreversibly inhibited centers (up to 50%).

To some extent, the oxygen-evolution activity in the Y160F mutant reflects the lower PS-II content than in the wild-type (see Figure 2). Moreover, it seems more sensitive than the wild type to photodamage as manifest by a rapid fall of the rate of O_2 evolution under continuous illumination (data not shown). Greater sensitivity to photoinhibition was recently reported in this mutant (Noren et al., 1991b). This may partly explain the lower oxygen-evolution activity detected in the PS-II-purified particles. The lower activity in the mutant seems to correlate to a lower relative amount of the 33-kDa protein and a greater contamination of PS-I as detected in the SDS gel (Figure 1). Nevertheless, the purified PS-II preparation exhibited a 4-fold increase of O_2-evolving activity over the thylakoids in the Y160F mutant and a 5–6-fold increase in the wild type.

Thermoluminescence. After a flash is given at low temperature, PS-II samples show a luminescence emission upon progressive heating as charge recombination occurs. Thermoluminescence measurements made after a single flash enable the detection and the relative quantitation of the $S_2Q_A^-$ and of the $S_2Q_B^-$ states (Rutherford et al., 1982). The $S_2Q_B^-$ luminescence was present in the PS-II particles (Figure 3B), confirming the presence of Q_B in its site. The $S_2Q_B^-$ band could be fitted by using one component in the simulation procedure. The fitting parameters were similar to those found in thylakoids (Figure 3A). A small $S_2Q_A^-$ band at (0–5 °C in Figure 2B) may be present arising from centers lacking functional Q_B as expected from the results on DCMU sensitivity described above. On comparing the $S_2Q_B^-$ band of the thylakoids (Figure 3A) and of the PS-II particles (Figure 3B), a slight shift of the maximum of the thermoluminescence peak to lower temperatures is observed (41 °C in thylakoids, 36 °C in PS-II particles). This indicates a minor change in the stability of the $S_2Q_B^-$ charge pair probably due to the detergent treatment. In samples which were frozen and thawed, a significant $S_2Q_A^-$ band (at 5 °C) was observed, indicating that some PS-II centers had lost functional Q_B (Figure 3C). The sensitivity of the Q_B site to freezing matches the observation on decreased DCMU sensitivity described in the previous section.

EPR Characterization of the PS-II-Enriched Particles. EPR spectroscopy has provided important information relevant

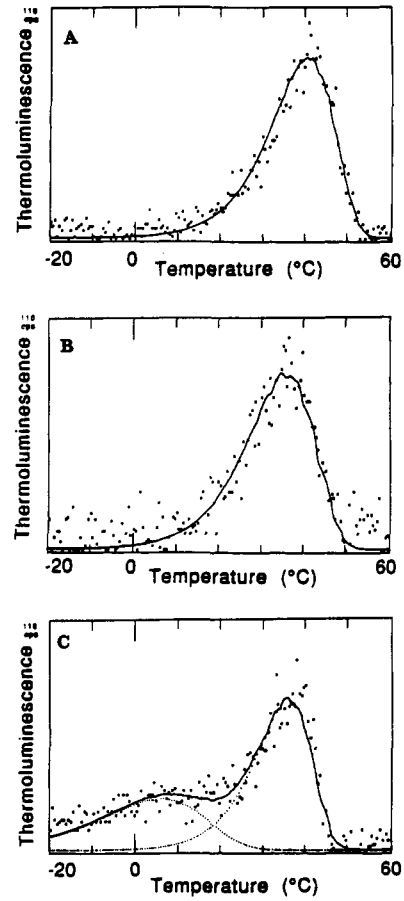

FIGURE 3: Thermoluminescence emission ($S_2Q_B^-$ band). After a flash has been given at −10 °C, samples were rapidly cooled in liquid nitrogen and then progressively heated (0.5 °C/s) up to 80 °C. Panel A: Thylakoids isolated from wild-type cells. Panel B: PS-II particles isolated from wild-type cells. The measurements were done immediately following the purification procedure. Panel C: PS-II particles isolated from wild-type cells which had been frozen and thawed before the illumination. The thermoluminescence signal (points) and the simulation of the bands (solid line) were obtained as described in Kirilovsky et al. (1991) and using the equation described in the same article. In Panel C, we also show the simulation of the two components of the signal (dotted line). The parameters used for the simulations were as follows. Activation energies (E_A): panel A, 1.05 eV; panel B, 0.95 eV; panel C, 0.5 eV and 1.15 eV. Trap number: panel A, 3294; panel B, 3403; panel C, 1383 and 2446. Frequency factor: panel A, 1.5×10^{13} s^{-1}; panel B, 6×10^{11} s^{-1}; panel C, 1.5×10^5 s^{-1} and 1.29×10^{15} s^{-1}.

to the structure and redox states of the components undergoing oxidation or reduction in PS-II. EPR signals from PS-II components were already described in PS-II particles from higher plants [reviewed in Miller and Brudvig (1991); Rutherford et al., 1991]. Most of these signals were also observed in PS-II preparations from thermophilic cyanobacteria [e.g., Atkinson and Evans (1983) and McDermott et al. (1988)] and from *Anacystis nidulans* (Aasa et al., 1987). EPR signals characteristic of PS-II components were monitored in the new PS-II preparation from *Synechocystis* 6803 in order to assess its suitability as a material for future in-depth EPR studies.

Electron Donor Side Components. The oxidized tyrosine 160 of the D_2 protein gives rise to a dark-stable EPR signal, centered at $g = 2$, known as signal II slow, or Tyr_D^+ [reviewed in Barry and Babcock (1987)]. The signal recorded in dark-adapted PS-II-enriched particles was essentially identical to that observed in whole cells. PS-II-enriched particles isolated from the Y160F mutant lacked the Tyr_D^+ EPR signal as observed earlier in whole cells (Debus et al., 1988a; Vermaas et al., 1988). As expected, only a small signal was present with

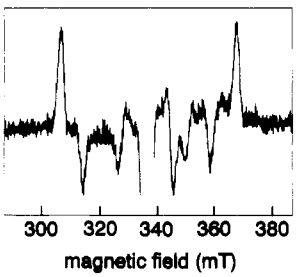

FIGURE 4: The triplet EPR signal in PS-II from *Synechocystis* 6803. The difference spectrum (spectrum recorded under illumination minus spectrum recorded in the dark after the illumination) is shown. Before illumination, the samples had been incubated for ≈30 min in the dark at room temperature in buffer B containing 40 mM Mops, pH 7.0, instead of 20 mM MES, pH 6.3, and 60% ethylene glycol, instead of glycerol. Also present were 1 mM EDTA, 20 mM sodium dithionite, and 0.1 mM benzyl viologen. Instrument settings: temperature, 4.2 K; microwave power, 63 μW; modulation amplitude, 25 G; microwave frequency, 9.44 GHz.

a *g*-value equal to 2.0025 and possibly due to a stable Chl^+ radical present in a small fraction of centers.

Illumination at the liquid helium temperature of PS-II centers incubated in the presence of dithionite and benzyl viologen gives rise to a triplet signal (van Mieghem et al., 1989) formed by charge recombination between P_{680}^+ and Ph^- (Rutherford et al., 1981). This signal is formed when Q_A is double-reduced prior to illumination (van Mieghem et al., 1989). The triplet signal can be observed in our preparation after treatment with dithionite and benzyl viologen (Figure 4), and its zero-field splitting parameters (D = 0.0287 cm^{-1}, E = 0.0044 cm^{-1}) are similar to those of the triplet state in PS-II preparations from higher plants (Rutherford et al., 1981). In addition, the orientation dependence of the triplet was similar to that observed in higher plants; i.e., the triplet z axis is oriented at approximately 60° to the membrane (data not shown; van Mieghem et al., 1991). No contamination from the reaction center triplet of PS-I was observed.

Two EPR signals have been attributed to the Mn complex in the S_2-state: the multiline signal (Dismukes & Siderer, 1981; Hansson & Andréasson, 1982; Brudvig et al., 1983) and the g = 4.1 signal (Casey & Sauer, 1984; Zimmermann & Rutherford, 1984). Spectrum b in Figure 5 shows the light-minus-dark spectrum recorded after a 198 K illumination of a dark-adapted PS-II preparation from wild-type *Synechocystis* 6803. Its shape is identical to that of the signal arising from the S_2-state of higher plants (spectrum a). Spectra a and b in Figure 5 were recorded in samples in which the PS-II concentration was adjusted to give the same level of the Tyr_D^+ signal. This corresponded to 1.1 mg of Chl/mL for the PS-II particles from *Synechocystis* 6803 and 4.5 mg of Chl/mL for the PS-II membranes isolated from spinach. Figure 5 shows that the amplitude of the multiline signal formed in PS-II isolated from *Synechocystis* 6803 was about 70% of that formed in PS-II membranes isolated from higher plants. The S_2-multiline signal in *Synechosystis* 6803 was recently reported (Noren et al., 1991a), but the present data represent a significant improvement in terms of resolution and noise.

During the course of this work, we noted a lack of correlation between the amplitude of S_2-multiline signal and the O_2-evolving activity. In early preparations, in which little oxygen evolution was observed, quite big S_2-multiline signals were observed. As improvements were made in the procedure, the activity improved without a proportional increase in the multiline EPR signal. The origin of this effect is unknown, but as the buffers used were essentially unchanged, the effect

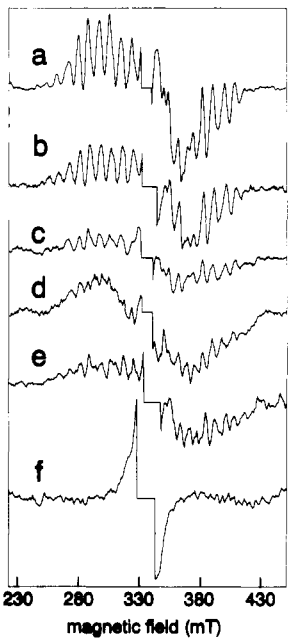

FIGURE 5: EPR signals from the S_2- and S_3-states in *Synechocystis* 6803. Spectra a, b, c, d and e are light-minus-dark spectra after illumination at 198 K given on dark-adapted PS-II isolated from: spinach, spectrum a; wild type of *Synechocystis* 6803, spectrum b; Tyr160Phe mutant of *Synechocystis* 6803, spectrum c; wild type of *Synechocystis* 6803 and treated with ammonia, spectrum d; wild-type of *Synechocystis* 6803 and reconstituted with Sr^{2+}, spectrum e. Spectrum f is the light-minus-dark spectrum after illumination at 0 °C given on dark-adapted PS-II isolated from wild type of *Synechocystis* 6803 and resuspended in the presence of EGTA. Due to the overlap with the large signal arising from Tyr_D^+ and/or Chl^+, the center parts of the spectra were deleted. Instrument settings: temperature, 10 K; modulation amplitude, 25 G; microwave power, 20 mW; microwave frequency, 9.44 GHz. Spectrum a contained 4.5 mg of Chl/mL; spectra b, c, d, e, and f, contained 1.1 mg of Chl/mL.

does not seem to involve Ca^{2+}, Cl^-, pH, etc. One possibility is that in some preparations the 33-kDa extrinsic polypeptide was partially released. This release is known to slow down oxygen evolution by a factor of 2–3 (Miyao et al., 1987).

Spectrum c in Figure 5 shows the light-minus-dark spectrum recorded after a 198 K illumination given on a dark-adapted PS-II preparation from the Y160F mutant of *Synechocystis* 6803. Amplitude of the multiline signal formed in the Y160F mutant corresponds to about 40% of that formed in the wild type on a per chlorophyll basis (spectrum b).

When NH_3, an inhibitor of oxygen evolution, interacts with PS-II in the S_2-state, the multiline EPR signal is modified (Beck et al., 1986). Upon addition of NH_3 to the PS-II-enriched membranes from *Synechocystis* 6803, the unmodified S_2-multiline signal could be observed immediately after illumination at 198 K (data not shown), but warming of the sample to 0 °C resulted in a modified signal shown in Figure 5d. The spectral modification and the temperature dependence of the ammonia effect are similar to those reported earlier in plants by Beck et al. (1986). The ammonia-modified S_2 has already been reported in the cyanobacterium *Anacystis nidulans* (Aasa et al., 1987).

Ca^{2+} is an obligatory cofactor in oxygen evolution [reviewed in Debus (1992); Rutherford et al., 1992; Yocum, 1991]. Its depletion inhibits charge accumulation at the S_3 to S_0 transition (Boussac et al., 1985; Boussac & Rutherford, 1988). It has been shown that the replacement of Ca^{2+} by Sr^{2+} resulted in a modification of the multiline EPR signal (Boussac & Rutherford, 1988). This was taken as spectroscopic evidence for a Ca^{2+} site close to the Mn cluster. Spectrum e in Figure

5 shows that replacement of Ca^{2+} by Sr^{2+} in PS-II of *Synechocystis* 6803 resulted in a modification of the multiline signal resulting in a smaller hyperfine spacing than in control sample as was observed in higher plants (Boussac & Rutherford, 1988).

Recently, an EPR signal has been observed in samples depleted of Ca^{2+} and illuminated at temperatures near 0 °C (Boussac et al., 1989, 1990b,c; Sivaraia et al., 1989; Ono & Inoue, 1990). This signal was interpreted as arising from the S_3-state and as resulting from the magnetic interaction between an oxidized histidine and the Mn cluster in the redox state that was present in the S_2-state (Boussac et al., 1989, 1990b,c). The S_3 signal could be also formed in the PS-II preparation from *Synechocystis* 6803 when it had been washed in a medium lacking Ca^{2+} and containing EGTA (Figure 5, spectrum f). In Ca^{2+}-depleted PS-II preparations from the plants, the width of the S_3 signal centered at $g = 2$ varied from 130 to 164 G in the absence or presence, respectively, of the extrinsic 17- and 23-kDa polypeptides (Boussac et al., 1990a). These polypeptides are absent in cyanobacteria (Stewart et al., 1985). Nevertheless, in *Synechocystis* 6803 the width of the S_3 signal appears to be less than 90 G (Figure 5, spectrum f). A similar 90-G-wide S_3 signal has been recently reported in Cl^--depleted PS-II membranes from plants (Boussac et al., 1992). The significance of this observation is under investigation.

In our conditions, we did not detect the $g = 4.1$ EPR signal. This is hardly surprising since (1) in plants, this signal cannot be observed after 200 K illumination in the presence of glycerol (Zimmermann & Rutherford, 1986); (2) the signal was not observed in other cyanobacteria (McDermott et al., 1988); (3) the $g = 4.1$ signal is absent in the PS-II preparations from higher plants when the 17- and 23-kDa extrinsic polypeptides are removed (dePaula et al., 1986) and cyanobacterial PS-II seems to lack both of these polypeptides (Stewart et al., 1985).

The similarities between the EPR spectra of the donor side components of *Synechocystis* 6803 and those of plants reinforce the idea that the oxygen-evolving enzyme functionally is essentially the same in both cases. This assertion extends to the role of Ca^{2+} since Sr^{2+} replacement and Ca^{2+} depletion also give lesions similar to those characterized in plants (both of these effects giving rise to EPR signals previously unreported in cyanobacteria). This conclusion is significant because the effects of Ca^{2+} depletion in cyanobacteria are usually considered to be different from those occurring in plants [e.g., Satoh and Katoh (1985), Debus (1991), and Yocum (1991)]. However, the present results are consistent with the recent proposal that the lesion caused by Ca^{2+} depletion is similar in both plants and cyanobacteria: i.e., a block of the $S_3Tyr_ZP_{680}{}^+$ to $S_3Tyr_Z{}^+P_{680}$ transition (Boussac et al., 1992).

Electron Acceptor Side Components. Q_A^- interacts magnetically with a non-heme Fe^{2+} ion. EPR signals at $g = 1.82$ (Nugent et al., 1981; Rutherford & Mathis, 1983) and $g = 1.90$ (Rutherford & Zimmermann, 1984) were attributed to the $Q_A^-Fe^{2+}$ interaction. Figure 6 (spectrum a) shows the $g = 1.9$ form of the $Q_A^-Fe^{2+}$ signal photoreduced in PS-II isolated from the wild type. In the presence of formate and DCMU, the $g = 1.82$ form was exclusively formed (Figure 6, spectrum c). Similar results have been seen in plants (Rutherford et al., 1984; Vermaas & Rutherford, 1984). The same signals could also be induced chemically by addition of dithionite (data not shown).

The Ph^- can also be detected by EPR. Magnetic interaction between Ph^- and $Q_A^-Fe^{2+}$ results in a signal that is split by approximately 30–50 G at $g = 2.0033$ (Klimov et al., 1980) depending on the pH (Rutherford & Zimmermann, 1984).

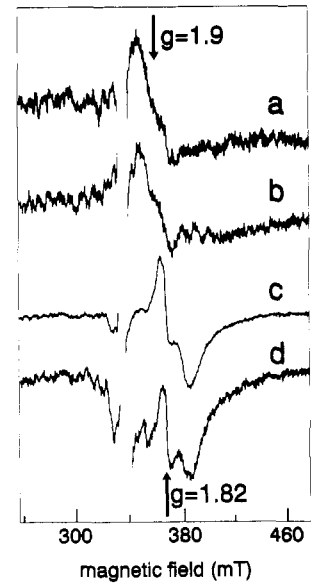

FIGURE 6: EPR signals from $Q_A^-Fe^{2+}$ in PS-II from *Synechocystis* 6803. Spectra a and c were recorded in PS-II isolated from the wild type and spectra b and d were from the G215W mutant. Amplitudes of the signals were approximately normalized to each other. Spectra a and b correspond to the light-minus-dark spectra after illumination at 77 K given on dark-adapted samples. The samples were previously incubated in buffer B at pH 6.8, instead of pH 6.3. The 0.1 mM PPBQ was at 0 °C. The samples were immediately frozen after illumination. The samples were previously incubated in buffer B at pH 6.8, and 50 mM sodium ascorbate, 1 mM EDTA, 2 mM DCMU, and 200 mM sodium formate were present. Instrument settings: temperature, 4.7 K; modulation amplitude, 22 G; microwave power, 32 mW; microwave frequency, 9.44 GHz.

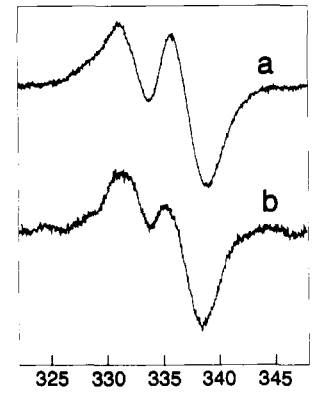

FIGURE 7: EPR signals from $Ph^-Q_A^-Fe^{2+}$ in PS-II from *Synechocystis* 6803. Spectra a and b correspond to the light-minus-dark spectra recorded after illumination at 77 K given on PS-II from the wild type (spectrum a) or from the G215W mutant (spectrum b). Amplitudes of the signals were normalized. Incubation of the samples was done in the same medium as in Figure 4 except that no benzyl viologen was added. Instrument settings: temperature, 4.5 K; modulation amplitude, 18 G; microwave power, 32 mW; microwave frequency, 9.44 GHz.

When PS-II particles from *Synechocystis* 6803 were chemically reduced in the presence of dithionite and were then illuminated at 198 K, the state $Ph^-Q_A^-Fe^{2+}$ was formed (Figure 7, spectrum a). The splitting was approximately 38 G.

We also investigated PS-II-enriched particles of the G215W mutant of *Synechocystis* 6803. This mutant has a modification in the D_2 protein which is expected to result in an alteration of the Q_A binding site (Vermaas et al., 1990b). An altered Q_A site might be manifest as modification in the signals from the $Q_A^-Fe^{2+}$ and $Ph^-Q_A^-Fe^{2+}$ states. Figures 6 (spectra b and

d) and 7 (spectrum b) show these signals from the PS-II preparation of the mutant. The signals from the G215W mutant showed a significantly lower amplitude compared to those of the wild type (approximately 2–4 times smaller on a Chl concentration basis). Amplitudes of the signals in the mutant were approximately normalized to those of the wild type in Figures 6 and 7. However, both the 1.9 and the 1.82 forms of the $Q_A^-Fe^{2+}$ signal in the mutant were similar in shape compared to those in the wild type. The shape and the splitting of the split signal were also similar in the wild type and in the mutant (Figure 7). Thus, the G215W mutation does not result in major spectral differences in the EPR spectra of the nearby electron-transfer components.

Conclusion

The modifications which we have introduced to the original preparation of Burnap et al. (1989) result in a preparation of oxygen-evolving PS-II particles that is useful for spectroscopic studies, not only being shorter and simpler but also providing higher yields and activities. The recently published preparation of Noren et al. (1991a) is purer with regard to PS-I contamination, but it is also lengthier. The Noren preparation also shows a higher O_2-evolution activity on a per chlorophyll basis; this is partly due to the smaller PS-I contamination, but also it is related to the fact that the cells used had higher O_2-evolution rates.

The catalog of EPR signals presented here demonstrates that the present preparation is well-suited for low-temperature EPR studies. At the same time, they emphasize the similarities between the PS-II in plants and cyanobacteria even at the level of the role of Ca^{2+}. In addition, we have found that our procedure is applicable to mutants which have site-directed changes in PS-II. This preparation should allow more rapid progress in the characterization of the large number of site-directed mutants already available.

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Synthesis and Biochemical Characterization of the New Sulfhydryl-Reactive ATP Analogue 8-Thiocyano-ATP. Its Interaction with Na,K-ATPase and Kinases†

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ABSTRACT: The synthesis of 8-thiocyano-ATP (CNS^8-ATP) is described. At 37 °C the ATP analogue inactivates Na,K-ATPase, hexokinase, and pyruvate kinase. In all three cases, inactivation can be prevented by the addition of ATP, thus indicating that CNS^8-ATP is recognized within the ATP binding site of the above enzymes. Incubation of the inactivated enzymes with dithiothreitol restores the catalytic activities. Therefore, it is likely that in these enzymes a mixed disulfide (E-S-S^8-ATP) is formed between a sulfhydryl in the ATP binding site (E-SH) and the ATP analogue:

$$\text{E-SH} + \text{CNS}^8\text{-ATP} \underset{K_D}{\leftrightarrow} [\text{E-SH}\cdot\text{CNS}^8\text{-ATP}] \xrightarrow[k_2]{} \text{E-S-S}^8\text{-ATP} + \text{HCN}$$

From the pseudo-first-order inactivation kinetics, a $K_D = 2.7\ \mu M$ with $k_2 = 0.142\ min^{-1}$ is calculated for the hexokinase and a $K_D = 40\ \mu M$ with $k_2 = 0.347\ min^{-1}$ is calculated for the pyruvate kinase interactions with the ATP analogue. At 4 °C, Na,K-ATPase recognizes CNS^8-ATP with a $K_D = 8.3\ \mu M$. At 37 °C, the enzyme becomes inactivated by the ATP analogue in a biphasic manner. Inactivation results in the incorporation of [α-^{32}P]8-CNS^8-ATP into the catalytic α-subunit of the enzyme. Limited tryptic digestion in the presence of 150 mM KCl results in the formation of a radioactive peptide of $M_r = 56\,000$, known to bear the purine binding domain of Na,K-ATPase. The results described in this article verify CNS^8-ATP as a sulfhydryl-reactive ATP analogue and characterize this new ATP analogue as a useful tool for structure/function studies on ATP-recognizing enzymes.

The catalytic α-subunit of Na,K-ATPase[1] contains 23 cyst(e)ine residues (Shull et al., 1985). At least one of them is thought to be localized within or very close to the ATP binding site of the enzyme (Skou, 1974; Patzelt-Wenczler et al., 1975; Jesaitis & Fortes, 1980; Schoner et al., 1987). This conclusion is based upon experiments performed with *N*-ethylmaleimide or sulfhydryl-reactive analogues of 6-

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[1] Abbreviations and enzymes: CNS^8-ATP, 8-thiocyanoadenosine 5′-triphosphate (8-thiocyano-ATP); 8-BrATP, 8-bromoadenosine 5′-triphosphate; 8-SH-ATP, 8-mercaptoadenosine 5′-triphosphate; 8-SH-ADP, 8-mercaptoadenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; SDS, sodium dodecyl sulfate; Na,K-ATPase, sodium and potassium activated adenosine 5′-triphosphatase (EC 3.6.1.37); hexokinase, ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1); pyruvate kinase, ATP:pyruvate 2-*O*-phosphotransferase (EC 2.7.1.40).

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