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Isolation and characterisation of oxygen evolving thylakoids from the marine prokaryote *Prochloron didemni*

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Abstract The present study describes the first successful attempt to isolate oxygen evolving thylakoids and thylakoid fragments from the marine prokaryote *Prochloron didemni*, a member of the recently discovered group of prochlorophytes. Oxygen evolving thylakoid membranes and fragments were isolated from seawater suspended cells of *Prochloron didemni* by passage of the cells through a Yeda press and subsequent differential centrifugation of the broken material. Three fractions were collected at 1000×*g*, 5000×*g*, and 30 000×*g* and identified by light microscopy as cells (and their fragments), thylakoids and membrane fragments, respectively. Pigment content, oxygen evolution rate and 77 K fluorescence spectra of these fractions were virtually identical. This finding indicates that the membrane fragments obtained are not enriched in photosystem II. The $P680^{+\bullet}$ reduction kinetics of thylakoid membrane fragments were determined by monitoring flash induced absorption changes at 830 nm and analysing the time course of their decay. The multiphasic relaxation kinetics and their modification by NH_2OH were found to be similar to those observed in cyanobacteria and plants. These findings provide an independent line of evidence for the idea of a high conservation of the basic structural and functional pattern of the water oxidising complex in all organisms that perform oxygenic photosynthesis.

Key words: *Prochloron*; Prochlorophyte; Oxygen evolution; Photosystem II; P680

1. Introduction

Oxygenic photosynthesis is a very ancient biological process dating back to nearly 3.5 billion years ago [1,2]. Little is known of the organisms which gave rise to this form of photosynthesis, although it is widely believed that they were cyanobacteria and that the evolution of these organisms involved the evolution of a photosystem (PS) II reaction centre core similar to that existing today [3–5]. PS II has been studied in a wide number of oxygenic photosynthetic organisms and with few exceptions is very similar: from the prokaryotic cyanobacteria to the chloroplasts of higher plants the essential components of the PS II core are identical [6–8]. The cyanobacteria were previously regarded as a unique homogeneous group of prokaryotic Eubacteria, that carried out oxygenic photosynthesis and contained chlorophyll (Chl) *a* and phycobiliproteins. The situation changed with the discovery of the prokaryotic oxygenic alga *Prochloron didemni* in 1975 [9,10]. This symbiotic prokaryotic alga was oxygenic but, in addition to Chl *a*, possessed Chl *b* and lacked phycobiliproteins. Since that time two other algae have been added to this group: *Prochlorothrix hollandica*, a freshwater filamentous alga [11], and *Prochlorococcus marinus*, a phytoplanktonic alga found in the world's oceans [12]. These algae have been placed in a phylum of their own, the Prochlorophyta [13]. However, phylogenetic tree reconstruction based on 16S rRNA and on the *rpoC* gene indicated that these organisms were unrelated and fell on different branches of the cyanobacterial clade [14,15]. While there has been some support for this proposal from the genes for the light harvesting complexes (LHCs) in these organisms [16,17], their PS II core has not been studied in detail.

An interesting question on the evolution of oxygen producing photosynthesising organisms is the development of PS II. Studies on the sensitivity to NH_2OH and the unusual hydrophobicity of the extrinsic PsbO protein led to the idea that the PS II donor side, where water oxidation to molecular oxygen takes place, differs in *Prochlorothrix hollandica* from that of cyanobacteria and higher plants [18,19]. This conclusion, however, is based on rather indirect evidence. It was recently shown that the multiphasic $P680^{+\bullet}$ reduction kinetics is a sensitive fingerprint of the reaction pattern of the PS II donor side (see [20,21] and references therein). Therefore this approach was used to address the above mentioned question.

2. Materials and methods

At present *Prochloron* cannot be cultured [13]. Colonies of the ascidian *Lissoclinum patella* containing the *Prochloron* cells as symbionts were collected in May 1998 from a depth of 2–4 m in the 'Blue pools' area of the Heron Island Reef (Great Barrier Reef, Australia). *Prochloron* cells were squeezed out of cut ascidian colonies into buffered seawater (0.1 M Tris, pH 9.2). The seawater was taken directly from Heron Island Reef and passed through a sterile filter prior to use. Contamination of the collected *Prochloron* cells with phycobilisome containing cyanobacteria was estimated to be less than 1% as judged from fluorescence microscopy. The cells were collected by low speed centrifugation (1000×*g*) and washed with buffered seawater (50 mM MOPS, pH 7.5). After another low speed centrifugation the collected cells were suspended in seawater (50 mM MOPS, pH 7.2, 300 mM sucrose) or aqueous buffer (50 mM MOPS, pH 7.2, 15 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose) and passed through a pre-chilled Yeda press at a pressure of about 100 MPa. The resulting Yeda press extracts are referred to as 'seawater' and 'water' samples, respectively. Unbroken cells and debris were removed from the Yeda press extracts

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Abbreviations: PS I, photosystem I; PS II, photosystem II; LHC, light harvesting complex; P680, primary donor of PS II; P700, primary donor of PS I; Y_Z, tyrosine 161 of the PS II D1 polypeptide; Chl, chlorophyll; WOC, water oxidising complex; BSA, bovine serum albumin; PPBQ, phenyl-*p*-benzoquinone; MOPS, 3-[*N*-morpholino]-propanesulphonic acid; Tris, tris-(hydroxymethyl)-amino methane

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by centrifugation with a GSA 3 rotor at 1000×*g* for 3 min at 4°C. The supernatant was rapidly frozen by dropping into liquid nitrogen and stored at −70°C until further use. All of the previously described steps were performed immediately after sample collection at the Heron Island Research Station (HIRS). Frozen *Prochloron* Yeda press extracts were transferred on dry ice to Sydney University for further sample treatment and analysis.

The samples were rapidly thawed and washed once with an aqueous buffer (10 mM MES, pH 6.5, 15 mM NaCl, 5 mM $MgCl_2$, 0.4 M sucrose, 0.2% BSA) and a second time with the same buffer but omitting BSA. The presence of BSA in the first washing step was required to minimise damage caused by protease activity. Thereafter the sample was homogenised and fractionated into three different subfractions of 1000×*g* (3 min, cells), 5000×*g* (5 min, thylakoid membranes), and 30 000×*g* (20 min, thylakoid membrane fragments) by means of centrifugation (JA-17 rotor, Beckman J2-21M/E centrifuge, 4°C).

2.1. PAM measurements

Photochemical quantum yield and electron transfer rates (ETR) of the cells were monitored by pulse-amplitude-modulated (PAM) fluorescence measurements with a Teaching-PAM fluorometer (Walz, Effeltrich, Germany). The cell suspension was dark adapted for 5 min prior to measurements. The relative quantum yield of photochemical energy conversion in PS II reaction centres was determined as the ratio F_v/F_m where $F_v = F_m - F_0$ with F_0 and F_m symbolising the fluorescence in the dark adapted state and after a saturating light pulse, respectively [22]. The ETR in units of μmol electrons m^{-2} s^{-1} was estimated from the effective PS II quantum yield as follows: $ETR(t) = \Delta F(t)/F_m'(t) \times c \times PAR$, where PAR denotes the quantum flux density of photosynthetically active radiation in μE m^{-2} s^{-1}, $\Delta F(t) = F_m'(t) - F(t)$ and c is an efficiency coefficient that was set at 0.42 in analogy to green leaves. For further details regarding this technique see Schreiber et al. [23].

2.2. Absorption measurements

Absorption spectra of the samples and their acetonic extracts (90% acetone) were recorded on a Varian Cary 1 spectrophotometer. Chlorophyll *a*, *b*, and *c* contents were estimated from the absorption spectra of acetone (90%) extracted pigments using a trichromatic equation [24].

2.3. 77 K fluorescence measurements

77 K fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer. The samples were suspended in a measuring buffer that contained about 50% glycerol and had an absorption of less than 0.03 at 430 nm. Samples were excited at 434 nm and the fluorescence emission was recorded between 650 and 750 nm.

2.4. Oxygen evolution measurements

Oxygen evolution was measured with a Hansatech oxygen electrode. The 1 ml sample volume contained 40 μg Chl, 1 mM $K_3Fe(CN)_6$ and 200 μM phenyl-*p*-benzoquinone (PPBQ).

2.5. 830 nm flash induced absorption changes

Flash-induced absorption changes at 830 nm were monitored with 1 ns time resolution using equipment previously described in [25]. A sample concentration of 27 μg Chl/ml was used and 1.5 mM $K_3Fe(CN)_6$ was added as an external electron acceptor. For the hydroxylamine treatment, the sample was incubated with 2 mM hydroxylamine for 5 min immediately prior to measurement.

3. Results and discussion

3.1. Characterisation of cells

Freshly harvested *Prochloron* cells were spun down and resuspended in four different isolation buffers. After 5 min dark adaptation fluorescence induction curves were recorded with a Teaching-PAM. Fig. 1 shows typical fluorescence traces for *Prochloron* cells in buffered seawater (50 mM MOPS, pH 7.2) and an osmotically equivalent aqueous medium (800 mM betaine, 300 mM sucrose, 50 mM MOPS, pH 7.2). The values of photochemical quantum yield (F_v/F_m) and average ETR gathered from the fluorescence measurements of the four cell suspensions are compiled in Table 1. An inspection of the data reveals that the F_v/F_m ratio is almost insensitive to cell suspension in different buffers whereas the ETR values exhibit a pronounced dependence. The invariance of F_v/F_m indicates that the formation of the 'stable' radical pair $Q_A^-{}^{\bullet}Y_Z^{OX}$ remains unaffected. On the other hand, the linear electron transfer under multiple turnover conditions becomes severely impaired when seawater is replaced by an aqueous buffer medium of equivalent (800 mM betaine, 300 mM sucrose) or lower (400 mM sucrose) osmolarity. The deleterious effects of the water treatment were complete within 5 min (minimum dark adaptation period) and could not be reversed by resuspension in seawater. These findings agree with previous results which show that oxygen evolution is severely inhibited after suspension of *Prochloron* cells in isoosmolar aqueous buffer solution. This phenomenon has been explained by a diffusive loss of components that are essential

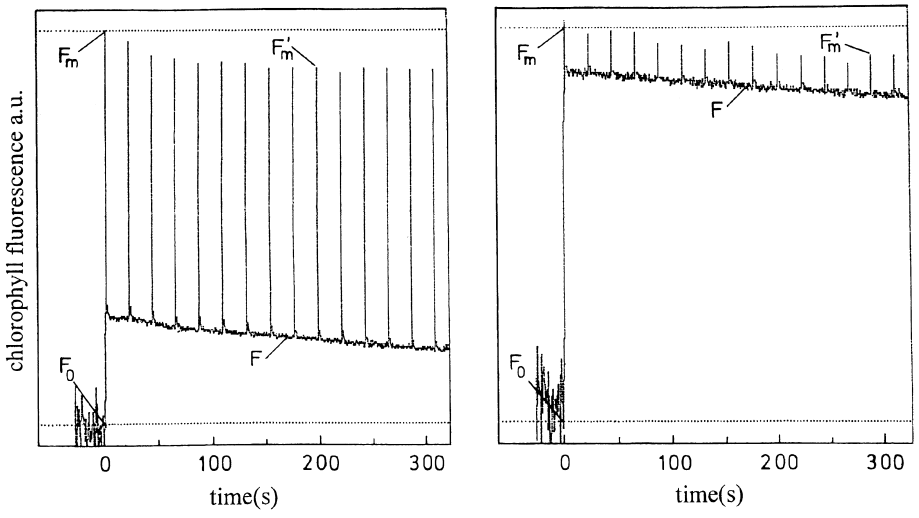

Fig. 1. Dark-light induction curves for *Prochloron* cells in seawater (left), and aqueous buffer+800 mM betaine+300 mM sucrose (right). The actinic light intensity (peak wavelength 660 nm) was 120 μmol quanta m^{-2} s^{-1}. The sweep time was 320 s.

Table 1
Average electron transfer rates (ETR) and F_v/F_m ratios for *Prochloron* cells determined in the indicated buffer media; all media contained 50 mM MOPS (pH 7.2)

Buffer medium	ETR (μM electrons m^{-2} s^{-1})	F_v/F_m
Seawater	28	0.76
800 mM betaine+300 mM sucrose	4	0.80
400 mM sucrose	6	0.79
Seawater+400 mM sucrose	14	0.80

for maintaining high ETR values and normally exchange between the cells and their host environment [26]. Metabolic soluble electron acceptors seem to be the most likely candidates. This idea is supported by the finding that addition of the artificial acceptors $K_3Fe(CN)_6$ and PPBQ leads to partial enhancement of the ETR values in aqueous buffer solution (data not shown).

For further characterisation, attempts were made to isolate thylakoids and thylakoid membrane fragments from *Prochloron*. A mechanical disruption by Yeda press treatment was applied to cells suspended either in seawater ('seawater sample') or in aqueous buffer ('water sample'). In both cases 400 mM sucrose was present as cryoprotectant.

3.2. Characterisation of Yeda press extracts

The oxygen evolution rates were measured under excitation with saturating light in the presence of $K_3Fe(CN)_6$ and PPBQ as artificial acceptors. Values of 135 ± 25 and 60 ± 15 μmol O_2 (mg Chl)$^{-1}$ h^{-1} were obtained for 'seawater' and 'water' samples, respectively. For fractionation of the raw material, thawed Yeda press extracts of both sample types were washed, homogenised and subjected to differential centrifugation. Three fractions were collected at $1000 \times g$, $5000 \times g$ and $30\,000 \times g$. Based on inspection by light microscopy these fractions were assigned to cells and cell fragments ($1000 \times g$ fraction), thylakoids ($5000 \times g$ fraction) and thylakoid membrane fragments ($30\,000 \times g$ fraction). The fractionation of the starting material exhibited a strikingly different pattern for 'seawater' and 'water' samples. In the former type more than 80% of the total chlorophyll content was found in the 5000 and $30000 \times g$ fractions and only less than 20% remained in the $1000 \times g$ fraction. In contrast, an entirely different distribution was obtained for the 'water' sample. In this case more than 95% of the total chlorophyll was collected in the $1000 \times g$ fraction.

The above mentioned results indicate that only 'seawater' samples can be used for the successful isolation of thylakoids and membrane fragments from *Prochloron*. Therefore the following investigations were performed exclusively with this material.

Fig. 2 shows in the top panel an overlay of the room temperature absorption spectra of acetonic extracts from *Prochloron* cells, thylakoids and thylakoid membrane fragments. The spectra are virtually the same for whole cells and the three Yeda press fractions. Evaluation of these spectra led to Chl *a*/*b* and Chl *a*/*c* ratios of 4.6 ± 0.1 and 30 ± 5, respectively. These values are somewhat larger than in a previous report [27]. This difference can be explained by the finding that pigmentation of *Prochloron* cells strongly depends on their natural illumination conditions [28].

The bottom panel of Fig. 2 shows that whole cells and thylakoid fragments of *Prochloron* exhibit identical fluorescence emission spectra at 77 K. The spectra are characterised by two peaks at 685 nm and 695 nm originating from PS II and a small shoulder of 715 nm. These spectral features are in agreement with the results of earlier studies [29]. One interesting consequence emerges from the lack of spectral differences: the mechanical disruption of cells and thylakoids by Yeda press does not lead to thylakoid membrane fragments with an enriched PS II content. This conclusion is highly supported by the finding that the oxygen evolution rates are the same within the experimental error for the three fractions of $1000 \times g$, $5000 \times g$ and $30\,000 \times g$. The values of 105 ± 15 μmol O_2 (mg Chl)$^{-1}$ h^{-1} obtained are somewhat below the activity of the raw Yeda press extract. These diminished rates probably originate from an activity loss of about 25% during the washing procedure.

With respect to the possibility of isolating PS II enriched membrane fragments, experiments were performed with detergents such as Triton X-100 and/or digitonin which are widely used to achieve this goal with thylakoids from higher plants. None of the attempts was successful. The failure to obtain PS II membrane fragments similar to those that were first isolated by Berthold et al. [30] is probably due to the poor thylakoid

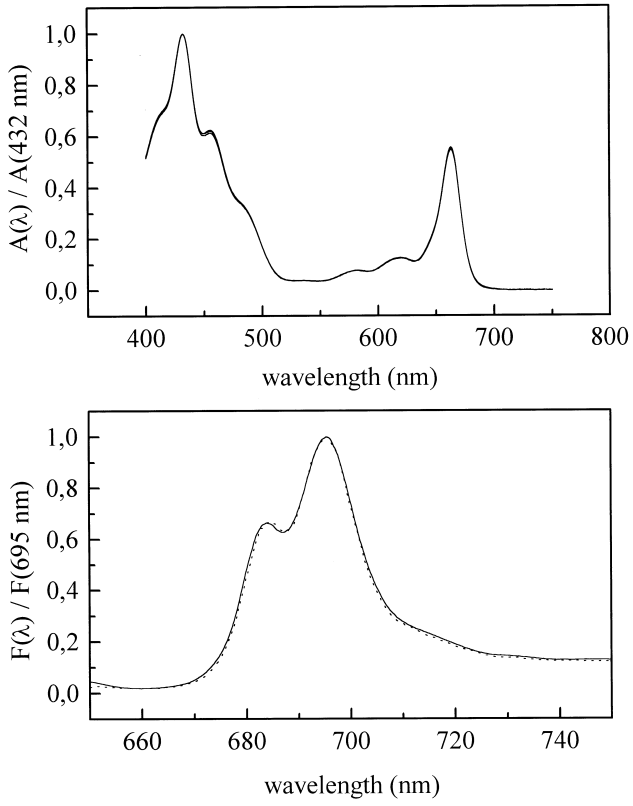

Fig. 2. Overlay of room temperature absorption spectra of acetonic extracts of *Prochloron* cells, thylakoids and thylakoid fragments normalised to the absorption maximum at 432 nm (upper panel) and 77 K fluorescence emission of *Prochloron* cells (solid line) and *Prochloron* thylakoid fragments (dotted line) normalised to the emission maximum at 695 nm. For further details see Materials and Methods, Section 2.

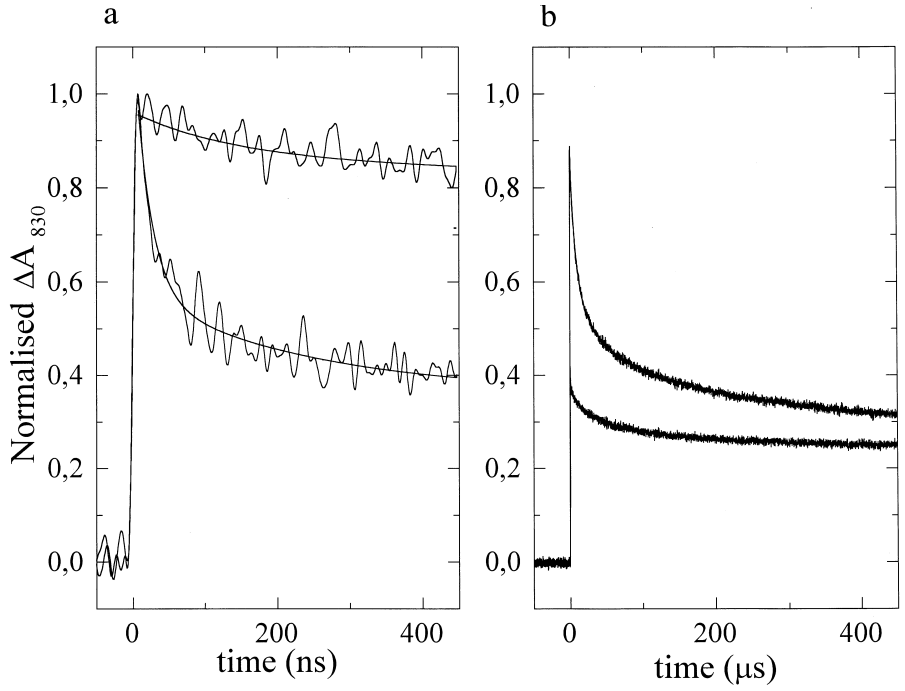

Fig. 3. Flash induced absorption changes at 830 nm in the nanosecond (a) and microsecond (b) time domains. In both panels the lower traces correspond to untreated *Prochloron* thylakoid fragments whereas the upper traces correspond to hydroxylamine treated *Prochloron* thylakoid fragments. Fifty signals were averaged per trace at a flash frequency of 2 Hz.

stacking between only two or three membrane layers in this species of *Prochloron* [31].

3.3. $P680^{+\bullet}$ reduction kinetics in membrane fragments from Prochloron

In order to optimise the scattering properties thylakoid membrane fragments ($30\,000\times g$ fraction) were used after passing through a 3 μm membrane filter. These samples contain both PS I and PS II, and therefore flash induced 830 nm absorption changes arise due to the turnover of both P680 and P700. However, a separation of these two different contributions can be achieved by the use of $K_3Fe(CN)_6$ at a sufficiently high concentration. In this case P700 becomes chemically oxidised [32] and reduction via linear electron transfer is comparatively slow. As a consequence, interference with the fast processes of $P680^{+\bullet}$ reduction is eliminated.

Fig. 3 shows in two different time domains traces of 830 nm changes induced by excitation with repetitive laser flashes. The traces on the left side were monitored with a high time resolution of 1 ns. The signals reveal that more than 50% of the flash induced absorption changes relax within 400 ns in oxygen evolving samples and that these fast kinetics are eliminated by addition of 2 mM NH_2OH. On the right side the traces are depicted in a 1000-fold wider time range. In this case the very fast relaxation of the untreated sample cannot be detected whereas the relaxation of the NH_2OH treated sample is well resolved. Furthermore, the absorption changes reach a similar level after about 500 μs. The remaining slow kinetics are predominantly ascribed to the turnover of P700 which is not affected by 2 mM NH_2OH. The general features of the signals in Fig. 3 closely resemble those measured in PS II membrane fragments from spinach [33,34] except for the significantly larger P700 contribution in the *Prochloron* samples. For a more detailed analysis the traces of both time domains were fitted by a multiphasic kinetics of five exponential decay components and a contribution that is nonrelaxing within a 1 ms sweep. The results obtained are compiled in Table 2. The relaxation kinetics of the flash induced 830 nm absorption of the untreated *Prochloron* thylakoid membrane fragments can be described by five components with lifetimes of 23 ns, 207 ns, 12 μs, 56 μs and 234 μs. These numbers and the values of the normalised amplitudes are in good agreement with those gathered from analogous measurements in PS II preparations from thermophilic cyanobacteria [35] and higher plants [25,33]. Likewise, the effect of 2 mM NH_2OH is very similar to that observed for PS II membrane fragments. The dominating 8 μs kinetics reflects the electron transfer from Y_Z to $P680^{+\bullet}$ in PS II complexes which are deprived of a functionally competent water oxidising complex (WOC) [36]. A significant extent of the 214 μs kinetics is indicative of a partial

Table 2
Result of a simultaneous fit of the traces of both time domains shown in Fig. 3 to five exponential decay components and a contribution that is nonrelaxing within a 1 ms sweep

Parameter	A_1	τ_1 (ns)	A_2	τ_2 (ns)	A_3	τ_3 (μs)	A_4	τ_4 (μs)	A_5	τ_5 (μs)	$A_{t>1ms}$
Untreated	0.39	23	0.21	207	0.03	12	0.06	56	0.04	234	0.24
NH_2OH treated	0.06	102	0.07	207	0.27	8	0.12	34	0.17	214	0.30

interruption of this electron transfer and its replacement by $P680^{+\bullet}Q_A^{-\bullet}$ recombination. This effect is well known from previous studies where the blockage of $P680^{+\bullet}$ reduction by Y_Z in NH_2OH treated samples was shown to originate from photoinhibition of the donor side [34,37]. After correction for the PS I contribution ($A_{t>1ms}$) the amount of $P680^{+\bullet}Q_A^{-\bullet}$ radical pair formation can be calculated from the amplitude of the flash induced absorption change at 830 nm. Based on a molar difference extinction coefficient of 6700 M^{-1} cm^{-1} at 830 nm [38] a value of 240 ± 25 is calculated for the ratio of chlorophyll molecules per $P680^{+\bullet}Q_A^{-\bullet}$ radical pair generated by a saturating laser flash.

4. Conclusion

The present study shows that oxygen evolving thylakoids and thylakoid membrane fragments can be isolated with high yield from seawater suspended *Prochloron* cells by Yeda press treatment and subsequent fractionation via differential centrifugation. On the other hand, no PS II enrichment in membrane fragments could be achieved by using detergents such as Triton X-100 and/or digitonin. This feature is most likely due to the lack of extended grana stacks and/or sufficient heterogeneity in the distribution of PS I and PS II. The thylakoid membrane fragments provided a suitable sample material for the first analysis of the $P680^{+\bullet}$ reduction pattern in *Prochloron*. A close similarity to the features of the corresponding reaction in cyanobacteria and higher plants was found. Since the multiphasic $P680^{+\bullet}$ kinetics provides a sensitive fingerprint for the reaction pattern of the PS II donor side [20,21] the present study highly supports the idea that the 'inner core' of PS II was highly conserved during evolution of oxygen evolving organisms [6] whereas the nature of regulating subunits underwent significant changes (see [39] and references therein).

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