The strontium inorganic mutant of the water oxidizing center (CaMn₄O₅) of PSII improves WOC efficiency but slows electron flux through the terminal acceptors

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Abstract

Herein we extend prior studies of biosynthetic strontium replacement of calcium in PSII-WOC core particles to characterize whole cells. Previous studies of Thermosynechococcus elongatus found a lower rate of light-saturated O₂ from isolated PSII-WOC(Sr) cores and 5–8 × slower rate of oxygen release. We find similar properties in whole cells, and show it is due to a 20% larger Arrhenius activation barrier for O₂ evolution. Cellular adaptation to the sluggish PSII-WOC(Sr) cycle occurs in which flux through the O₆Q₆ acceptor gate becomes limiting for turnover rate in vivo. Benzoquinone derivatives that bind to Q₆ site remove this kinetic chokepoint yielding 31% greater O₂ quantum yield (QY) of PSII-WOC(Sr) vs. PSII-WOC(Ca). QY and efficiency of the WOC(Sr) catalytic cycle are greatly improved at low light flux, due to fewer misses and backward transitions and 3-fold longer lifetime of the unstable S₃ state, attributed to greater thermodynamic stabilization of the WOC(Sr) relative to the photoactive tyrosine Y₅. More linear and less cyclic electron flow through PSII occurs per PSII-WOC(Sr). The organismal response to the more active PSII centers in Sr-grown cells at 45 °C is to lower the number of active PSII-WOC per Chl, producing comparable oxygen and energy per cell. We conclude that redox and protonic energy fluxes created by PSII are primary determinants for optimal growth rate of T. elongatus. We further conclude that the (Sr-favored) intermediate-spin S = 5/2 form of the S₂ state is the active form in the catalytic cycle relative to the low-spin S = 1/2 form.

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1. Introduction

Oxygenic photosynthesis is responsible for the vast majority of the Earth’s biomass [1]. Photosystem II (PSII) powers oxygenic photosynthesis by using light to drive the formation of a proton gradient and the chemical reduction of plastoquinone (PQ) by the net reaction:

2H₂O + PQ → O₂ + 2PQH₂ + 4H⁺ + 4(inside) → O₂ + 2PQH₂ + 4H⁺ + 4(inside).

PSII is highly conserved across a wide range of organisms that contribute to this global process, both in terms of the protein subunit composition and many specific residues comprising the active site of water oxidation, the metal-oxo cluster known as the water-oxidizing complex (WOC) [2]. The WOC is comprised of an inorganic cluster, Mn₄CaO₅, coordinated by conserved amino acid residues, that delivers electrons to the PSII reaction center via a photooxidizable tyrosine residue, Y₅. The WOC is universally found throughout organisms which rely on PSII. The only known inorganic alteration to this cluster which results in a catalytically active WOC is the replacement of calcium (Ca) by strontium (Sr) [3], although Sr-substitution has not yet been found in vivo.

Over thirty years ago it was first observed that substitution of strontium for calcium by reconstitution in the PSII-WOC partially reactivated oxygen evolution activity in isolates of both cyanobacteria [4] and higher plants [5]. Shortly thereafter, the site was localized to the Mn cluster [7,8]. Historically, the isolated PSII core with strontium and artificial electron acceptors has been studied to investigate the function of calcium relative to the WOC [5,7,9], its
relationship to S-state transition times [10], restructuring events [11, 12], and free energy gaps [13], and especially the effect of substitution on the overall rate of oxygen production [57,14,15].

The WOC inorganic cluster is unstable when removed from its protein scaffold. However, it can be reassembled from the individual elements in vitro, starting with the cofactor-depleted apo-WOC PSII complex, plus free Mn$^{2+}$, Ca$^{2+}$, and HCO$_3^-$ in the presence of light and electron acceptors. This in vitro process is called photoassembly and is believed to mimic biogenesis and repair in vivo [3,16–19]. Ca$^{2+}$ was shown to be essential for forming the functional WOC cluster by restricting the number of photooxidizable Mn$^{2+}$ to precisely 4.0 per site [20]. In the absence of Ca$^{2+}$, Mn$^{2+}$ photooxidation incorporates as many as 20 Mn$^{2+}$ and eventually forms an amorphous oxyhydroxydioxide polymer containing Mn$^{3+}$ and Mn$^{4+}$, [Mn$_{Ox}$(OH)$_y$]$_n$ with $y=20$, that is catalytically inactive [21,22]. This inactivity is due to suppression of formation of the catalytically active form of Mn. Therefore, Ca$^{2+}$ plays an indispensable role in templating the formation of the correct structure of the Mn$_{Ox}$WOC core.

Curiously, strontium replacement of calcium accelerates the rate of photoassembly of the WOC [23]. Sr$^{2+}$ was shown to bind selectively to the Ca$^{2+}$ effector site of spinach apo-WOC-PSII particles, causing a 5-fold acceleration of the rate of cluster photoassembly compared to Ca$^{2+}$, as measured by the recovery of O$_2$ evolution [23]. Mechanistically, it was found that this occurs because Sr$^{2+}$ is five times faster than Ca$^{2+}$ in accelerating the net flux through the first two sequential assembly steps: $k_1$ – the photo-oxidation of the first Mn$^{2+}$ – Mn$^{3+}$, and $k_2$ – the subsequent dark step in which Ca$^{2+}$ affinity increases by 10× and forms the first stable intermediate, Ca$^{2+}$([OH])$_2$Mn$^{4+}$. Thus, this second (dark) process is rate-limiting overall for recovery of O$_2$ evolution functionality. A protein conformational change was postulated to occur on the dark step, but not shown directly [10]. Deactivation by charge recombination from this intermediate was shown to be 2× slower in the presence of Sr$^{2+}$ than Ca$^{2+}$, and postulated to arise from its greater thermodynamic stability. The resulting photoassembled Sr-WOC-PSII has a 65% lower O$_2$ evolution yield per flash than Ca$^{2+}$ samples, which is also similar to the slower light-saturated O$_2$ evolution rate in the Sr-exchanged holo-enzyme, noted above [7]. No metal ion other than Sr$^{2+}$ has been found to functionally replace Ca$^{2+}$ in water oxidation. Neither Mg$^{2+}$ nor Ba$^{2+}$ bind to the Ca$^{2+}$ effector site, while VO$^{2+}$ and Cd$^{2+}$ do bind, but do not activate O$_2$ evolution activity.

The mechanism by which Sr creates these major functional changes and whether the influence it has extends also to controlling flux through the electron acceptor side of PSII remain unclear. However, detailed structural information has appeared recently which can contribute to this understanding. The crystal structures of PSII with calcium or strontium in the WOC (PDB IDs: 3WU2 and 4IL6, respectively) have been determined with relatively high resolution for Thermosynechococcus vulcanus (the nearest relative of our selected strain) [24,25]. A somewhat lower resolution structure of the native calcium PSII core from Thermosynechococcus elongatus is also available (PDB ID: 4V62) [26,27]. These data provide a structural platform for prediction of function and analysis of spectroscopic data. In particular, detectable structural shifts of the location of Sr$^{2+}$ closer to Y$_2$ may cause redox energy changes for the WOC (S-states) relative to Y$_2$. Specifically, since the WOC is closer to Y$_2$, the substitution can be expected to increase the electrical potential gradient and the electronic coupling, thereby facilitating forward electron transfer and slowing charge recombination between the acceptor and donor sides. This prediction is contrary to the observation of slower light saturated O$_2$ evolution. Redox potential shifts within the WOC across the S2/S3 transition and between Q$_b$ and Q$_a$ stemming from Sr$^{2+}$ substitution have previously been demonstrated [13].

In the present manuscript, we extend the earlier functional studies to examine for the first time the kinetics of electron/proton transfer in native Ca and Sr-substituted WOCs in vivo, within intact cells of T. elongatus. This approach, although more challenging, eliminates both alterations caused by biochemical isolation in detergents and the use of artificial electron acceptors essential for in vitro studies. Kinetic studies of WOC turnover can now be extended to intact cells by application of a Chi fluorescence method that allows detection of the WOC catalytic cycle [28–31]. Together, these methods allow precise determination of PSII turnover energetics and kinetics in its native environment. As such, we can examine the influence of the electron acceptor side on PSII flux from water oxidation and its potential regulation of WOC turnover.

2. Materials and methods

All cultures used were grown at 45 °C in replete or Sr-substituted (380 μM Ca or Sr) DTN medium [32] in volumes of 150 mL with bubbling of 1.0% CO$_2$-supplemented air under 40 μmol m$^{-2}$ s$^{-1}$ continuous light from Philips Silhouette cool white fluorescent lamp, following the method of Sugiura with modifications [33]. All cultures were derived from a Thermosynechococcus elongatus His-tagged CP-47 mutant strain provided by Dr. Sugiura (henceforth referred to as T. elongatus). Our method here varies from that of Sugiura in that T. elongatus is very light-sensitive and grown at below normal growth temperatures (48–55 °C normal). In Sr-containing medium cells only grow stably at lower light intensities. For both cultures, we had good success at ~40–45 μmol m$^{-2}$ s$^{-1}$ continuous light at a growth temperature of 45 °C. All samples were taken from culture in mid-exponential growth. For determination of growth phase of sampled cultures, optical density (OD) was measured at 730 nm using a Thermo Scientific Evolution-60 spectrophotometer. Chlorophyll a (Chl a) concentration was determined using this instrument, after methanol extraction from culture [34].

Quantification of the D1 subunit of PSII was carried out via two methods (Fig. S1). Polyacrylamide gel electrophoresis and Western blot were performed against a D1 protein standard (Agrisera) using primary antibodies targeted to a conserved PSII sequence and horseradish peroxidase secondary antibody/luminoil detection. Western blots were conducted in quadruplicate and results averaged. All samples were adjusted to equivalent chlorophyll concentrations of 3 μg/mL before blotting for ease of comparison to other data. Quantification of the tyrosine-D radical was performed by EPR spectroscopic measurements against a standard curve of Freny’s salt (potassium nitrosodisulfonate) using a Bruker Elexsys E580 spectrometer; conditions: 10 mW microwave power, 70 dB receiver gain, and 100 kHz modulation frequency at 100 K. Oxygen was detected electrochemically by custom-made Clark-type electrodes using single turnover flashes (STF) of 20 μs duration applied at a range of flash frequencies [31]. The LED spectral range was 660 ± 20 nm FWHM, and the light intensity was 32,000 μmol photons per second per square meter. Individual flash oxygen traces were integrated to obtain absolute quantum yields per flash by comparing to a standard of known concentration. Steady-state oxygen yields from multiple flashes in a train of flashes following 2 min dark incubation were also averaged and plotted separately as in Fig. 4. Lastly, for comparing the relative oscillations in oxygen yield from different pulse trains, as in Fig. 2, the individual flash yields were normalized to the same steady-state value after oscillations decayed to zero.

The absolute O$_2$ quantum yields (mol O$_2$ per flash/mol PSII-D1) were obtained by normalizing to the number of PSII centers measured by Chl a concentration and EPR spectroscopy. Current generated by the reduction of O$_2$ following a single flash was integrated to obtain the total charge, which corresponds to O$_2$ consumed at the electrode (4 e$^-$ per molecule).

S-state decay lifetimes were determined by advancing a dark-acclimated culture to the desired state using flashes, followed by dark incubation for variable time in this state, followed by rapidly advancing to oxygen evolution via flashes [28,30,31,35]. The populations were corrected using the WOC inefficiency parameters listed in Table 1. For certain measurements, cultures were supplemented with 250 μM
dimethylbenzoquinone (DMBQ) in order to bypass any effects of strontium substitution localized to the acceptor side of PSII [28–31].

All WOC cycle parameters and S-state populations [36] derived from flash oxygen measurements were obtained by data fitting using the STEAMM algorithm to solve the Markov matrix representing the theoretical WOC cycle. The VZAD model of the WOC cycle was used based on four inefficiency parameters, as shown in Fig. S2 [37]. The absolute O₂ yield was normalized to unity and not used to generate the fits, as all centers that pass from S₂ to S₀ are assumed to produce the same O₂ yield and either recycle or inactivate (ε). The squared difference between the observed and simulated O₂ amplitude was minimized. Another index of the accuracy of the model that we used to assess quality of the fits is based on agreement between the WOC cycle period (obtained by Fourier transformation), P₉, and the calculated model-dependent period, Pcalc. [37]. Fourier transforms of oxygen evolution measurements described above were determined using a fast Fourier transform algorithm available as part of the VZAD software suite (available online at: http://chem.rutgers.edu/dismukes-developed-software-available-research).

FRFR measurements were performed using a custom-made fluorometer [28] and samples of the same live cultures used in oxygen evolution measurements. Chl a fluorescence emission at 660 nm was measured using trains of 50 flashes at 100 Hz.

To determine the temperature dependence of steady-state oxygen evolution rate, cultures were subjected to continuous saturating red light (120 μEin/m²/s) with continuous stirring in an air-tight, temperature-controlled chamber using a Hansatech Oxygraph Plus oxygen electrode. All rates of oxygen evolution were corrected for respiration by subtracting the rate of oxygen consumption at measurement temperatures in total darkness.

Growth medium in which Sr replaced Ca was analyzed for Ca content by ICP-OES and contained 330 mM residual Ca as contaminant. Native (Ca) growth medium contained 60 mM Sr contaminant. These contaminant levels, being < 0.1% of the other cation by molarity, can be treated as insufficient to induce significant interference, and indeed others have shown higher levels of contamination have produced samples which may be differentiated [14,38,39]. Furthermore, T. elongatus does not commonly exchange calcium and strontium in assembled PSI [38]. Thus, after many generations it can be expected that no functional Ca-WOCs remain in Sr-culture and vice versa.

3. Results

3.1. Cell growth rate and pigments

Sr-culture was grown in Sr-substituted medium for three months (1624 fold dilution) prior to any measurements in order to fully adapt to conditions. The growth rate of adapted cells at 45 °C is shown in Fig. S3. All experiments were conducted on culture in mid-exponential phase growth (between 3 and 4 days since inoculation), at which point the two conditions produce equal growth rates.

Both chlorophyll (Chl a) and phycocyanin content were affected by Sr growth, indicating an altered antenna structure. A significant amount of Chl a is present as PSI, and the effects of whole-cell Sr-substitution on PSI content have not been reported. We therefore normalized oxygen evolution data to PSI reaction centers. The chlorophyll:D1 ratio was determined by Western blot and independently by EPR (Fig. S1). In both methods, a linear calibration series was obtained as a function of cell content. The chlorophyll:D1 ratio was determined to be 1504 ± 88 molecules by WB and 1474 ± 29 molecules by EPR per PSII-D1(Ca) and 2142 ± 131 and 2036 ± 45 molecules, respectively, per PSII-D1(Sr).

Due to lower variance, the EPR values were used in all following calculations reliant on PSII-D1 content.

Sr-substitution also causes a decrease in phycobilin content. We determined the phycocyanin content by the method of Lawrenz et al. [40] and normalized to the PSII-D1 content. In the native culture, there are 6.7 phycocyanin molecules per PSII-D1, whereas the Sr-substituted culture contains 5.4 per PSII-D1. Because of the different antenna sizes we determined the light saturation curves for both cultures. The results are given in Fig. S4. Under growth conditions, both cultures exhibit similar light usage, while measurements of maximum oxygen yield given in Fig. 5 were performed under saturating light. It must be noted that this strain does not grow under saturating light conditions and the Sr cultures are appreciably more light sensitive.

3.2. Flash O₂ quantum yields

Oxygen quantum yields (QYs) were measured by LED pulses of saturating intensity and 20 μs duration at room temperature, as shown in Fig. 1A. The amplitude and number of the oscillations are visibly larger for the Sr-grown cultures (4 cycles vs. 3). The values plotted are the absolute quantum yield relative to PSII-D1 subunit content as determined by EPR spectroscopy. The quantum yield after decay of the oscillation (at steady-state) is given in Fig. 1. For the Sr-grown cultures this initial QY is 58% of the Ca-grown cultures (Fig. 1A), and agrees with previously reported yields for PSII core complexes of T. elongatus. [3,7,14,15]. As shown in Fig. 1C, the average QY in both samples decreases with continued flashing over 90 min and the difference in QYs between Sr and Ca remains quite consistent (Sr is 60% of Ca at 90 min).

The lower O₂ yield in Sr-grown cells vs. Ca-grown cells is reversed upon addition of benzoquinone derivatives that are permeable to the outer membrane [31]. In the presence of DMBQ (Fig. 1D), the integrated O₂ yield per WOC is approximately 31% greater in Sr-substituted culture than in Ca-grown, indicating that the lower yield of O₂ seen in the absence of DMBQ in Sr-grown cells (Fig. 1C) is due to an acceptor-side limitation. Over the 100-minute measurement period, the O₂ yield in both samples first increases and then decreases, due to the time for DMBQ to equilibrate with the acceptor side and for light induced reduction to DMBQH₂, respectively [31].

The absolute quantum yield per flash was determined to be 60.5 mmol O₂/mol D1 flash in Ca-grown culture without DMBQ, as opposed to 34.2 mmol O₂/mol D1 in Sr-grown culture. These yields correspond to 24.2% and 13.7% quantum efficiency, respectively. Addition of DMBQ resulted in peak quantum yields of 67.0 mmol O₂/mol D1 in Ca-grown culture and 87.9 mmol O₂/mol D1 in Sr-grown culture, corresponding to 26.8% and 35.2% quantum efficiency, respectively. In the presence of DMBQ, the quantum efficiency of Sr-grown culture therefore increases by 157%, a factor significantly greater than observed as a result of quinone addition in vivo in any other strain to date [31].

As shown in Fig. 2B, addition of the electron acceptor DMBQ produces much stronger oscillations in O₂ yield in both Sr-grown and Ca-grown cultures, extending over 10 and 7 cycles, respectively. Titrations with DMBQ were done to determine the optimal concentration (250 μM), in a manner observed for all other cell types that we
and others have investigated [29,31,41]. DMBQ introduces some photoinactivation seen as a decreasing slope. This loss appears in the modeling as the inactivation parameter ($\varepsilon$) discussed later. Oscillations in the Ca-grown culture remain shallower.

The Fourier transform of these data, shown in Fig. 2C and D, provides a model independent representation of the WOC cycle period for O$_2$ production at this flash frequency (P$_{FT}$). Upon adding DMBQ to cells, the entire distribution shifts from high period, indicative of high inefficiency, to lower periods. The most probable cycle period decreases from P$_{FT}$ = 5.27 to 4.16 (Ca-grown) and from P$_{FT}$ = 4.72 to 4.14 (Sr-grown). Without DMBQ the Sr-grown culture has a significantly shorter peak period, and the relative contribution from higher period oscillations is less, reflecting the higher efficiency of the WOC cycle in Sr-grown cells. These differences almost completely disappear in the presence of DMBQ, with the exception that the FT of the oscillations have lower amplitude in Ca-grown cells, as expected reflecting their lower QY. Another subtle distinction introduced by DMBQ is the presence of a new peak at period-2, particularly in the Sr-grown culture (addressed below).

Fits of the oxygen flash yields to the VZAD model are given in Fig. 2A and B, and parameters are summarized in Tables 2A and 2B for room and growth temperature, respectively. The WOC cycle peak period can be calculated from the WOC inefficiency parameters using the theoretical expression which relates them in the VZAD model [31,37]. These values are given in Tables 2A and 2B, and are plotted on the experimental plot in Fig. 2C and D, without and with DMBQ, respectively. The close agreement should be noted, further confirming the accuracy of the VZAD model. Without DMBQ, both cultures show different dark-stable S state populations, typically Ca/Sr = 50/40 for S0 and 50/60 for S1. The Sr-grown culture thus shows an initial distribution somewhat closer to the ideal Kok model: 0.25:0.75:0:0, reflecting less reduction of S1 in the dark. Addition of DMBQ typically increases the population of the dark-stable S2 and S3 states. Particularly in Ca-grown cells the S2 state is populated, while in Sr-substituted cells it still decays under these conditions. However, the S3 state is more stable in Sr-substituted cells. These altered S state populations can be attributed to changes in the kinetics of decay of S2 and S3, as shown next.

The measured decay kinetics of S2 and S3 in whole cells are given in Fig. 3, together with fits to a biexponential decay model (Table 1). There is a significant increase, approximately three-fold, in the lifetime of the S3 state in Sr-substituted culture, indicating that Sr-substitution significantly slows electron back-flow to the WOC in this state. Sr-substitution has little net effect on the lifetime of the S2 state. Addition of DMBQ increases the lifetimes of both the S2 and S3 states in both cultures (Table 1), but the increase is quite small for S2, 29% for Ca and 22% for Sr, while the S3 lifetime increases substantially by 49% (Sr) and 129% (Ca). This large difference between S2 and S3 means that the yield of charge recombination, which is known to occur via plastosemiquinone forms of QA and QB, is less influenced by DMBQ in the S2 state versus S3. Furthermore, since DMBQ acts by binding to the QB site, the implication is that plastosemiquinone(B) is less readily oxidized or displaced by DMBQ in the S2 state.

3.3. Inefficiency parameters of the WOC cycle

The WOC cycle parameters obtained from fits to the VZAD model are reported at flash rate 0.5 Hz and 23 °C (Table 2A) and 45 °C (Table 2B). The data show that in the absence of exogenous electron acceptor the
Sr-grown cultures have significantly smaller inefficiency parameters ($\alpha$, $\beta$, $\delta$). At 23 °C (45 °C) the misses ($\alpha$) are a full 10% (14%) fewer, indicating that capturing of charge separation events is correspondingly more efficient than for Ca-grown cultures. Backward transitions ($\delta$) are 6% (12%) fewer, indicating that addition of an electron is correspondingly less probable. Double hits ($\beta$) are small in both cases. Effectively, the Sr-substituted PSII is operating 17% (28%) more efficiently per-WOC cycle. Loss of centers by photoinactivation ($\varepsilon$) is small at 3% (1%), but also 3-fold larger than for Ca-grown cells. Since photoinactivation ($\varepsilon$) is presumed to be irreversible, in the long run, this difference amounts to a major disadvantage for Sr cultures.

Addition of DMBQ significantly reduces the two main inefficiency parameters ($\alpha$, $\delta$). Misses decrease by nearly 3-fold, as do backward transitions. The absolute improvement in performance is more substantial for Ca-grown cells. DMBQ increases double hits substantially in Ca-grown cells (5% and 11.6% at 23 °C and 45 °C, respectively), while much less so in Sr-grown cells, suggesting the latter acceptor side is more tolerant to oxidation of the non-heme iron, the common source of double hits [42]. These changes, together with the increase in oxygen yield, indicate that DMBQ removes the blockage of electron/proton flow on the acceptor side that limits PSII turnover in both cell types.

Measurements at the growth temperature (45 °C, Table 2B) show there is a general decrease in misses, while backward transitions are somewhat increased (Ca) or unchanged (Sr). Double-hits are only observed in Ca-grown cells with DMBQ added and quite substantial (11.6%), indicating that Sr suppresses oxidation of the non-heme iron substantially relative to Ca [29,31,41] [42]. Measurement at growth temperature reveals the initial dark S-state populations in the Sr-grown culture retain a significant fraction (5%) of WOCs in the S3 state with DMBQ added, but not in the S2 state, consistent with the slower decay of S3 (Table 1). In summary, the Sr-culture shows a more ideal period closer to 4, lower miss and backward transition parameters, and a more stable S3 population (slower recombination), indicating a more efficient culture even without DMBQ. These parameters are far better than observed in the Ca-grown culture of most lower-temperature species [31].

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**Table 2A**

WOC cycle parameters and dark S-state populations at measurement temperature (A) 23 °C and (B) at 45 °C. Flash rate 0.5 Hz; dark preincubation time 180 s.

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<th>$\beta$</th>
<th>$\delta$</th>
<th>$\varepsilon$</th>
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<th>S3</th>
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**Fig. 2.** Relative flash-oxygen yields from Ca- and Sr-grown cultures: A) in the absence of exogenous electron acceptors, and B) supplemented with 250 μM DMBQ. Data are normalized at the steady-state for comparison and are the average of 30 flash trains at flash frequency (0.5 Hz). The fits to the VZAD program are shown in red. Residual values are plotted and represent the difference between experimental values and VZAD fits. For clarity, they have been offset by — 0.1 for Ca-grown culture and — 0.2 for Sr and do not exceed 4% of average yield. Fourier transforms of experimental data from A) and C) are given in B) and D), respectively.
3.4. Rate of PSII turnover

Fig. 5 shows the temperature dependence of the maximum PSII turnover rate, obtained by measuring the light-saturated O2 evolution rate, after correcting for the (low) respiration rate, and normalizing to D1 content. We modeled the data using the Arrhenius formula in the range 15–50 °C. At 55 °C and above, O2 evolution sharply declines due to denaturation of PSII, while below 15 °C the rate is negligibly slow for our instrumentation to detect.

Both cell types exhibit a nearly linear Arrhenius plot over this temperature range, but with different slopes. The PSII turnover rate in Sr-grown cells has a 21% larger "activation energy", 86.8 vs. 71.9 kJ/mol, resulting in a lower rate of oxygen evolution at room temperature, where *T. elongatus* is often studied, but higher oxygen evolution rate at growth temperature. Thus, the blockage on the acceptor side which limits the yield of O2 per PSII in Sr-grown cells relative to Ca-grown cells is substantially reduced at 45 °C.

3.5. Site of acceptor side inhibition

The locus of inhibition which limits electron flux on the acceptor side more severely in Sr-substituted cells was investigated using two methods (Fig. 6). The functional size of the PQ pool was estimated from the number of flashes in a train that reduce the yield of Fv/Fm by 50%. When the flash rate exceeds the rate of PQH2 reoxidation, the pool fills and Fv/Fm decreases [28]. This experiment is shown in Fig. 6A for both cultures and shows that the Sr-grown culture naturally
possesses approximately 28 PQ molecules per PSII, as compared to 17 in the Ca-grown culture. It should be stressed these are kinetically determined, and are upper estimates of the actual number of available PQ molecules. The second method we tested is blockage of the QB site using the specific inhibitor DCMU. Addition of DMBQ in the presence of 2 μM DCMU does not restore the yield of Fv/Fm in either Sr or Ca cells (Fig. 6B), indicating that DMBQ acts by oxidizing at the QB site or downstream, and that QA− is not directly involved.

4. Discussion

4.1. Flash O2 WOC efficiency

Prior observation of the slower light-saturated turnover rate of PSII in Sr-substituted core particles at room temperature had been attributed to changes within the Mn4SrO5 core itself that slow the terminal O2 release step [14]. It therefore came as a surprise to us to learn from the present experiments that the slower PSII turnover in Sr cells under saturating intensity actually results from blockage on the electron acceptor side, rather than from slower turnover of the WOC. This was established by the data in Figs. 1 and 2, demonstrating that removal of this blockage upon addition of DMBQ results in the light-saturated turnover rate increasing from 58% of that of Ca-grown cells to 131%. Likewise, increasing the measurement temperature from room to 47 °C overcame this kinetic blockage (Fig. 5), again consistent with a rate-limiting activation process such as diffusion within the QB/PQ pool exchange site. Once the acceptor blockage is removed, the electron/hole pairs generated in PSII can be captured with greater efficiency by the WOC(Sr), with fewer misses and fewer backward transitions (Tables 2A and 2B). The lower miss parameter and longer S3 state lifetime (Table 1) indicate that the WOC(Sr) is more efficient in both capturing holes and holding on to them in the dark, respectively. The latter property indicates a higher efficiency at low light intensity for WOC(Sr) than for WOC(Ca). This distinction is analogous to the benefit gained by the low-light and high-light isoforms of the D1 subunit of PSII, which are differentially expressed at low vs. high light intensities, respectively [28,30,43].

4.2. Backward transitions

are appreciably less probable in WOC(Sr) than WOC(Ca), 8% vs. 20% at the growth temperature, respectively, and are eliminated by populating QB with DMBQ. The function of the backward transition is different than recombination. We have previously shown that inclusion of the backward transition parameter is essential for accurate fitting of the O2 oscillations and can reach quite a large fraction of centers in some phototrophs (~0.24) [31,37]. Backward transitions occur when electrons from outside the water oxidation cycle enter the cycle by reduction of the WOC in S2 (and possibly S3). We have proposed that they are a manifestation of cyclic electron flow within PSII (CEF-PSII) [31]. CEF-PSII has been previously proposed to occur in other phototrophs [44–47]. Although the mechanism of backward transitions and CEF-PSII remain to be fully elucidated, it is distinct from classical cyclic electron flow around PSI. The function of CEF-PSII is energy conversion by proton pumping across the thylakoid membrane, the transfer of a proton from outside to inside using light energy to convert previously

Fig. 5. Temperature dependence of the O2 evolution rate in Sr- and Ca-grown cultures. All measurements were taken under continuous saturating (120 μEin/m²/s) red light in well-stirred culture. Rates of O2/PSII-D1 are plotted vs. temperature in (A) and as an Arrhenius plot, ln rate vs. 1/T, in (B). The data above 50 °C are influenced by thermal deactivation and thus not used in B. "Activation energies" for oxygen evolution are calculated from the Arrhenius plot and reflect the entire WOC cycle. The data are the average of 3 measurements.

Fig. 6. FRR fluorometric measurements of Ca- and Sr-grown cultures. (A) Comparison of trains of 50 pulses with PQ pool limits denoted. (B) Effect of addition of DCMU to culture. All measurements were done at 100 Hz and 23 °C.
stored energy of chemical bonds (PO₄H₂) into a proton gradient. This vectorial proton pumping process differs mechanistically from scalar proton evolution that takes place when water is oxidized, and serves the purpose of converting stored redox energy into ion gradient energy and ultimately ATP.

4.3. Recombination lifetimes

The 2.4-fold longer S₃ state recombination half-life in WOC(Sr) provides evidence for a larger Sr-dependent reorganization barrier (structural rearrangement) upon S₃ → S₂ recombination. The decreased DMBQ effect on this rate indicates that the larger reorganization barrier slows the rate sufficiently such that electron recombination from other sources occurs (independent of the Qb site where DMBQ acts). XAS evidence provides strong evidence for a large structural rearrangement during the S₂ → S₃ transition for both WOC(Ca) and WOC(Sr) in core particles [48,49], and this is likely true in whole cells too based on the large Arrhenius activation barrier we found for O₂ production. EPR and XAS evidence indicate a minor reshaping of the S₂ state in WOC(Sr) [11,30,50–58], with the closed S₂ configuration (intermediate spin S = 5/2 ground state) favored by Sr-substitution, while the open configuration (low spin S = 1/2 ground state) is favored in Ca cultures [14,51,59].

4.4. The quality factor

Q = 1/(α + β + δ + ε) is convenient for depicting the relative efficiency for completing the full transit through the four step WOC cycle, as a function of flash rate, irrespective of which type of inefficiency occurs [28,37]. This data format, although model dependent, makes clear the dramatic benefit in WOC efficiency afforded by Sr-substitution at all measured flash rates. It also illustrates the significant downregulation of flux through PSII-WOC controlled by the electron acceptor side, which can be removed by oxidizing the electron acceptor PQ pool (addition of DMBQ). This illustrates a major form of regulation that occurs in vivo in both types of cells. This result points to a possible strategy for improving light to redox energy conversion by increasing the size of the PQ pool or by removing its regulation. However, as DMBQ addition also causes loss of backward transitions, this strategy will have negative consequences on CEF-PSII. In vivo measurement of Q by O₂ flashes is restricted to slow rates. Q can also be measured at much higher flash rates using Chl fluorescence, but emission intensity is low in T. elongatus cells (see Fig. 6). This limitation has two sources: Strong absorption by phycoobilins in both cultures and the more efficient WOC(Sr) photochemistry causing lower Fv emission.

4.5. Growth rate and PSII-WOC

At the optimal growth conditions for Sr-substituted T. elongatus cells (approximately 40 μE/m²/s light intensity and 45–50 °C), the WOC turnover rate is not the limiting factor in electron transport. The rate limitation lies on the acceptor side. At this light intensity, it would be able to complete all S-state transition more quickly than it would receive enough light to advance through the next cycle [10]. Moreover, because of the greater efficiency of the WOC(Sr) cycle, more centers advance and more O₂ is formed at this relatively low light intensity. The 8-fold slower O₂ release kinetic of PSII-WOC(Sr) versus PSII-WOC(Ca) [14] is not a limiting factor at this low light intensity. At high light intensity where this difference could influence light saturated growth rate, Sr cultures do not grow owing to severe photoinhibition. Even at low light intensity the larger photoinactivation parameter (ε) reflects this greater light sensitivity of WOC(Sr).

As noted in Fig. 5, the Sr-grown culture generates more O₂ per PSII-D1 at the growth temperature (45 °C) and in the absence of quinones. However, this falls off sharply above 50 °C, unlike Ca-grown cells which are proportionately stable up to 65 °C. Additionally, the lower relative concentration of PSII-D1 in Sr-grown cells effectively offsets the efficiency advantage at 45 °C and the highly similar growth rates observed for both Sr and Ca cells support this result (Fig. S3). Thus, we may reconcile the observed effects of Sr substitution on growth and O₂ evolution. The organismal response to the more active PSII centers in Sr-grown cells at 45 °C is to lower the number of active PSII centers per Chl. This suggests that the energy flux created by PSII is the primary determinant of growth rate of T. elongatus.

4.6. Period-2 oscillations

We observe that Sr-substitution increases the probability of period-2 oscillations in O₂ flash yield (Fig. 2C, D). This occurs in many phototrophs we have studied and typically appears when the PQ pool is oxidized using a benzoquinone electron acceptor [31]. This point to the influence of the two-electron gate, Q₆Q₇, through binary modulation of the recombination probability. As we used a constant (S state independent) miss parameter, this feature is not captured in the VZAD model. S state dependent miss parameters have been previously shown to improve fits to a Kok model [60–63]. The double-hit parameter in Sr-substituted culture is extremely low (Tables 2A and 2B), even in the presence of DMBQ, and cannot explain the degree to which period-2 oscillations are observed, especially given that the five-fold higher rate of double-hits in Ca-grown culture is concurrent with a lower degree of period-2 oscillation (Table 2A) [36,37]. Furthermore, a period-2 oscillation in O₂ yield can only be generated by this method in the event of two consecutive double-hits from S₀ to S₂ and S₂ to S₄ [36,37]. Unless double-hits are strongly preferred in these particular states, it is more likely that a larger period-3 peak would be observed, simply because only one double hit to the S₀, S₁, or S₂ state is required. As no significant period-3 peak is observed, this phenomenon cannot be attributed to double-hits in the conventional sense.

An alternative explanation for period-2 oscillations in O₂ yield could be a two-electron oxidation to hydrogen peroxide (H₂O₂) instead of four-electron oxidation to O₂, followed by dismutation, H₂O₂ → O₂ + H₂O, external to the WOC cycle [64–66]. PSII(SZ) has been previously shown to both produce hydrogen peroxide by oxidizing water [65–68] and further oxidize it to oxygen [64], so it is feasible that some PSIIIs are preferentially performing this function. This could also account for the slight increase in the inactivation rate of the WOC (ε) observed in Sr-substituted culture (Tables 2A and 2B), as hydrogen peroxide readily forms hydroxyl radicals upon one-electron reduction (Fenton chemistry). Why peroxide is potentially being produced is controversial given its toxicity. Recent evidence indicates that hydrogen peroxide, produced as a result of imbalances in redox state, acts as a signaling molecule to activate classic CEF in higher plants in vivo [69]. At present we have no direct evidence for increased hydrogen peroxide formation in WOC(Sr).

4.7. Thermodynamic model

To help interpret the results herein, a thermodynamic model is given in Scheme 1B, which is supported by the structural model in Scheme 1A taken from the 2.1 Å resolution X-ray diffraction (XRD) structure of T. elongatus PSII-WOC(Sr) and the 1.9 Å resolution structure of PSII-WOC(Ca) [24,25]. However, much of the following interpretation is associated with features conserved across other PSII-WOCS [30,57]. We start by considering the properties of the alkaline earth ions. Sr²⁺ has a 0.14–0.18 Å larger ionic radius than Ca²⁺ complexes with coordination number 8 and 6 (11–16% larger). These have a closed shell (noble metal) electronic configuration and inaccessible ionization potentials to the 3 + state and thus act solely as spherical ionic cations. The experimental gas phase dehydrogenation enthalpy to remove one water molecule from Ca²⁺ (Sr²⁺) coordinated to 5, 6 or 7 water molecules is 26.7 (23.9), 22.0 (20.9), and 17.7 (17.1) kcal/mol, respectively [70]. These
data provide a quantitative estimate of the available energy difference for restructuring the first coordination shell.

As depicted in Scheme 1A, the XRD structure of *T. elongatus* PSII-WOC indicates that Sr is displaced toward Yz relative to the position of Ca, decreasing the YZ(O)-Sr distance by $-0.26 \text{ Å}$ and increasing all three Sr-O(core) distances by an average of $+0.09 \text{ Å}$. Both water molecules W3 and W4 remained coordinated to Sr and consistently are shifted toward YZ (W3 by the substantial distance of 0.30 Å). These shifts were recently supported by QM/MM modeling by Vogt et al. [71]. Based on these largest of all structural changes and assuming ionic potentials, a change in the relative reduction potentials is expected, with that for Yz$^+/Yz$ increasing and that for Mn$_4$O$_5$(Si$^+$/Si) decreasing. This thermodynamic shift predicts a change in population based on the Nernst equation, as given in Scheme 1B. This prediction is completely in line with our experimental data for PSII-WOC(Sr), demonstrating shifted populations favoring hole transfer from the reaction center to the WOC: 1) decrease in Fv/Fm, 2) shift in dark S state populations from S0 to S1, 3) greater stability of the S3 state (substantially longer decay time), 4) fewer misses. The predicted free energy was calculated using the Nernst equation $\Delta G = -RT \ln K_{eq}$.

\[ K_{eq} = \frac{k_F}{k_B} \tag{1} \]
\[ \Delta G = -RT \ln K_{eq} = -RT \ln \left( \frac{K_{Sr}}{K_{Ca}} \right) \tag{2} \]

Assuming $Y_z^+ = 1/(1 + \alpha)$. The predicted $\Delta G$ $= -2.3 \text{ kJ/mol}$ favors a lower energy of the WOC relative to Yz. This value is comparable to the difference in hydration energies for removal of a water molecule bound to 7-coordinate Sr$^2+$/Ca$^2+$ in the gas phase, as noted above. A shift in reduction potential of QA$^-$/QA in the S2 state resulting from Sr-substitution was previously determined by Kato et al. [13].

4.8. Implications for the mechanism of water oxidation

The present work informs about the chemical mechanism of water oxidation by PSII-WOCs. The present work makes it absolutely clear that WOC(Sr) is a far better catalyst than WOC(Ca) at low turnover rates where O$_2$ release is not rate-limiting. The improvement in efficiency and quantum yield arise from the decrease in charge recombination caused by the thermodynamic stabilization of the WOC(Sr) relative to Yz$^+$. A consequence of this stabilization is the larger activation barrier to transit through the full catalytic cycle, and another is the resulting slower kinetics of O$_2$ release.

Sr-substitution favors the intermediate spin state of the S2 state WOC(Sr) characterized by the g4.1 EPR signal [7], while the S2 state of WOC(Ca) can exist in either a low spin $S = 1/2$ ground state (resting S1 precursor) or the g4.1 form corresponding to a S = 5/2 ground state (active S1 precursor). The EPR data are in excellent agreement with magnetic susceptibility changes measured for the full catalytic cycle at room temperature, showing that two magnetically distinct
forms of the WOC(Ca) exist, corresponding to a low spin (resting form) and intermediate spin (active form) [8]. The intermediate spin S2 state has been attributed to the “closed” structural form of the WOC, in which O₅S is part of the SrO₂Mn₃ cluster and does not bridge to Mn₄#4 [72,73]. This assignment is consistent with the reported structural change upon Sr-substitution in the S1 state which has the “closed” WOC form in which Mn₄#4 is further away from Sr than Ca by + 0.22 Å (Scheme 1A). The EPR and magnetic susceptibility data for S2 are consistent with this assignment. We can now say with confidence based on our studies of WOC(Sr) that the intermediate spin form of the S2 state possessing the “closed” cubane subcluster is the active form in the catalytic cycle, exhibits higher quantum efficiency for O₂ production, and has slower recombination rate than the low spin S = ½ form.

4.9. Site of acceptor side inhibition

While the models in Scheme 1A/B serve to explain the observed effects very thoroughly, and generally complement prior in vitro studies of Sr-substitution [3–7,9,14,15,51], some phenomena were observed which warrant further investigation. The locus of inhibition which limits electron flow on the acceptor side of Sr-substituted cells can be securely placed beyond Qₘ. Evidence localizing the site of flux inhibition to the Qₘ site comes from the two experiments given in Fig. 6. Sr-grown cells synthesize a significantly larger PQ pool size, likely as an attempt to overcome the limitation which limits the WOC(Sr). Only when DMBQ is added does the forward flow of electrons through Qₘ increase while the backward flux is eliminated. It is possible that growth on Sr negatively impacts some PSIIIs at the acceptor side [2,4–6,9,35,74]. Recently, Khan et al. [75] demonstrated a potential function of the glutamate-rich loops near the non-heme iron of spinach PSII membranes in maintaining a balance of cations in that region [29,31,33]. It is possible that calcium is present in this cation balance, and indeed Khan et al. showed inhibition of light driven electron transport to a cobalt-modified PSII upon binding of calcium to the glutamate sites. However the inhibition by Ca²⁺ is weak (2 mM KD) and was the same for Sr²⁺, suggesting a quite different non-specific origin than what is reported herein. More likely causes for inhibition involve modification of redox potential at the acceptor side resulting from strontium substitution [13] or modification of the redox state of the PQ pool itself resulting from strontium substitution elsewhere in the cell.

5. Conclusions

We have demonstrated that O₂ evolution rates increase in cultures containing WOC(Sr) on a per-PSII basis, provided a concurrent limitation on the acceptor side is removed. We posit that this limitation is primarily based on cycling of plastoquinone between the Qₘ site, the PQ pool and cytochrome b₅₆₃, which is the primary limitation to PSII flux. The WOC(Sr) is thermodynamically stabilized relative to Yz, resulting in an increased activation barrier for oxygen evolution, but also increased activity in the optimal growth temperature range of 45–50 °C. At low light intensity, the culture responds to this increase in WOC cycle efficiency by decreasing the PSII concentration, suggesting that energy flux from PSII determines cell growth rate. The advantages of Sr-substitution are increased efficiency of WOC oxidation and a slower charge recombination from the S3 state, which enable better performance at low light intensity. However, Sr-substitution results in a slower O₂ release step, a greater photoinduction rate (even at low light intensity), suffers from bleaching at high light intensities, has a lower temperature range for growth, and may possibly form hydrogen peroxide, all making it less robust than Ca-culture.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2016.06.004.

References


[33] S. Petrie, R. Stranger, R. Jace, Rationalising the geometric variation between the a and b monomers in the S-1 angstrom crystal structure of photosystem II, Biochim. j. 21 (2015) 6780–6792.


[38] G. Han, F. Mamedov, S. Styling, Misses during water oxidation in photosystem II are State-dependent, J. Biol. Chem. 287 (2012) 13422–13428.


