



## Influence of the PsbA1/PsbA3, Ca<sup>2+</sup>/Sr<sup>2+</sup> and Cl<sup>-</sup>/Br<sup>-</sup> exchanges on the redox potential of the primary quinone Q<sub>A</sub> in Photosystem II from *Thermosynechococcus elongatus* as revealed by spectroelectrochemistry

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### ABSTRACT

Ca<sup>2+</sup> and Cl<sup>-</sup> ions are essential elements for the oxygen evolution activity of photosystem II (PSII). It has been demonstrated that these ions can be exchanged with Sr<sup>2+</sup> and Br<sup>-</sup>, respectively, and that these ion exchanges modify the kinetics of some electron transfer reactions at the Mn<sub>4</sub>Ca cluster level (Ishida *et al.*, J. Biol. Chem. 283 (2008) 13330–13340). It has been proposed from thermoluminescence experiments that the kinetic effects arise, at least in part, from a decrease in the free energy level of the Mn<sub>4</sub>Ca cluster in the S<sub>3</sub> state though some changes on the acceptor side were also observed. Therefore, in the present work, by using thin-layer cell spectroelectrochemistry, the effects of the Ca<sup>2+</sup>/Sr<sup>2+</sup> and Cl<sup>-</sup>/Br<sup>-</sup> exchanges on the redox potential of the primary quinone electron acceptor Q<sub>A</sub>, E<sub>m</sub>(Q<sub>A</sub>/Q<sub>A</sub><sup>-</sup>), were investigated. Since the previous studies on the Ca<sup>2+</sup>/Sr<sup>2+</sup> and Cl<sup>-</sup>/Br<sup>-</sup> exchanges were performed in PsbA3-containing PSII purified from the thermophilic cyanobacterium *Thermosynechococcus elongatus*, we first investigated the influences of the PsbA1/PsbA3 exchange on E<sub>m</sub>(Q<sub>A</sub>/Q<sub>A</sub><sup>-</sup>). Here we show that i) the E<sub>m</sub>(Q<sub>A</sub>/Q<sub>A</sub><sup>-</sup>) was up-shifted by ca. +38 mV in PsbA3-PSII when compared to PsbA1-PSII and ii) the Ca<sup>2+</sup>/Sr<sup>2+</sup> exchange up-shifted the E<sub>m</sub>(Q<sub>A</sub>/Q<sub>A</sub><sup>-</sup>) by ca. +27 mV, whereas the Cl<sup>-</sup>/Br<sup>-</sup> exchange hardly influenced E<sub>m</sub>(Q<sub>A</sub>/Q<sub>A</sub><sup>-</sup>). On the basis of the results of E<sub>m</sub>(Q<sub>A</sub>/Q<sub>A</sub><sup>-</sup>) together with previous thermoluminescence measurements, the ion-exchange effects on the energetics in PSII are discussed.

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### 1. Introduction

In oxygenic photosynthetic organisms, Photosystem II (PSII), one of the two large pigment–protein complexes, performs the light-driven water oxidation, leading to evolution of proton and dioxygen. The catalytic site for water oxidation is a Mn<sub>4</sub>CaO<sub>5</sub> cluster [1], which

**Abbreviations:** PSII, Photosystem II; Phe *a*, pheophytin *a*, Q<sub>A</sub>, primary quinone electron acceptor; Q<sub>B</sub>, secondary quinone electron acceptor; Y<sub>Z</sub>, redox active tyrosine residue; Chl, chlorophyll; TL, thermoluminescence; SrCl-PSII, Ca<sup>2+</sup>/Sr<sup>2+</sup> exchanged PSII; CaBr-PSII, Cl<sup>-</sup>/Br<sup>-</sup> exchanged PSII; CaCl-PSII, untreated PSII; 43H, *T. elongatus* strain with a His-tag on the C terminus of CP43; WT\*3, *T. elongatus* strain with a His-tag on the C terminus of CP43 and in which the *psbA1* and *psbA2* genes are deleted; OTTLE, optically transparent thin-layer electrode; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea

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acts as a device accumulating oxidizing equivalents. Light-induced charge separation results in the formation of a radical pair that quickly leads to the formation of the P680<sup>+</sup>Phe *a*<sup>-</sup> state, where P680 stands for a weakly coupled chlorophyll dimer and Phe *a* for pheophytin *a*. The oxidative power generated with P680<sup>+</sup> then allows the oxidation of the Mn<sub>4</sub>CaO<sub>5</sub> cluster via a redox active tyrosine residue called Y<sub>Z</sub>. The oxidation of the Mn<sub>4</sub>CaO<sub>5</sub> cluster occurs sequentially forming the S<sub>*n*</sub> states where *n* varies from 0 to 4 [2,3]. Two water molecules are oxidized during the transition from the S<sub>3</sub> state to S<sub>0</sub> via the intermediate S<sub>4</sub> state. Meanwhile, the electron of Phe *a*<sup>-</sup> is transferred to the plastoquinone pool via the primary and secondary quinone electron acceptors called Q<sub>A</sub> and Q<sub>B</sub>, respectively.

Ca<sup>2+</sup> is an essential element for the water oxidation [4,5]. X-ray crystallographic data [1] revealed that Ca<sup>2+</sup> is linked to all four manganese ions by oxo bridges. In Ca<sup>2+</sup> depleted PSII the highest oxidation state that can be formed is the S<sub>2</sub>Y<sub>Z</sub><sup>\*</sup> state, and thus water oxidation is inhibited [6,7]. The lost activity can be restored by the addition of Ca<sup>2+</sup>. Repletion with Sr<sup>2+</sup> also fully restores the water oxidation mechanism but the oxygen evolution rate of Sr<sup>2+</sup>-substituted PSII is significantly

lower than that with  $\text{Ca}^{2+}$  [8,9]. So far  $\text{Sr}^{2+}$  has been the only divalent cation allowing such substitution and restoration abilities [4,5,8–10].

$\text{Cl}^-$  is also indispensable for the turnover of the S-state transitions [4,11]. X-ray crystallographic studies recently identified two  $\text{Cl}^-$ -binding sites in the vicinity of the cluster and thus suggested that the two anions may contribute to coordination structure of the cluster and also be involved in either proton exit channels or water inlet channels [1,12–14]. Some anions, for example,  $\text{Br}^-$ ,  $\text{I}^-$ ,  $\text{NO}_3^-$ , and  $\text{NO}_2^-$ , can substitute for  $\text{Cl}^-$  [15,16]; a recent study demonstrated that  $\text{Br}^-$  can support oxygen evolution almost as effectively as  $\text{Cl}^-$  [13].

The effect of the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  and/or  $\text{Cl}^-/\text{Br}^-$  exchange(s) on the oxygen-evolving activity of PSII is reflected by alteration in kinetics and thermodynamics of the S-state transitions. Measurements of time-resolved UV–visible absorption changes and thermoluminescence (TL) revealed that the  $\text{S}_3$  to  $\text{S}_0$  transition accompanying reduction of  $\text{Y}_Z^{\cdot}$  is significantly slowed down by the ion exchanges, and this slowing down has been rationalized by the decrease in the energy level of  $\text{S}_3$  assuming that the ion exchanges affect mainly the electron donor side [17,18]. The  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange has also been shown to decrease the entropic component in the formation of the transition state in the  $\text{S}_3\text{Y}_Z^{\cdot}$  to  $\text{S}_0\text{Y}_Z$  transition [19]. However, it was demonstrated by flash induced fluorescence decay measurements that the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange might also decelerate the electron transfer rate from  $\text{Q}_A^-$  to  $\text{Q}_B$  [20]. Several studies have shown in addition that  $\text{Q}_A$  to  $\text{Q}_B$  electron transfer is influenced by the modification on the donor side of PSII [21,22]. In particular  $\text{Ca}^{2+}$  depletion has been shown to shift the redox potential of  $\text{Q}_A$  [23–25]. In the present work, to clarify the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  and  $\text{Cl}^-/\text{Br}^-$  exchange effects on the redox properties of the acceptor side, we measured the redox potential of  $\text{Q}_A$ ,  $E_m(\text{Q}_A/\text{Q}_A^-)$ , in the biosynthetically ion-exchanged PSII complexes from the thermophilic cyanobacterium *Thermosynechococcus elongatus* [17] by using spectroelectrochemistry. This approach has recently proved very powerful as it revealed the spread of the  $E_m(\text{Q}_A/\text{Q}_A^-)$  values between species [26,27]. In the light of these results, previous investigations of the redox properties of the  $\text{Mn}_4\text{CaO}_5$  by TL measurements [17] are also discussed.

Most of the experiments dealing with the biosynthetic ion exchanges were performed in a *T. elongatus* strain in which the *psbA1* and *psbA2* genes, two of the three genes encoding the D1 protein of PSII, had been deleted [28]. Cyanobacteria generally have a *psbA* gene family with 1–6 gene copies (for a review, see Ref. [29]) and *T. elongatus* has three *psbA* genes [30]. *PsbA1* is dominantly expressed under normal growth conditions, whereas *psbA3* is markedly induced under stress conditions such as high light illumination [31–33]. It has also been shown that transcription of *psbA2* is induced by micro-aerobic conditions [34]. The amino acid sequences of these three PsbA proteins are not identical: The processed PsbA1 (344 amino acid residues) differs by 31 and 21 residues from the PsbA2 and PsbA3, respectively. These substitutions cause partially functional differences, particularly in the electron transfer, the water oxidation, and photoprotection [31,33,35–37]. In the present work, to avoid possible complications due to a PsbA exchange when cultivating the cells under abnormal conditions, such as in the presence of  $\text{Sr}^{2+}$  and  $\text{Br}^-$ , we used the strain lacking both *psbA1* and *psbA2* [28] and compared the results to those obtained with PsbA1-PSII.

## 2. Materials and methods

Biosynthetically  $\text{Ca}^{2+}/\text{Sr}^{2+}$  or  $\text{Cl}^-/\text{Br}^-$  exchanged PSII (hereafter, SrCl-PSII and CaBr-PSII) as well as untreated PSII (CaCl-PSII) complexes were isolated, as described previously [17], from a *T. elongatus* WT\*3 strain [28], which is a His-tagged CP43 strain (43-H) [38] with genetic modification to possess only the *psbA3* gene. The WT\*3 cells were cultured in a DTN medium containing 0.8 mM  $\text{CaCl}_2$ ; for the isolation of SrCl-PSII or CaBr-PSII, the culture medium was supplemented with 0.8 mM  $\text{SrCl}_2$  or  $\text{CaBr}_2$ , respectively, instead of  $\text{CaCl}_2$  [17]. Oxygen-

evolving activities of the purified PSII complexes were 5000–6000, 1800–2600, and 3200–3600  $\mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$  for CaCl-PSII, SrCl-PSII, and CaBr-PSII, respectively [17]. The purified PSII complexes were stored in liquid nitrogen at a concentration of about 1.5–2.0 mg  $\text{Chl mL}^{-1}$  in a medium containing 10% glycerol, 1 M betaine, 40 mM MES-NaOH (pH 6.5), 15 mM  $\text{CaCl}_2$  and 15 mM  $\text{MgCl}_2$  (instead,  $\text{CaBr}_2$  and  $\text{MgBr}_2$  for CaBr-PSII), until they were used.

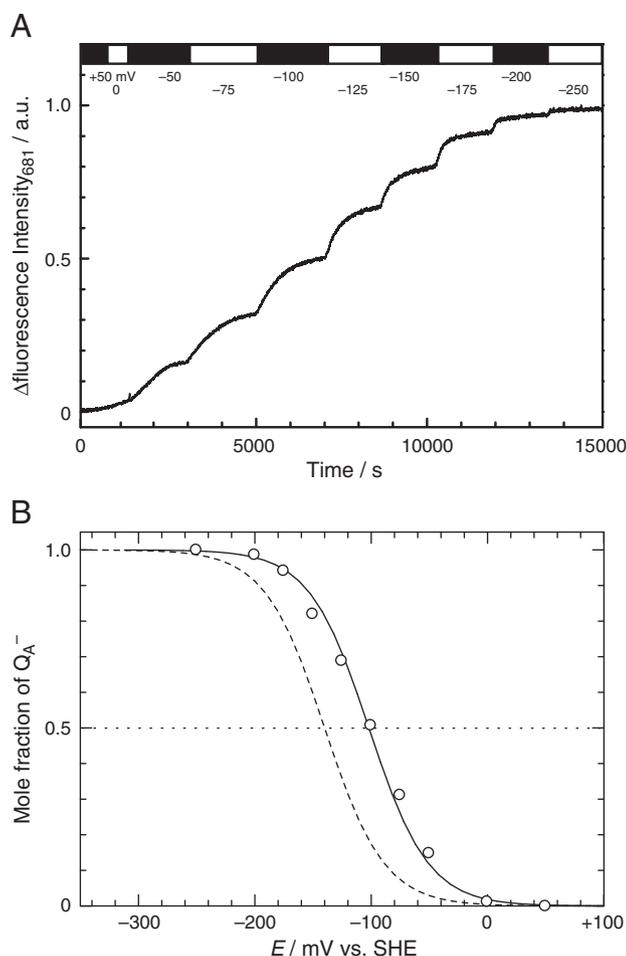
Spectroelectrochemical measurements have been carried out in essentially the same manner as in previous work [26]. The PS II samples were suspended at a Chl *a* concentration of 150  $\mu\text{M}$ , corresponding to 4.3  $\mu\text{M Q}_A$ , in a medium containing 50 mM MES-NaOH (pH 6.5), 0.1% dodecyl- $\beta$ -D-maltoside, 1 M glycine-betaine, 1% taurine, 15 mM  $\text{CaCl}_2$ , 15 mM  $\text{MgCl}_2$ , and 200 mM KCl; for CaBr-PSII,  $\text{CaBr}_2$ ,  $\text{MgBr}_2$  and KBr were used instead. A combination of the following redox mediators were added into the medium: 50  $\mu\text{M}$  anthraquinone-2-sulfonate ( $E_m = -195 \text{ mV}$ ), 50  $\mu\text{M}$  2-hydroxy-1,4-naphthoquinone ( $E_m = -100 \text{ mV}$ ) and 100  $\mu\text{M}$  *N,N,N',N'*-tetramethyl-*p*-phenylenediamine ( $E_m = +300 \text{ mV}$ ). A sample solution was transferred into an optically transparent thin-layer electrode (OTTLE) cell equipped with a gold mesh (100 mesh/inch) working electrode, a Pt black counter electrode and a Ag–AgCl reference electrode [39]. The electrode potential was controlled with a potentiostat Model 2020 (Toho Technical Research). The electrode potential is hereafter referred to the standard hydrogen electrode SHE (0 mV vs. Ag–AgCl is equivalent to +199 mV vs. SHE). The Chl fluorescence was excited with a weak monochromatic beam of 430 nm light, and the emission in line with the measuring beam was detected at the backside of the OTTLE cell placed in a sample chamber of a spectrofluorometer (FP6500/JASCO). The spectroelectrochemical measurements were performed at 14 °C. For the measurements of the ratio of fluorescence maximum level to minimum one ( $F_m/F_0$ ) of the PSII sample solutions, a double-modulation fluorometer model FL-3500 (Photon Systems Instruments) was used.

## 3. Results

### 3.1. Redox potential of $\text{Q}_A$ in CaCl-PSII with *PsbA3* as the D1 protein (*PsbA3*-PSII)

Previous spectroelectrochemical measurements have been performed with PsbA1-PSII [26]. Even though exchanging D1 encoded by *psbA1* for that encoded by *psbA3* is not expected to significantly alter the properties of  $\text{Q}_A$  since it is bound by  $\text{D}_2$ , TL and temperature dependence of the fluorescence decay performed on both PSII suggested that the  $E_m(\text{Q}_A/\text{Q}_A^-)$  might differ [35]. We thus first characterized the CaCl-PSII purified from WT\*3 (CaCl-PsbA3-PSII).

Fig. 1A shows the evolution of the fluorescence intensity at 681 nm resulting from stepwise potential changes. As shown in Ref. [26] this wavelength corresponds to the peak of the fluorescence emission spectrum and its intensity reflects the redox state of  $\text{Q}_A$ . To allow the comparison of these spectroelectrochemical results with the previous ones [26], we avoided freeze-thawing that has been shown to induce a shift in the  $E_m(\text{Q}_A/\text{Q}_A^-)$  value [24], i.e., after purification the PsbA3-PSII samples were kept at 4 °C until use. Assuming that the fluorescence change resulting from electrochemical reduction is proportional to the fraction of  $\text{Q}_A$  reduced to  $\text{Q}_A^-$ , Nernstian plots were constructed for the magnitude of the fluorescence intensity against the electrode potential (Fig. 1B, see also Fig. S1, Supplementary Material). The data obtained for PsbA3-PSII are nicely fitted with a theoretical one-electron redox process, and three independent measurements yielded a value of  $-102 \pm 2 \text{ mV}$  for  $E_m(\text{Q}_A/\text{Q}_A^-)$  in PsbA3-PSII. This is 38 mV more positive than the previous value obtained for  $E_m(\text{Q}_A/\text{Q}_A^-)$  in PsbA1-PSII ( $-140 \pm 2 \text{ mV}$ , Ref. [26]). Even considering possible deviations for a linear relationship between the fluorescence yield and the redox state of  $\text{Q}_A$  [27,40], which can be estimated on the basis of the  $F_m/F_0$  values (3.7 and 5.9



**Fig. 1.** Spectroelectrochemical outcome from redox reaction of  $Q_A$  in PsaA3-PSII isolated from *T. elongatus*: (A) Fluorescence intensity change at 681 nm during a potential journey from +50 mV to –250 mV for the PSII complexes; (B) Nernstian plot based on the relative values of the fluorescence intensity. The solid curve in (B) represents a theoretical one for one-electron redox process with  $E_m = -102$  mV; the dashed curve is drawn for a theoretical one-electron redox process with  $E_m = -140$  mV, which is the result for PsaA1-PSII from *T. elongatus* (cf. Ref. [26]). For the measurements, PSII samples without freeze-thawing were used.

for PsaA1-PSII and PsaA3-PSII, leading to the deviations of 16 and 19 mV, respectively; see Table 1), the difference in the  $E_m(Q_A/Q_A^-)$  values found here can be considered as significant.

Spectroelectrochemical measurements of  $E_m(\text{Phe } a/\text{Phe } a^-)$  for both PSII [35,41] previously revealed that the  $E_m(\text{Phe } a/\text{Phe } a^-)$  value of PsaA3-PSII is 17 mV more positive than that of PsaA1-PSII

**Table 1**  
Redox potential values of  $Q_A/Q_A^-$  and experimental parameters for PSII complexes from *T. elongatus*.

PSII	$E_m(\text{exp})^a/\text{mV vs. SHE}$	$E_m(R=1)^b/\text{mV vs. SHE}$	$R (F_m/F_0)$
PsaA3-PSII	$-102 \pm 2$	$-83 \pm 2$	5.9
PsaA1-PSII <sup>c</sup>	$-140 \pm 2$	$-124 \pm 2$	3.7

<sup>a</sup> Experimental values determined by spectroelectrochemistry: The value for PsaA3-PSII is an average of three independent measurements; the value for PsaA1-PSII is an average of four independent measurements [26]. For the measurements, PSII samples without freeze-thawing were used.

<sup>b</sup> “True” values estimated from  $E_m(\text{exp})$  by considering possible deviation from a linear relationship between the fluorescence yield and the redox state of  $Q_A$ . The deviations can be estimated on the basis of values of  $F_m/F_0$  (cf. [27,40]).

<sup>c</sup> The values cited from the previous work [26,27].

( $-522 \pm 3$  mV and  $-505 \pm 6$  mV for PsaA1-PSII and PsaA3-PSII, respectively). This difference in the redox potential can be ascribed to the difference in the amino acid residue at position 130 of PsaA (D1–130), which is located at a hydrogen bond distance from the 13<sup>1</sup>-keto C=O group of Phe *a* [1]: The residue in PsaA1 is a Gln and a Glu in PsaA3. A recent FTIR study together with theoretical calculations [42] showed that the Glu side-chain expectedly provides a stronger hydrogen bond to the 13<sup>1</sup>-keto C=O group of Phe *a*<sup>−</sup> anion, leading to a more positive value of  $E_m(\text{Phe } a/\text{Phe } a^-)$ . As regards to redox potentials, the difference between PsaA1-PSII and PsaA3-PSII is larger for  $Q_A$  than for Phe *a* and, even more importantly, these respective shifts in  $E_m$  result in an increase in the absolute of  $\Delta E_m$ , rather than a decrease as would be expected from the stabilization of Phe *a*<sup>−</sup> by the D1–Q130E substitution.

Before the development of the spectroelectrochemical measurements described earlier [26] and here, which provide means to assess the redox potentials of the cofactors of interest, TL measurements have been performed to investigate the effects of the PsaA1 to PsaA3 substitution on the energetics [31,33,35]. Outcomes of TL, namely peak temperature and intensity of an emission band, depend on the free energy gaps between the excited state of P680 (P680\*) and the charge separated states, [P680<sup>++</sup> Phe *a*<sup>−</sup>],  $S_2Q_A^-$  or  $S_2Q_B^-$  [43,44]. Though TL data thus reflect total changes in free energy gaps ( $\approx$  redox potential differences) of these cofactors, one can refer to site-directed mutagenesis studies on this position in *Synechocystis* sp. PCC 6803, as a model case concerning the D1–Q130E substitution, that demonstrated the relationship between the shift of  $E_m(\text{Phe } a/\text{Phe } a^-)$  and the TL data changes [45–47]: Time resolved absorption measurements lead to an estimation of +33 mV [46] up-shift for the  $E_m(\text{Phe } a/\text{Phe } a^-)$  resulting from the D1–Q130E substitution, and on the other hand, +30–38 mV [47] was calculated from a change in TL intensity. Further, the theoretical effects of the  $E_m(\text{Phe } a/\text{Phe } a^-)$  shift on the TL data were satisfyingly simulated in terms of intensities and peak temperatures [44]. A lower intensity in the TL B-band together with a small down-shift of the peak temperature has also been reported in *T. elongatus* whole cells containing PsaA3-PSII induced by cultivation under strong light illuminations [31] instead of PsaA1-PSII and in deletion mutants with only the *psbA<sub>3</sub>* gene when compared to a deletion mutant with only the *psbA<sub>1</sub>* and *psbA<sub>2</sub>* genes [33]. These variations were similar to those observed in *Synechocystis* 6803 but their extent of the difference in *T. elongatus* was smaller, suggesting a +18–20 mV shift of  $E_m(\text{Phe } a/\text{Phe } a^-)$ ; this was explained by the compensatory effects of some of the substituted amino acid residues between PsaA1 and PsaA3 other than D1–Q130E, such as D1–Leu151Val and D1–Ser124Phe, being located in the vicinity of the Phe *a*. On the other hand, TL measurements made on PsaA1-PSII and PsaA3-PSII complexes isolated from *T. elongatus* yielded contradictory results to those obtained with the *T. elongatus* cells [33,35]: The peak intensity obtained with PsaA3-PSII was found larger while the peak temperature was similar for both PSII. These results are inconsistent with the idea that only the  $E_m(\text{Phe } a/\text{Phe } a^-)$  would be shifted. In view of this, the unexpected shift of  $E_m(Q_A/Q_A^-)$  found in the present work might contribute to the contradictory TL results. Hence we attempted to analyze the TL data of the two types of PSII [35] on the basis of the recent theory [44] applied with their  $E_m(\text{Phe } a/\text{Phe } a^-)$  and  $E_m(Q_A/Q_A^-)$  shifts (See Fig. S2, Supplementary Material).

A simulation assuming only the  $E_m(\text{Phe } a/\text{Phe } a^-)$  shift by +17 mV indicates that the peak temperature of the TL band should be lower and the intensity should be smaller (green-solid curve in Fig. S2) when compared with the control, namely PsaA1-PSII (black-solid curve); this assumption reproduces qualitatively the result obtained from the site-directed mutation of the D1–Q130E in *Synechocystis* 6803 [47]. Meanwhile, assuming the  $E_m(\text{Phe } a/\text{Phe } a^-)$  and  $E_m(Q_A/Q_A^-)$  are shifted by +17 mV and +40 mV, respectively, simulations show that the peak temperature and intensity should be higher and smaller, respectively (blue-solid curve), at variance with experimental results for the PsaA1-PSII and PsaA3-PSII complexes [35]. As a

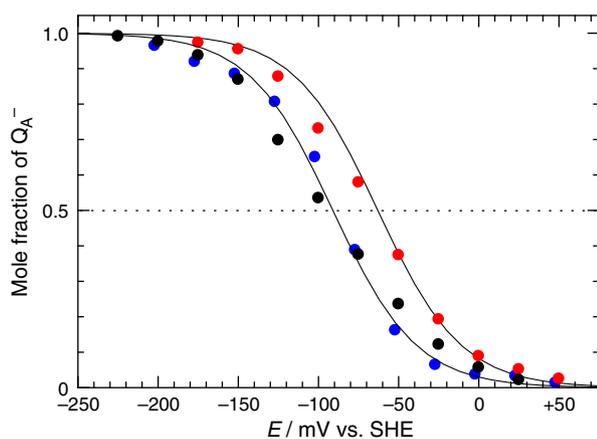
consequence, though the TL data cannot be rationalized even with the  $E_m(\text{Phe } a/\text{Phe } a^-)$  and  $E_m(\text{Q}_A/\text{Q}_A^-)$  shifts, the latter shift, reported here, might contribute partly to the almost similar peak temperature observed in the TL experimental curves.

### 3.2. Biosynthetic $\text{Ca}^{2+}/\text{Sr}^{2+}$ or $\text{Cl}^-/\text{Br}^-$ exchange influences on the redox potential of $\text{Q}_A$ in PSII composed of PsbA3

As shown in Ref. [17,18], *T. elongatus* cells can be photoautotrophically grown in  $\text{Sr}^{2+}$ -containing media instead of  $\text{Ca}^{2+}$  and also in  $\text{Br}^-$ -containing media instead of  $\text{Cl}^-$ , resulting in the biosynthetically ion-exchanged PSII complexes, SrCl-PSII and CaBr-PSII. A previous study showed that the substituted  $\text{Sr}^{2+}$  in the  $\text{Mn}_4$ -cluster ion is not spontaneously exchangeable for  $\text{Ca}^{2+}$  ions in  $\text{Ca}^{2+}$ -containing media [18]; on the other hand, the exchange of  $\text{Br}^-$  for  $\text{Cl}^-$  in the  $\text{Cl}^-$ -binding sites occurs [12,13,17]. Therefore, the spectroelectrochemical measurements for CaCl-PSII and SrCl-PSII were performed with solutions containing Cl-salts as electrolyte, and CaBr-PSII were studied with solution containing Br-salts.

Fig. 2 shows the Nernstian curves for the redox reaction of  $\text{Q}_A$  based on the spectroelectrochemical outcomes, as obtained as Fig. 1A (see also Supplementary Material, Figs. S3–S5A), for the three types of PSII, i.e., CaCl-PSII, CaBr-PSII and SrCl-PSII all composed of PsbA3. Since measurements on CaBr-PSII and SrCl-PSII were done with once-frozen samples we have also determined the redox potential of  $\text{Q}_A$  in a once-frozen CaCl-PSII sample. The three sets of data could be satisfyingly fit by a one-electron theoretical Nernstian curve. However, as shown in Figs. S3–S5B (Supplementary Material), the slopes of data sets in a semi-logarithmic plot were 67–71 mV per decade, i.e., larger than the theoretical value of 57 mV at 14 °C. In contrast, the slopes of the data points for the non-exchanged PsbA3-PSII (CaCl-PSII) samples without freeze-thawing (Fig. S1) were  $55 \pm 3$  mV per decade ( $58 \pm 3$  mV per decade for PsbA1-PSII [26]), i.e., close to the theoretical value. This suggests that the deviations from the Nernst equation might stem from freeze-thawing that would induce heterogeneity in the sample and yield a less negative  $E_m(\text{Q}_A/\text{Q}_A^-)$  value of  $-89 \pm 2$  mV for CaCl-PSII than  $-102 \pm 2$  mV for the non-frozen samples.

Krieger and co-workers [24] reported that freezing and thawing of oxygen-evolving PSII samples at low potentials where  $\text{Q}_A$  is reduced induces an irreversible change in the  $E_m(\text{Q}_A/\text{Q}_A^-)$  value to over 150 mV more positive values owing to structural perturbation at the donor side of PSII. The rather small redox potential difference



**Fig. 2.** Nernstian plot for the redox reaction of  $\text{Q}_A$  in the PSII complexes based on the relative values of fluorescence intensity at 681 nm; for CaCl-PSII (black), CaBr (blue), SrCl (red). The lines represent a theoretical one for a one-electron redox process with  $E_m = -62$  mV or  $-89$  mV.

(13 mV) reported here shows that in the present case with PSII from *T. elongatus* the freezing-induced shift here is not so influential.

In once-frozen samples, i.e., with samples comparable to those used in all the previous spectroscopic studies, the results clearly indicate that the  $E_m(\text{Q}_A/\text{Q}_A^-)$  value of  $-88$  mV for CaBr-PSII is almost the same as that for CaCl-PSII ( $-89$  mV), whereas the  $E_m(\text{Q}_A/\text{Q}_A^-)$  value for SrCl-PSII ( $-62$  mV) is 27 mV more positive, as summarized in Table 2. We did not find any significant difference for the  $E_m(\text{Q}_A/\text{Q}_A^-)$  when measured in the presence of Br<sup>-</sup> salts rather than Cl<sup>-</sup> salts (data not shown). The difference in the  $E_m(\text{Q}_A/\text{Q}_A^-)$  values between CaCl-PSII and SrCl-PSII is significant even in light of deviations for the linear relationship between the fluorescence yield and the redox state of  $\text{Q}_A$  (see Table 2). It can thus be concluded that modification in the  $\text{Ca}^{2+}$ -binding site, but not in the  $\text{Cl}^-$ -binding site, influences on  $E_m(\text{Q}_A/\text{Q}_A^-)$ .

## 4. Discussion

We report here that the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange at the  $\text{Mn}_4\text{CaO}_5$  cluster of PsbA3-PSII from *T. elongatus* up-shifts  $E_m(\text{Q}_A/\text{Q}_A^-)$  by ca. 27 mV. This is at variance with  $\text{Cl}^-/\text{Br}^-$  exchange that hardly influences  $E_m(\text{Q}_A/\text{Q}_A^-)$ . As mentioned in the Introduction, the effects of the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  and/or  $\text{Cl}^-/\text{Br}^-$  exchange(s) on the oxygen-evolving activity of PSII have been observed by many authors. More precisely, it has been proposed that the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange affects the redox properties of the oxygen-evolving  $\text{Mn}_4\text{CaO}_5$  cluster because of the difference in physico-chemical properties like the atomic radius and Lewis acidity of the two cations, as suggested by EPR [18,48], FTIR [49–51], EXAFS spectroscopy [52,53] and so on. There are in addition several pieces of evidences pointing to effects of the ion exchange on the thermodynamics and kinetic properties of  $\text{Q}_A$  and/or  $\text{Q}_B$ . Kargul et al. demonstrated, on the basis of the flash induced fluorescence decay measurements [20], that the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange induces a slowing down of the  $\text{Q}_A$  to  $\text{Q}_B$  electron transfer. Further, an EPR study [48] showed that the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange possibly induces slight change in the environment of the non-heme iron located between  $\text{Q}_A$  and  $\text{Q}_B$ . Saliently, removal of the Mn and/or Ca ions from PSII induces a ca. 150 mV positive shift of  $E_m(\text{Q}_A/\text{Q}_A^-)$  [23–25]. The structural rationales behind these long distance effect remains to be identified, but a likely hypothesis is that the membrane spanning helices mediate the structural change [23]. In view of these studies and the present result, it can be proposed that the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  exchange probably induces slight structural change on the stromal (cytoplasmic) side. Yet, the extent of these changes is presumably smaller than that induced by the removal of  $\text{Ca}^{2+}$ , and so is the resulting shift of  $E_m(\text{Q}_A/\text{Q}_A^-)$  (+27 mV instead of +150 mV). As regards to  $\text{Cl}^-$ ,  $\text{Cl}^-$ -depletion hardly affects  $E_m(\text{Q}_A/\text{Q}_A^-)$  [25]. Consistent with this, we did not find any change in  $E_m(\text{Q}_A/\text{Q}_A^-)$  upon  $\text{Cl}^-/\text{Br}^-$  exchange.

The consequences of the  $\text{Ca}^{2+}/\text{Sr}^{2+}$  or  $\text{Cl}^-/\text{Br}^-$  exchange on the overall energetics of PSII have been previously discussed on the basis

**Table 2**

Redox potential values of  $\text{Q}_A/\text{Q}_A^-$  and experimental parameters for CaCl-PSII (PsbA3-PSII),  $\text{Ca}^{2+}/\text{Sr}^{2+}$  or  $\text{Cl}^-/\text{Br}^-$  biosynthetically exchanged PSII complexes.

PSII	$E_m(\text{exp})^a/\text{mV}$ vs. SHE	$E_m(R=1)^b/\text{mV}$ vs. SHE	$R (F_m/F_0)$	TL in $\text{S}_2\text{Q}_B^c$
SrCl	-62	-44	5.7	52
CaBr	-88	-69	6.0	50
CaCl	-89	-70	5.9	48

<sup>a</sup> Experimental values obtained by spectroelectrochemistry: The value for SrCl-PSII is an average of two independent measurements,  $-65$  mV and  $-58$  mV; the value for CaBr-PSII is an average of three independent measurements with a S.D. of  $\pm 2$  mV; the value for CaCl-PSII is an average of three independent measurements with a S.D. of  $\pm 2$  mV.

<sup>b</sup> "True" values estimated from  $E_m(\text{exp})$  by considering possible deviation from a linear relationship between the fluorescence yield and the redox state of  $\text{Q}_A$ . The deviations can be estimated on the basis of values of  $F_m/F_0$  (cf. [27,40]).

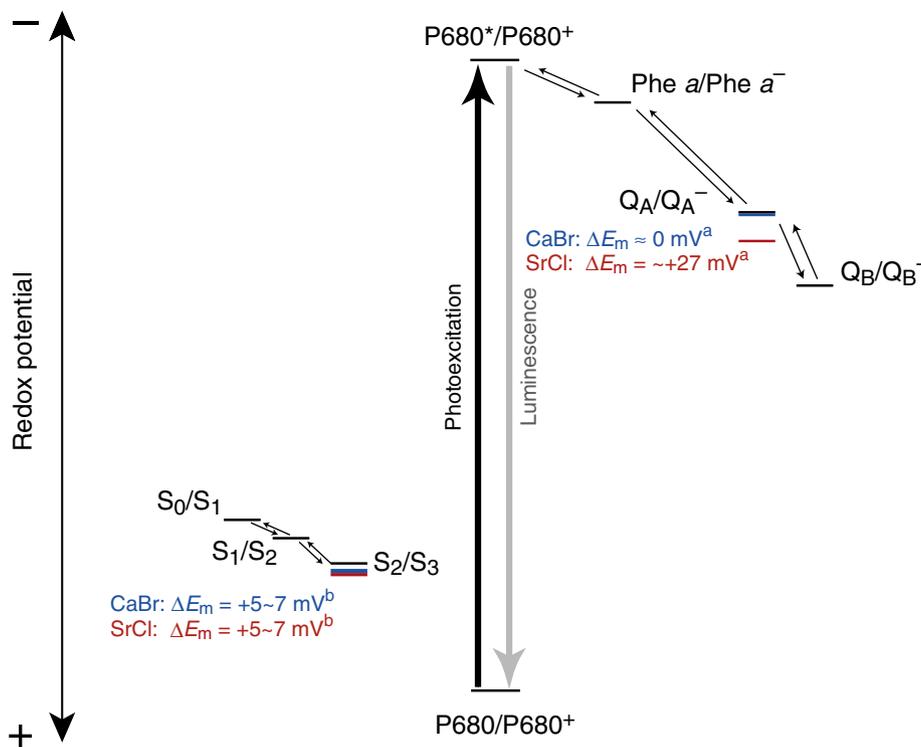
<sup>c</sup> Temperature peak position of the thermoluminescence glow curves from the  $\text{S}_2\text{Q}_B$  charge recombination; the values cited from Ref. [17].

of TL data [17]. However, the here-reported shift of the  $E_m(Q_A/Q_A^-)$  upon  $Ca^{2+}$  to  $Sr^{2+}$  exchange was not known at this time and hence not taken into account. This issue thus needs revision in the light of the present results. According to literature data, the redox potential difference between the  $Q_A$  and  $Q_B$  acceptors in isolated thylakoids is ca. 70 mV [54,55]. Under comparable conditions the temperature difference between the corresponding Q and B TL bands is ca. 20 °C [55]. As regards to the donor side, it has been shown in [17] that, in *T. elongatus* PSII, the TL arising from the  $S_3Q_B^-$  is shifted by 6 °C with respect to that of  $S_2Q_B^-$ , a shift with should reflect the 20 meV increase in energy level of  $S_3$  with respect to  $S_2$  (for a review, see Ref. [56]). These figures lead to an empirical relationship of 0.3–0.4 K meV<sup>-1</sup> (for a recent review on this issue, see Ref. [44]). On the basis of these estimates and of the here-reported shift of the  $E_m(Q_A/Q_A^-)$  one can attempt to draw an accurate picture of the energetic consequences of the  $Ca^{2+}$  to  $Sr^{2+}$  exchange. The TL peak temperature for the  $S_2Q_B^-$  charge recombination has been shown to be up-shifted by  $\approx 2$  °C upon the  $Ca^{2+}/Sr^{2+}$  exchange [17]. Since the redox properties of the  $Mn_4$  cluster in the  $S_2$  state were not modified by this exchange [18] it seems likely that the up-shift by  $\approx 2$  °C results from the contribution of the increase in the  $E_m(Q_A/Q_A^-)$  value in the  $S_2Q_B^-$  charge recombination. Expectedly, this 2 °C shift is smaller than expected if one applies the 0.3–0.4 K meV<sup>-1</sup> coefficient to the 27 mV shift in redox potential because what mostly determines the TL curve in this case is the free energy gap between  $Q_B^-$  and  $Phe a^-$ .

It has been shown that the  $Ca^{2+}/Sr^{2+}$  exchange induces a decrease in the energy level of the  $S_3$  state [17]. In addition it induces an up-shift of the TL peak by  $\approx 4$  °C [17]. Taking into account the 2 °C originating from the contribution of the  $E_m(Q_A/Q_A^-)$  the remaining 2 °C likely reflects the decrease in the energy levels of the  $S_3$  state in SrCl-PSII when compared to CaCl-PSII. Applying the 0.3–0.4 K meV<sup>-1</sup> coefficient, this translates into a  $\approx 5$ –7 mV up-shift in the redox potential of the  $S_3/S_2$  couple in the SrCl-PSII compared to CaCl-PSII. As a consequence,

the  $Ca^{2+}/Sr^{2+}$  or  $Cl^-/Br^-$  exchange effects on the energetics in PSII are summarized in Fig. 3. In  $Ca^{2+}/Sr^{2+}$  and also  $Cl^-/Br^-$  exchanged PSII complexes (SrBr-PSII), the shift of the peak temperature of the TL curve resulting from the  $S_3Q_B^-$  charge recombination is 6 °C [17], which, following the same reasoning as above, translates into a  $(6 - 2)/0.3 - 0.4 = 10$ –14 mV shift of the  $S_3/S_2$  couple redox potential resulting from the CaCl to SrBr substitution. In relation to the result of Kargul et al. [20], our results also suggest that the deceleration of the electron transfer rate between  $Q_A$  and  $Q_B$  induced by the  $Ca^{2+}/Sr^{2+}$  exchange might stem from the decrease in the free energy change of the electron transfer, the extent of which should correspond to the  $E_m(Q_A/Q_A^-)$  difference in the two types of PSII.

We also found out that the  $E_m(Q_A/Q_A^-)$  value of PsbA3-PSII is ca. 40 mV more positive than that of PsbA1-PSII which is dominantly expressed under usual growth conditions for *T. elongatus* (Fig. 1 and Table 1). At first sight, this shift in  $E_m(Q_A/Q_A^-)$  may seem unexpected because  $Q_A$  is bound to PsbD (D2) rather than PsbA (D1). However,  $Q_A$  is linked to the  $Q_B$  binding site, made by the D1 protein, through the  $Q_A$ -His214(D2)-Fe-His215(D1)- $Q_B$  molecular bridge. A FTIR study together with docking calculations [57] suggested that the hydrogen bond strength between D1-His215 and  $Q_B$  influences the hydrogen bond strength between D2-His214 and  $Q_A$  through this molecular bridge, so that any change in the  $Q_B$  site may propagate through this H-bond wire to  $Q_A$  and possibly lead to a shift of  $E_m(Q_A/Q_A^-)$ . Notably, switching from PsbA1 to PsbA3 results in an amino acid substitution at position 270 (Ser in PsbA1, Ala in PsbA3) [31], which might modify the structure of the  $Q_B$  site by changing the hydrogen bond strength with D1-His215, and hence shift  $E_m(Q_A/Q_A^-)$ . As mentioned in [35], D1-S270A substitution may also contribute to loosen the hydrogen bond with the head group of a lipid sulfoquinovosyldiacylglycerol (SQDG) located in the  $Q_B$  site and may be the rationale behind the difference of the binding characteristics of herbicides such as DCMU or bromoxynil to the  $Q_B$  site. In addition, an EPR study [58] indicated that



**Fig. 3.** Schematic diagram for the ground and charge separated states in PSII based on the redox potentials: (a) The differences in the  $E_m(Q_A/Q_A^-)$  values between CaCl-PSII (PsbA3-PSII) and biosynthetically ion-exchanged PSII, SrCl-PSII or CaBr-PSII, were determined by the spectroelectrochemical measurements in the present work; (b) Taking into account a part of the peak temperature difference of the thermoluminescence glow curves for  $S_3Q_B^-$  charge recombination [17] originating from the contribution of the  $E_m(Q_A/Q_A^-)$  shift, the difference in the  $E_m(S_2/S_3)$  values is estimated empirically (for detail, see text).

$E_m$  of the non-heme iron, which is a link in the molecular bridge, is more positive in PsbA1-PSII than in PsbA3-PSII.

It is remarkable that the difference in  $E_m(Q_A/Q_A^-)$  (ca. 40 mV) between PsbA1-PSII and PsbA3-PSII is larger than that of  $E_m(\text{Phe } a/\text{Phe } a^-)$  (17 mV) which is directly, within a hydrogen bond distance, linked to the D1-Q130E substitution. In *T. elongatus*, the PsbA3 (D1) protein that differ by 21 amino acids from PsbA1 is expressed under high light conditions [26,33], and hence this PsaA substitution has been proposed to contribute to photoprotection [33,35,37,42]. Indeed, PSII can undergo photo-damage and this is considered to originate, at least in part, from the production of harmful  $^1\text{O}_2$  accompanying the decay of  $^3\text{P680}$  (for reviews, see Refs. [59–61]). The critical parameter is thus whether or not  $^3\text{P680}$  is formed during charge recombination. It has been pointed out that the decrease in the energy gap between  $\text{Pheo}_{D1}^+\text{Q}_A$  and  $\text{Pheo}_{D1}\text{Q}_A^-$  in PsbA3-PSII resulting from the shift in  $E_m(\text{Phe } a/\text{Phe } a^-)$  induced by the D1-Q130E substitution would make the repopulation of the  $\text{Pheo}_{D1}^+\text{Q}_A$  state easier in PsbA3-PSII than in PsbA1-PSII [59] and thus disfavor the direct charge recombination between  $\text{P680}^{++}$  and  $\text{Q}_A^-$ . Since formation of  $^3\text{P680}$  involves the formation of the  $^3[\text{P680}^{++}\text{Pheo}_{D1}^-]$ , this paradoxically would imply that PsbA3-PSII would be more prone to photo-damage than PsbA1-PSII. To get round this contradiction, it has been proposed that owing to its very large driving force ( $\sim 1.6$  eV), the charge recombination from  $^1[\text{P680}^{++}\text{Pheo}_{D1}^-]$  to  $\text{P680Pheo}_{D1}$  operates in the inverted region of the Marcus curve where the rate of the electron transfer reactions increases when the driving force decreases [59]. This means that the charge recombination from  $^1[\text{P680}^{++}\text{Pheo}_{D1}^-]$  (to the detriment of the population of the  $^3[\text{P680}^{++}\text{Pheo}_{D1}^-]$  state) would be faster in PsbA3-PSII than in PsbA1-PSII. Further, Rutherford et al. [61] recently proposed that the expected accumulated state under high light conditions is  $\text{Pheo}_{D1}^+\text{Q}_A^-$  rather than  $\text{Pheo}_{D1}\text{Q}_A^-$  so that the energy gap between the  $\text{Pheo}_{D1}\text{Q}_A^-$  and  $\text{Pheo}_{D1}^+\text{Q}_A^-$  states would not be the most relevant parameter and that the tuning of the respective yield of the various recombination pathway would reside in the  $^1[\text{P680}^{++}\text{Pheo}_{D1}^-]$  to  $\text{P680Pheo}_{D1}$  charge recombination. In any case the here-reported 40 mV up-shift of the  $E_m(Q_A/Q_A^-)$  associated with the switch from PsbA1-PSII to PsbA3-PSII is an additional parameter that needs being taken into account. This up-shift results in a larger free energy gap between  $\text{Pheo}_{D1}\text{Q}_A^-$  and  $\text{Pheo}_{D1}^+\text{Q}_A^-$  and this will contribute to prevent the formation of the  $^3[\text{P680}^{++}\text{Pheo}_{D1}^-]$  state under light conditions that do not yield the accumulation of  $\text{Pheo}_{D1}^+\text{Q}_A^-$ . In addition, the lower energy level of  $\text{P680}^{++}\text{Phe } a^-$ , resulting from the more positive  $E_m(\text{Phe } a/\text{Phe } a^-)$ , should increase the quantum yield of PSII as shown in the case of site-directed mutants [45,46,62]. Consistent with this, we found a larger  $(F_m - F_0)/F_m$  ratio in PsbA3-PSII than in PsbA1-PSII (0.83 versus 0.73; cf. Table 1). Yet, we acknowledge the fact that the rationale for increasing the quantum yield under high, i.e. non limiting light intensity is not obvious. This may not be a functional requirement but merely the consequence of the fine tuning required to minimize  $^3\text{P680}$  formation. In conclusion, in *T. elongatus*, PsaA3 has many reasons to be preferentially expressed under high light conditions, while it should remain to be cleared whether photoprotection induced by PsaA substitution accompanying the  $E_m(Q_A/Q_A^-)$  shift as shown here for *T. elongatus* may be generalized to other cyanobacteria or not.

In light of the discrepancy between the TL experimental results and the simulation based on the  $E_m(\text{Phe } a/\text{Phe } a^-)$  and  $E_m(Q_A/Q_A^-)$  shifts (Fig. S2), the redox properties of the other redox cofactors might be modified; as pointed out previously [35], the TL intensity also reflects fraction of PSII with  $\text{Q}_B^-$  in the dark, these centers being silent after one flash in TL experiment. Moreover, the addition of DCMU to PSII centers with  $\text{Q}_B^-$  shifts to the right the equilibrium of  $\text{Q}_A\text{Fe}^{\text{II}}\text{Q}_B^- \leftrightarrow \text{Q}_A^-\text{Fe}^{\text{II}}\text{Q}_B$  [58,63,64] resulting in closed reaction centers. An EPR study [58] also demonstrated that upon the addition of DCMU the shift of  $\text{Q}_A\text{Fe}^{\text{II}}\text{Q}_B^-$  toward  $\text{Q}_A\text{Fe}^{\text{III}}\text{Q}_B\text{H}_2$ , silent in TL, also occurred in PsaA3–PSII but not in PsaA1-PSII. These multiple modifications of the redox cofactors as well as the revealed differences in  $E_m(\text{Phe } a/\text{Phe } a^-)$  and in  $E_m(Q_A/Q_A^-)$

likely explain the TL experimental results for the two types of PSII and also contribute to the higher quantum yield.

In any case, the present findings show that the tuning of the redox potentials of cofactors involved in PSII function is exquisitely subtle. Slight structural modifications at the donor side may affect the redox potential of a quinone located more than 25 Å apart and shifting from PsaA3 to PsaA1 have multiple functional consequences. This likely illustrates the necessary shaping, imposed by the oxygen rich environment, of the relative yield of forward and productive reactions, on the one hand, and of the energy wasting ones on the other hand [61]. From a more general stand point, it shows that the rationale behind the gene regulation that control the expression of the *psaA3* and *psaA1* genes in response to different light regimes does not sum up to the effect of a single amino acid substitution and that further studies, including X-ray approaches, will be required to fully understand the physiological significance of this regulation.

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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.bbabi.2012.06.006>.

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