Biochimica et Biophysica Acta 1847 (2015) 276–285

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Assembly of oxygen-evolving Photosystem II efficiently occurs with the apo-Cytb_{559} but the holo-Cytb_{559} accelerates the recovery of a functional enzyme upon photoinhibition

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ARTICLE INFO

Article history:
Received 14 October 2014
Received in revised form 17 November 2014
Accepted 26 November 2014
Available online 3 December 2014

Keywords:
Photosystem II
Cytb_{559}
Haem axial ligands
Photoinhibition

ABSTRACT

Cytb_{559} in Photosystem II is a heterodimeric b-type cytochrome. The subunits, PsbE and PsbF, consist each in a membrane α-helix. Roles for Cytb_{559} remain elusive. In *Thermosynechococcus elongatus*, taking advantage of the robustness of the PSII variant with PsbA3 as the D1 subunit (WT*3), 4 mutants were designed hoping to get mutants nevertheless the obligatory phototrophy of this cyanobacterium. In two of them, an axial histidine ligand of the haem-iron was substituted for either a methionine, PsbE/H23M, which could be potentially a ligand or for an alanine, PsbE/H23A, which cannot. In the other mutants, PsbE/Y19F and PsbE/T26P, the environment around PsbE/H23 was expected to be modified. From EPR, MALDI-TOF and O_2 evolution activity measurements, the following results were obtained: Whereas the PsbE/H23M and PsbE/H23A mutants assemble only an apo-Cytb_{559} the steady-state level of active PSII was comparable to that in WT*3. The lack of the haem or, in PsbE/T26P, conversion of the high-potential into a lower potential form, slowed-down the recovery rate of the O_2 activity after high-light illumination but did not affect the photoinhibition rate. This resulted in the following order for the steady-state level of active PSII centers under high-light conditions: PsbE/H23M ≈ PsbE/H23A << PsbE/Y19F ≤ PsbE/T26P ≤ WT*3. These data show i) that the haem has no structural role provided that PsbE and PsbF are present, ii) a lack of correlation between the rate of photoinhibition and the E_m of the haem and iii) that the holo-Cytb_{559} favors the recovery of a functional enzyme upon photoinhibition.

The light-driven oxidation of water in Photosystem II (PSII) is the first step in the photosynthetic production of most of biomass, fossil fuels and O_2 on Earth. PSII in cyanobacteria is made up of 17 membrane protein subunits and 3 extrinsic proteins. Although the PsbY subunit was not detected in [1] it was seen in [2,3]. Altogether these 20 subunits bear 35 chlorophylls, 2 pheophytins (Phe), 2 haems, 1 non-haem iron, 2 plastoquinones (Q_A and Q_B), a Mn_4CaO_5 cluster, 2 Cl^-, 12 carotenoids and 25 lipids [1]. The excitation resulting from the absorption of a photon is transferred to the photochemical trap that undergoes charge separation. The positive charge is then stabilized on P_{680} which is composed of four chlorophyll *a* molecules, P_{D1}/P_{D2} and Chl_{D1}/Chl_{D2}, and two pheophytin *a* molecules, Phe_{D1}/Phe_{D2}. Then, $P_{680}^{+\bullet}$ oxidizes Tyr_Z, the Tyr161 of the D1 polypeptide, which in turn oxidizes the Mn_4CaO_5 cluster. On the electron acceptor side, the electron is transferred to the primary quinone electron acceptor, Q_A, and then to Q_B, a two-electron and two-proton acceptor, *e.g.* [4–6]. The Mn_4CaO_5 cluster accumulates oxidizing equivalents and acts as the catalytic site for water oxidation. The enzyme cycles sequentially through five redox states denoted S_n where *n* stands for the number of stored oxidizing equivalents. Upon formation of the S_4 state two molecules of water are rapidly oxidized, the S_0 state is regenerated and O_2 is released, *e.g.* [5–12].

Two hemoproteins associate with PSII complex [1]. One is the extrinsic Cytc_{550} found in red algae and cyanobacteria, reviewed in [13], the second is Cytb_{559} that is present in all photosynthetic species and is located in the membrane at the periphery of PSII [1]. Cytb_{559} is a heterodimer with two subunits, α and β (encoded by the *psbE* and *psbF* genes), of ≈ 9 kDa and ≈ 5 kDa, respectively, both having an α-helix spanning

Abbreviations: PSII, Photosystem II; Chl, chlorophyll; MES, 2–(N–morpholino) ethanesulfonic acid; P_{680}, chlorophyll dimer acting as the electron donor; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; Cm, chloramphenicol; Sm, streptomycin; Sp, spectinomycin; 43H, *T. elongatus* strain with a His–tag on the C terminus of CP43; PQ, plastoquinone 9; WT*3, cells containing only the *psbA*$_3$ gene; $Pheo_{D1}$, pheophytin; P_{D1} and P_{D2}, Chl monomer of P_{680} on the D1 or D2 side, respectively; Cyt, cytochrome; PPBQ, phenyl *p*–benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; β–DM, *n*–dodecyl–β–maltoside; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; HP, IP, LP, forms are high-potential, intermediate-potential and low-potential forms of Cytb_{559}

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http://dx.doi.org/10.1016/j.bbabio.2014.11.009
0005-2728/

the thylakoid membrane (Fig. 1A). In the thermophilic cyanobacterium *Thermosynechococcus elongatus*, the two axial ligands of the haem iron are PsbE/His23 and PsbF/His24.

The precise role(s) for these two cytochromes is(are) still today not completely understood despite considerable amount of researches that cannot be all cited here, see however [13–34] and references therein. Among the roles that have been proposed for the Cytb_{559} it has been suggested that it could have a protective role against photoinhibition by taking electron(s) from reduced quinones or Pheo [20–25] thus avoiding the formation of the deleterious singlet oxygen. It has also been proposed that it could be involved in a secondary electron transfer pathway thus avoiding a too large oxidizing power on the electron donor side of PSII [20]. The implication of Cytb_{559} in the repair of PSII after photoinhibition has also been discussed, *e.g.* [34].

Many site-directed mutations in Cytb_{559} have been constructed and studied either in plant PSII, *e.g.* [24], in *Chlamydomonas reinhardtii*, *e.g.* [26–28,34], in the cyanobacterium *Synechocystis* 6803, *e.g.* [29–33] and in *Thermosynechococcus elongatus* [21] (see Fig. S1 in the Supplementary Material for the location of the amino acids already mutated in the literature). One of the challenges was to get mutants with an assembled PSII but lacking the haem with the hope to identify either a structural role for the haem or the electron transfer steps which could be inhibited and therefore would require the haem. Unfortunately, in most of the studied cases, the mutants lacking the haem axial ligands of Cytb_{559} were unable to assemble the PSII making such a study impossible. There was however one exception in which mutants lacking the haem assembled. Indeed, in [26,34] the authors succeeded in making mutants in *C. reinhardtii* on the axial ligand of haem iron of the α subunit: the PsbE/His23Met, PsbE/His23Tyr [26] mutants and the PsbE/His23Cys mutant [34]. Whereas these three mutants could not grow autotrophically, they were able to assemble O_2 evolving PSII up to ≈ 15 % of the wild-type level whereas the purified PsbE/His23Met and PsbE/His23Tyr mutants contained no haem [26] and the PsbE/His23Cys mutant contained a haem at sub-stoichiometric levels [34]. These results lead the authors to conclude that the haem was not absolutely required for the PSII assembly. However, in [26], one question raised by the authors was that the haem content was determined by EPR in purified PSII but not in thylakoids due to the low level of PSII in the membrane. So, since Met and Tyr in [26] and Cys in [34] could be potentially an axial ligand of the haem iron, the possibility that the haem was lost during the PSII purification procedure due to an altered ligation could not be discarded.

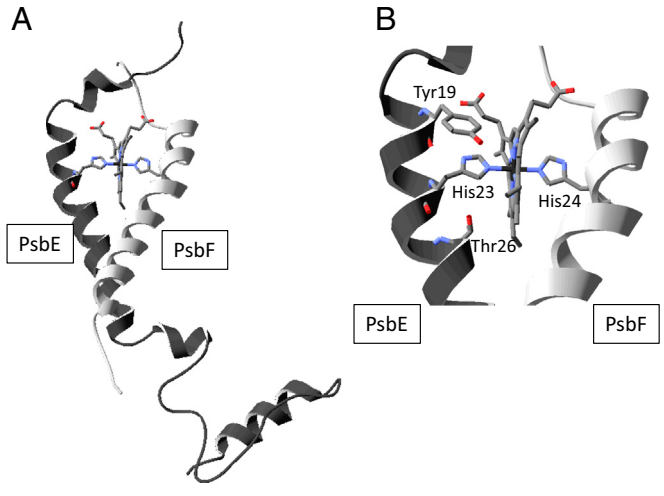

Fig. 1. (A) Structure of Cytb_{559} consists with PsbE, PsbF helicies and a haem from the 1.9 Å model of Umena et al. (1). His23 of PsbE and His24 of PsbF are axial ligand of the haem-Fe. (B) Structure around the haem of Cytb_{559}. Tyr19, His23 and Thr26 of PsbE were amino acid residues that were substituted by Phe, Ala or Met, and Pro for PsbE/Tyr19Phe, PsbE/His23Ala, PsbE/His23Met and PsbE/Thr26Pro, respectively in this study. Structures in Panel A and B were drawn with Swiss Pdb Viewer with the PDB 3ARC structure (1).

It is generally agreed that the oxygen evolution activity in purified PSII from cyanobacteria, in particular from the thermophilic *T. elongatus*, is much more stable than in PSII purified from *C. reinhardtii*. Another advantage of *T. elongatus* is that the structure has been resolved in a very similar cyanobacterium, *T. vulcanus*. However, the great disadvantage of *T. elongatus* is its inability to grow in heterotrophic conditions in contrast to *C. reinhardtii* and *Synechocystis* 6803. Since, so far, this latter species is however unable to assemble PSII in mutants lacking the haem of Cytb_{559} we have nevertheless attempted to make site directed mutants lacking the Cytb_{559} haem in *T. elongatus* with the hope that the robustness of PSII variant with PsbA3 as the D1 subunit would be an asset.

Four mutants have been designed and studied (Fig. 1B). In the PsbE/Tyr19Phe and the PsbE/Thr26Pro mutants, the environment of the PsbE/His23, one of the two haem iron axial ligands, was expected to be modified. Indeed, Tyr19 is involved in the H-bond network around His23 and in the Thr26Pro mutant the PsbE α-helix is expected to be strongly disrupted. In the two other mutants, the PsbE/His23 haem iron axial ligand was substituted for either a Met which potentially could be also a ligand or for an Ala which cannot. We found that the four mutants were able to grow in photoautotrophic conditions. This allowed us to monitor the photoinhibition rate under high-light conditions by measuring the O_2 evolution activity. Moreover, thylakoids and then PSII could be purified with a yield similar to that one in the wild type. This allowed us to study these mutants by MALDI-TOF mass spectrometry and EPR spectroscopies. Indeed, the Cytb_{559} exhibits different redox potential forms: a high-potential (HP) form with a midpoint redox potential (E_m) around +400 mV and forms with lower potentials, ≤ + 280 mV, *i.e.* either intermediate potentials (IP) or low potential (LP), *e.g.* [16,35,36] and references therein). It is of note that each of the IP and LP notations may refer to different potentials depending on the authors. The HP form is labile and easily converted to lower potential forms by treatments that alter the PSII integrity [35,36]. In *T. elongatus* wild type, the E_m of both the HP (≈ + 390 mV) and IP (≈ + 260 mV) forms are not affected by pH, while a pH-dependent behavior has been described for the LP form in plant PSII, [16] and references therein.

At least four EPR forms of Cytb_{559} are detectable; the HP and LP forms both in a non-relaxed and relaxed state, [16,37] and references therein. The non-relaxed HP form of Cytb_{559} with $g_z \approx 3.08$, $g_y \approx 2.16$ and $g_x \approx 1.41$ can be induced by low-temperature (<140 K) illumination of an O_2-evolving sample. The relaxed high-potential form exhibits slightly different g values with $g_z \approx 3.03$, $g_y \approx 2.19$ and $g_x \approx 1.44$ and is induced by an annealing of the sample above 200 K [37]. The non-relaxed LP form of Cytb_{559}, with $g_z \approx 3.05$, $g_y \approx 2.18$ and $g_x \approx 1.46$, is observed in Mn-depleted PSII after illumination at low temperature (<200 K), whereas the relaxed state with $g_z \approx 2.95$, $g_y \approx 2.25$ and $g_x \approx 1.51$ is observed after an annealing of the sample above 200 K. The relaxation process has been interpreted as being possibly a reorientation of the imidazole plan of at least one of the two histidine ligand [37]. Since the IP forms exhibit *g* values intermediate between those of the HP and LP forms there is an apparent correlation between the redox potential of Cytb_{559} and its EPR characteristics, *e.g.* [35,37]; larger is the g_z value higher is the redox potential. This correlation is likely because a high g_z value is synonym of a more distorted structure (*i.e.* a more constrained structure) in the oxidized state, *e.g.* [38] and reference therein. So, the presence of the haem in the four mutants studied here and possibly the redox properties and oxidation level of Cytb_{559} were extrapolated from the EPR characteristics in thylakoids and then in purified PSII.

From these measurements it is confirmed that the haem is not at all required for the PSII assembly since in both the PsbE/His23Met and the PsbE/His23Ala mutants the PSII was fully active whereas the haem (but not the PsbE and PsbF subunits) was lacking. However, in these