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The role of the high potential form of the cytochrome *b*559: Study of *Thermosynechococcus elongatus* mutants

Fernando Guerrero [a,b,1], Jorge L. Zurita [a,b,2], Mercedes Roncel [a], Diana Kirilovsky [b], José M. Ortega [a,*]

[a] Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Américo Vespucio 49, 41092 Seville, Spain
[b] Laboratoire de Bioénergétique Moléculaire et Photosynthèse, Institut de Biologie et de Technologies de Saclay (iBiTec-S), CEA Saclay, 91191 Gif-sur-Yvette cedex, France

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ABSTRACT

Cytochrome *b*559 is an essential component of the photosystem II reaction center in photosynthetic oxygen-evolving organisms, but its function still remains unclear. The use of photosystem II preparations from *Thermosynechococcus elongatus* of high integrity and activity allowed us to measure for the first time the influence of cytochrome *b*559 mutations on its midpoint redox potential and on the reduction of the cytochrome *b*559 by the plastoquinone pool (or Q_B). In this work, five mutants having a mutation in the α-subunit (I14A, I14S, R18S, I27A and I27T) and one in the β-subunit (F32Y) of cytochrome *b*559 have been investigated. All the mutations led to a destabilization of the high potential form of the cytochrome *b*559. The midpoint redox potential of the high potential form was significantly altered in the αR18S and αI27T mutant strains. The αR18S strain also showed a high sensitivity to photoinhibitory illumination and an altered oxidase activity. This was suggested by measurements of light induced oxidation and dark re-reduction of the cytochrome *b*559 showing that under conditions of a non-functional water oxidation system, once the cytochrome is oxidized by $P680^+$, the yield of its reduction by Q_B or the PQ pool was smaller and the kinetic slower in the αR18S mutant than in the wild-type strain. Thus, the extremely positive redox potential of the high potential form of cytochrome *b*559 could be necessary to ensure efficient oxidation of the PQ pool and to function as an electron reservoir replacing the water oxidation system when it is not operating.

1. Introduction

In higher plants, algae and cyanobacteria, the primary steps of photosynthetic light conversion take place in a large pigment–protein complex named photosystem II (PSII), which drives light-induced electron transfer from water to plastoquinone (PQ) with the concomitant production of molecular oxygen (for a review see [1]). The reaction center (RC) of PSII is composed of an heterodimer formed by the D1 and D2 membrane proteins that bears all the cofactors needed for charge separation and stabilization: six chlorophylls (Chl) (including four of the Chl center, P680), two pheophytins, two plastoquinones Q_A and Q_B, one non-heme iron atom, one tetranuclear manganese cluster (Mn_4CaO_5) and two redox active tyrosines. A hemeprotein, named cytochrome *b*559 (Cyt *b*559), appears also tightly bound to RC in all photosynthetic oxygen-evolving organisms.

Cyt *b*559 is one of the essential components of PSII RC, but its function although widely investigated, still remains unclear (for a review see [2,3]). The resolution of the 3-D structure of PSII has clearly established that Cyt *b*559 is a heme-bridged protein heterodimer with two subunits (α and β, encoded by *psbE* and *psbF* genes) of 9 and 4 kDa, respectively [4–7]. Each polypeptide chain forms a transmembrane α-helix, the heme being located near the stromal side and perpendicular to the membrane plane. A bis-histidine axial coordination for the heme is largely supported by these structural studies.

Cyt *b*559 is a *b*-type cytochrome with peculiar physicochemical properties. It is found in PSII preparations in three different states: a high potential form (HP) with a midpoint redox potential (E_m) of ≈ +400 mV, another intermediate potential form (IP) with an E_m of ≈ +200 mV and a low potential form (LP) whose E_m ranges from +80 and 0 mV depending on the organisms [3,8–12]. Many authors have proposed that the HP

Abbreviations: B band, thermoluminescence emission due to $S_{2/3}Q_B^-$ charge recombination reaction; Chl, chlorophyll; Cm, chloramphenicol; CP43, a proximal antenna complex of photosystem II; Cyt, cytochrome; E_h, redox potential of the solution; E_m, midpoint redox potential; HP, IP and LP, high, intermediate and low potential forms; HTL, high temperature thermoluminescence; MES, 2-(N-morpholino) ethanesulfonic acid; OEC, oxygen evolving complex; P680, primary electron donor in PSII; PSII, photosystem II; Q_A and Q_B, the primary and secondary quinone acceptors of the reaction center of PSII; RC, reaction center; S_2 and S_3, oxidized states of the manganese oxygen-evolving complex of PSII; Sm, streptomycin; Sp, spectinomycin; TL, thermoluminescence

* Corresponding author. Tel.: +34 954489573; fax: +34 954460065.
E-mail addresses: Fernando.guerrero@helsinki.fi (F. Guerrero), jorge.zurita@juntadeandalucia.es (J.L. Zurita), mroncel@us.es (M. Roncel), kirilovsky@cea.fr (D. Kirilovsky), ortega@us.es (J.M. Ortega).
[1] Present address: Institute of Biotechnology, University of Helsinki, Viikinkaari 1, 00014 Helsinki, Finland.
[2] Present address: Fundación Progreso y Salud, Junta de Andalucía, Américo Vespucio 5, 41092 Seville, Spain.

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form is the physiological and functional state of Cyt *b*559 [3,10]. However, the HP form is labile and easily converted to the LP/IP forms by treatments that alter the membrane structure or during the process of PSII isolation [8,10]. In plants, algae and cyanobacteria, different kinds of PSII preparations with very variable percentages of the HP form, peaking at about 80% have been described [8,10,11,13,14].

A great number of hypotheses have been put forth in order to explain the function of Cyt *b*559. Most of them propose that this protein may participate in secondary electron transfer pathways protecting PSII against oxidative damage [15–18]. In these proposals, Cyt *b*559 would donate electrons, via a β-carotene molecule, to reduce highly oxidizing chlorophyll radicals generated in PSII RC under donor-side photoinhibitory conditions [17,19,20]. On the other hand, Cyt *b*559 may accept electrons from the acceptor side of PSII (Q_B or PQ) to prevent the formation of damaging singlet oxygen species under acceptor-side photoinhibitory conditions [8,21,22].

Different new roles as oxygen reductase, superoxide reductase, superoxide oxidase and plastoquinol oxidase have been recently proposed for Cyt *b*559 [12,22–25]. The role as plastoquinol oxidase, taking electrons from the plastoquinone pool, has been supported by the existence of a third quinone, Q_C, different from the quinone sites, Q_A and Q_B, at 15 Å only from the heme of Cyt *b*559 [7]. Some authors have postulated the existence of three quinones, besides Q_A, in PSII core complex preparations of *Thermosynechococcus (T.) elongatus* [26,27]. A function for Q_C associated with a secondary electron transfer along the Cyt *b*559 in PSII has been proposed by Guskov et al. [7]. Therefore, a role for Cyt *b*559 as part of a connecting channel between the plastoquinone pool and Q_B and Q_C sites would be possible.

Most of the site-directed mutagenesis studies of the α- and β-subunits of Cyt *b*559 have been carried out in *Chlamydomonas reinhardtii* and *Synechocystis* sp. PCC 6803. No stable PSII was assembled when the heme-coordinating histidines were replaced by leucines [28], neither when the histidine of the α-subunit was replaced by tyrosine or methionine [29], or in deletion mutants of the α-subunit [30] and of both α- and β-subunits [31]. In fact, all these mutants were impaired in the functional PSII assembly and therefore not useful for determining the function of Cyt *b*559 in PSII.

Recently, a set of site-directed mutants of Cyt *b*559 in *Synechocystis* sp. PCC 6803 that grew photoautotrophically and accumulated stable PSII RC's has been constructed [14,32,33]. Mutants with changes on heme coordination (His22 of the α- or β-subunit) and on charged residues of the cytoplasmic side of Cyt *b*559 (R7Eα, R17Eα and R17Lβ) showed a higher sensitivity to photoinhibition than the WT strain. These results have led the authors to propose a functional role of Cyt *b*559 in protection of PSII under photoinhibition conditions [14]. They also suggested that the electrostatic interactions between these arginine residues and the heme propionates of Cyt *b*559 are essential in the maintenance of the structure and redox properties of the cytochrome. However, the *Synechocystis* sp. PCC 6803 strain used in this work seems to present an important limitation for the study of the Cyt *b*559 role because the PSII preparations contained very low proportions of the HP form [14,33].

The thermophilic cyanobacterium *T. elongatus* has become in the last years a new model organism for photosynthesis researches, since genetic engineering techniques have been developed [34,35] and several 3-D structures of PSII have been solved from it [4,6,7,36]. In 2003, our group was already able to obtain PSII preparations from *T. elongatus*, 50% containing the HP form of cytochrome *b*559 and 50% an IP form, both in their reduced state [11]. No LP form was present in these preparations. The HP form in *T. elongatus* showed the typical redox properties of the HP form in higher plants: (1) it has a highly oxidizing E_m, which is not affected by pH [11] and (2) it is partially converted into a form of lower potential (IP) by treatments such as washing with Tris or gentle heating [11]. Recently, PSII preparations with a higher degree of integrity and activity have been prepared in our groups using a new His-tagged CP43 *T. elongatus* strain ([37] and this work). This is essential to study the properties of the Cyt *b*559 HP form. The high rates of O_2 evolution obtained in these preparations were good indicators of the integrity of the donor side of PSII ([37] and Table 1). The 1:1 ratio of the concentration of Cyt *b*559 regarding Cyt *c*550 [37] indicates that Cyt *c*550 is not released from PSII during the isolation process, also a good indicator of donor side integrity. The most important characteristic of these isolated PSII complexes is that 85% of them presented Cyt *b*559 in the reduced HP form with an E_m value of about +400 mV. Thus, our PSII preparations are an excellent material for the study of the effect of changes in the E_m values and on the percentage of HP/IP forms on the function of Cyt *b*559.

A series of site-directed mutants of Cyt *b*559, each carrying a single amino acid substitution, has been constructed in the thermophilic cyanobacterium *T. elongatus*, in order to modify the redox potential of the heme without altering the assembly properties of PSII. In this work we have analyzed in depth six of these mutant strains: five in the α-subunit (I14A, I14S, R18S, I27A and I27T) and one in the β-subunit (F32Y). All mutants grew photoautotrophically and assembled functionally PSII RC. For the first time, the redox potential of site-directed mutants of Cyt *b*559 has been determined. The E_m of the HP form of Cyt *b*559 was significantly altered in two of the mutant strains: αR18S and αI27T. The αR18S strain also showed a high sensitivity to photoinhibitory illumination. The results support the proposal that the HP form of Cyt *b*559 may function in a secondary electron transfer cycle in order to protect PSII when the water oxidation system is impaired.

2. Material and methods

2.1. Construction of plasmids for the mutation of cytochrome b559

The genome region containing the genes *psbE*, *psbF*, *psbL* and *psbJ* (forming an operon) (Fig. 1) was amplified by PCR using the oligonucleotides *cytb559BssHIIa* and *cytb559BssHIIb* (Supplementary Table I). The amplified region which includes part of the *tlr1540* gene encoding a serine–threonine kinase was digested by BssHII and cloned. The *tlr1540* gene was interrupted by insertion of a 2.2 kb DNA fragment containing the *aadA* gene from Tn7, conferring resistance to spectinomycin (Sp) and streptomycin (Sm) [38], in the unique KpnI restriction site of the *tlr1540* gene. Site-directed mutagenesis of the plasmid containing the interrupted *tlr1540* gene was performed using the QuikChange XL site-directed mutagenesis kit of Stratagene as recommended by the manufacturer. The synthetic mutagenic oligonucleotides used to create the different point mutations in the *psbE* and *psbF* genes (encoding for the α and β-subunits of the Cyt *b*559, respectively) are shown in Supplementary Table II. The mutagenic oligonucleotides also introduced a silent mutation creating or eliminating restriction enzyme sites for mutant screening.

2.2. Transformation of T. elongatus cells and genetic analysis of mutants

The construction of WT' strain of *T. elongatus*, containing a His-tagged CP43 for isolation of PSII complexes with high activity, was described in Guerrero et al. [37]. The plasmids containing the site-mutated *psbE* and *psbF* genes were introduced in WT' by electroporation according to Mühlenhoff and Chauvat [34] with slight modifications as described in [39]. To create the control strain WT", a plasmid containing the *psbE* and *psbF* genes (without mutations) and the *tlr1540* gene interrupted by the Sp/Sm resistance cassette was introduced into WT' cells.

After electroporation, cells were rapidly transferred to 2 ml of DTN-medium [34] and incubated for 48 h in a rotary incubator at 45 °C under low light conditions. Then, the cells in 0.1–0.2 ml aliquots were spread on plates containing chloramphenicol (Cm) (2 μg ml^{-1}) or Sp/Sm (12 μg ml^{-1}/6 μg ml^{-1}) and incubated at 45 °C, under dim light and a humidified atmosphere. Once green