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Biochimica et Biophysica Acta 1708 (2005) 333 – 341
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Extinction coefficients of cytochromes b559 and c550 of *Thermosynechococcus elongatus* and Cyt b559/PS II stoichiometry of higher plants

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Received 9 December 2004; received in revised form 21 March 2005; accepted 3 May 2005
Available online 26 May 2005

Abstract

"Reduced minus oxidized" difference extinction coefficients $\Delta\varepsilon$ in the α-bands of Cyt b559 and Cyt c550 were determined by using functionally and structurally well-characterized PS II core complexes from the thermophilic cyanobacterium *Thermosynechococcus elongatus*. Values of 25.1 ± 1.0 mM^{-1} cm^{-1} and 27.0 ± 1.0 mM^{-1} cm^{-1} were obtained for Cyt b559 and Cyt c550, respectively. Anaerobic redox titrations covering the wide range from −250 up to +450 mV revealed that the heme groups of both Cyt b559 and Cyt c550 exhibit homogenous redox properties in the sample preparation used, with E_m values at pH 6.5 of 244 ± 11 mV and -94 ± 21 mV, respectively. No HP form of Cyt b559 could be detected. Experiments performed on PS II membrane fragments of higher plants where the content of the high potential form of Cyt b559 was varied by special treatments (pH, heat) have shown that the α-band extinction of Cyt b559 does not depend on the redox form of the heme group. Based on the results of this study the Cyt b559/PSII stoichiometry is inferred to be 1:1 not only in thermophilic cyanobacteria as known from the crystal structure but also in PSII of plants. Possible interrelationships between the structure of the Q_B site and the microenvironment of the heme group of Cyt b559 are discussed.

Keywords: Photosystem II; Cyt b559; Cyt c550; Redox potential; Extinction coefficient

1. Introduction

Photosytem II (PSII) is a multimeric pigment–protein complex anisotropically incorporated into the thylakoid membrane and acting as water-plastoquinone oxidoreductase. The overall pattern of this complex has been thoroughly analysed during the last three decades (see Refs. [1–3] for recent reviews). On the other hand, detailed structural information became available only recently when suitable crystallization of PSII core complexes from the thermophilic cyanobacteria *Thermosynechococcus (T.) elongatus* and *T. vulcanus* was successful and X-ray structure analysis performed [4–7]. These data confirm that the cofactors required for PQH_2 formation and oxidative water cleavage are incorporated into a heterodimeric protein matrix consisting of polypeptides D1 and D2. Apart from the components of the main electron transport pathway, all PSII complexes contain cytochrome b559 (Cyt b559) with a heme group axially coordinated by the two histidine residues provided by the polypeptides PSII-E and PSII-F (for reviews, see Refs. [8–11]).

The PSII of oxygen evolving cyanobacteria contains a second type of a bis-His ligated heme group which is covalently bound to polypeptide PSII-V. This heme con-

Abbreviations: PS II, photosystem II; Cyt, cytochrome; WOC, water-oxidizing complex; Chl, chlorophyll; Car, carotenoid; Pheo, pheophytin; E_m, midpoint potential; HP, high potential; IP, intermediate potential; LP, low potential; $\Delta\varepsilon$, difference "reduced minus oxidized" extinction coefficient; MES, 2-[N-Morpholino]ethanesulfonic acid; β-DM, n-dodecyl β-D-maltoside; FWHM, full width at half maximum

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doi:10.1016/j.bbabio.2005.05.002

taining subunit called Cyt c550 exerts regulatory functions on the water-oxidizing complex (WOC) in cyanobacteria [12,13]. It has been replaced by a nonheme subunit during the evolutionary development [13,14] without significantly affecting the reaction coordinates of oxidative water cleavage [15,16]. As a result of this event, Cyt b559 is the only heme protein in PSII complexes of plants.

Cyt b559 is characterized by a striking heterogeneity of its redox properties. Typical values of the midpoint potentials reported in the literature for the different redox forms are about +400 mV (high potential form, HP), +250 mV (intermediate potential form, IP) and +100 mV (low potential form, LP) [17–23]. Among these forms, the HP Cyt b559 with its unusually high reduction potential is unique for b type cytochromes (for a review, see Ref. [24]). HP Cyt b559 dominating in intact chloroplasts and thylakoids is very labile and prone to transformation into lower potential form(s). As a consequence, this form is already missing at the level of PS II core complexes from higher plants [22]. HP Cyt b559 is probably more stable in *T. elongatus* because solubilized PS II core complexes from this organism were recently reported to be characterized by a significant content of this form [25].

The molecular basis of the different redox forms of Cyt b559 is not yet clarified, although several proposals have been made to explain the origin of this striking phenomenon: mutual orientation of the planes of the His ligands [26], the pattern of H-bonding of the axial ligands [23,27], the mode of their protonation [17,23,28] or the nature of the heme coordination [22]. Likewise, the function of Cyt b559 is not fully understood [8–11]. It seems most likely that Cyt b559 participates in a cyclic electron flow with a protective role to photoinhibition, especially for developing PSII under greening conditions where a functionally competent WOC is not yet established [10,11,29–31]. The function of Cyt b559 as both alternative electron donor and acceptor in PSII was demonstrated in a recent study [31], where dehydration of spinach PSII membrane fragments was shown to block electron transfer from Q_A^- to Q_B and water oxidation at the acceptor and donor side, respectively, thus giving rise to a pronounced light induced turnover of Cyt b559. The structural and functional relevance of Cyt b559 has been recently nicely illustrated for the example of a single site mutation in the PSII-F subunit. The replacement of residue Phe 26 by Ser was shown to induce marked ultrastructural changes (much less grana formation), a drastic decrease of the PSII content and dark reduction of the PQ pool [32].

Apart from the problems in unravelling the function of Cyt b559 and the relevance of the different redox forms, an apparently much simpler question was a matter of controversial discussion during the last three decades: how many copies of Cyt b559 are bound to a native PSII complex? Values in the range between 1 and 2 were obtained for the Cyt b559/PSII stoichiometry in different oxygen evolving PSII preparations (see reviews [8–11] and Refs. [22,33]). One of the major obstacles for an unambiguous answer of this question is the determination with sufficient precision of the difference extinction coefficient $\Delta\varepsilon$ of Cyt b559 bound to PSII complexes. Based on measurements of reduced minus oxidized difference spectra in the α-band, $\Delta\varepsilon$ values of Cyt b559 were determined for spinach Cyt b559 attaining the LP redox form either in the isolated heme protein [8,34–36] or bound within D1–D2–Cyt b559 complexes and PS II core complexes [36]. The following numbers are reported: $\Delta\varepsilon^{red}$ (559–570)=15 mM^{-1} cm^{-1} [8], $\Delta\varepsilon^{red}$ (559–600)=21 mM^{-1} cm^{-1} [34], $\Delta\varepsilon$ (559.5–570)=17.5 mM^{-1} cm^{-1} [35] and $\Delta\varepsilon$ (559–543,575)[1]=23.4 mM^{-1} cm^{-1} [36]. For all calculations on the stoichiometry presented so far, the $\Delta\varepsilon$ values were assumed to be the same for the different redox forms of Cyt b559.

A breakthrough in obtaining an unambiguous Cyt b559/PSII stoichiometry was the X-ray structure analysis of PSII core complexes from *T. elongatus* [4,6,7] and *T. vulcanus* [5]. It was found that these thermophilic cyanobacteria contain a single Cyt b559 per PSII monomer. This 1:1 stoichiometry, however, must not necessarily pertain in PSII of other oxygen evolving organisms (for discussion, see Ref. [31])

Therefore, the present study addresses the problem of Cyt b559/PS II stoichiometry in higher plants. Factors that might be responsible for controversial results on the Cyt b559/PS II stoichiometry are thoroughly analysed. On the basis of the $\Delta\varepsilon$ value of "reduced minus oxidized" Cyt b559 determined for highly purified PS II core complexes from *T. elongatus*, we conclude that in higher plant PS II membrane fragments the Cyt b559/PS II ratio is close to 1. In addition to this major topic also the difference extinction coefficient of "reduced minus oxidized" Cyt c550 is reported for the first time for the cyanobacterium *T. elongatus*.

2. Materials and methods

PS II membrane fragments were obtained from sugar beet according to the method of Berthold et al. [37] with some modifications [38]. All assays with these samples were performed in a buffer containing 100 mM MES (pH 6.5), 0.4 M sucrose, 15 mM NaCl, 10 mM $CaCl_2$ and 10% glycerol (standard medium).

For a conversion of the HP Cyt b559 into lower potential redox forms, the procedures of high pH treatment [22] or heat treatment were carried out. In the first case, oxygen-evolving PS II membrane fragments were incubated at room temperature for 15 min in a medium containing 25 mM CHES (pH 9.2), 0.4 M sucrose, 15 mM NaCl, 10 mM $CaCl_2$

[1] The absorbance in the maximum was taken relative to a straight line connecting the points at 543 and 575 nm.

and 0.5 mM $K_3[Fe(CN)_6]$. The pH of this suspension was subsequently shifted back to pH 6.5 by the addition of 150 mM MES buffer. Finally, the sample was washed twice in a standard buffer medium. For heat treatment, samples of PS II membrane fragments were incubated for 10 min in a water bath at 55 °C in the dark.

Oxygen-evolving PS II core complexes from the thermophilic cyanobacterium *T. elongatus* were isolated by using β-DM for solubilization and ion exchange chromatography as outlined in detail in Ref. [39]. PS II core complexes were isolated in form of homodimers. The assays were performed in a buffer containing 100 mM MES (pH 6.5), 0.3 M sucrose, 10 mM NaCl, 10 mM $CaCl_2$ and 0.02% β-DM.

Difference absorbance spectra of Cyt b559 and Cyt c550 in their α-bands were measured in a Cary 4000 spectrophotometer with an optical slit width of 2 nm in case of PS II core complexes or 2.5 nm in case of PS II membrane fragments. The optical path length was 1 cm. The amplitudes of the α-band signals of Cyt c550 (at 549 nm) and Cyt b559 (at 560 nm) were determined relative to lines connecting the points 535 nm, 570 nm and 543 nm, 573 nm, respectively. Difference extinction coefficients of Cyt b559 and Cyt c550 were calculated on the basis of a Chl/PS II ratio of 36 in preparations of PS II core complexes from *T. elongatus* [6,7,39]. The chlorophyll *a* concentration in the samples was determined on the basis of $\Delta\varepsilon_{663.6-750}=76.8$ mM^{-1} cm^{-1} of Chl a in 80% acetone according to Ref. [40]. The average number of Chls per PS II of 230±20 in PS II membrane fragments from sugar beet was taken from the data obtained for analogous spinach preparation by measurements of the flash induced oxygen yield under repetitive excitation [41]. This value is in agreement with most reports in the literature [42–45].

Anaerobic redox titrations of Cyt b559 and Cyt c550 were performed at 20 °C by measuring difference absorbance spectra at different ambient redox potentials of the suspension [22] in the presence of the following set of redox mediators: 1,4-benzoquinone, 2,3,5,6-tetramethyl-p-phenylenediamine, 2,5-dimethyl-1,4-benzoquinone, $FeSO_4$–EDTA complex, 2-methyl-1,4-naphtoquinone, 2-methoxy-1,4-naphtoquinone and anthraquinone-2-sulfonate at concentrations of 50 μM; 2-hydroxy-1,4-naphtoquinone at a concentration of 20 μM; N-methylphenazonium methosulfate, N-methylphenazonium ethosulfate and 2-amino-3-chloro-1,4-naphtoquinone at concentrations of 10 μM; benzyl viologen at a concentration of 3μM. Both reductive and oxidative redox titrations were performed in each experiment. The initial concentration of $K_3[Fe(CN)_6]$ in reductive titration of Cyt b559 was 0.5–1 mM. Anaerobic conditions were achieved by addition of 0.25 mg/ml glucose oxidase, 0.05 mg/ml catalase and 5 mM glucose in the sample medium and by a constant flow of argon through the spectroscopic cuvette above the sample solution. Potentiometric Nernst curves were analysed by a nonlinear curve-fitting Origin program.

3. Results

The "reduced minus oxidized" difference spectrum of isolated Cyt b559 in the α-band is characterized by a peak at 559 nm and isobestic points at 538 nm, 548 nm and 570 nm ([35], see also [8,34]). A precise determination of zero points of this difference spectrum, however, is complicated for Cyt b559 bound to the PS II complex because of usual baseline distortions due to possible contributions from other pigments (Chl, Car, Pheo) and/or caused by particles sedimentation. It is clear that the choice of appropriate reference points for the baseline is of high significance in order to account properly for these effects and to diminish their contributions. Therefore, we carefully examined the difference spectra of PS II samples measured at different redox potentials.

Fig. 1 presents typical "reduced minus oxidized" difference spectra of intact PS II membranes in the wavelength region from 520 to 590 nm, recorded in the course of the reductive titration of Cyt b559 and with the absorbance spectrum measured at +480 mV as the reference. The data show that in a wide range of redox potentials from +480 mV to +40 mV, the reduction of Cyt b559 takes place and gives rise to a progressive absorbance increase with the main peak at 560 nm. It is also seen that at redox potentials lower than about +100 mV the reduced minus oxidized difference spectrum of Cyt b559 is distorted by two types of additional signals, i.e., the bending of the baseline and the appearance of a derivative-like bandshift at the blue end of the spectrum (curves d and e in Fig. 1). The baseline bending artefact also reported in previous studies (see [18,22,44,46]) represents

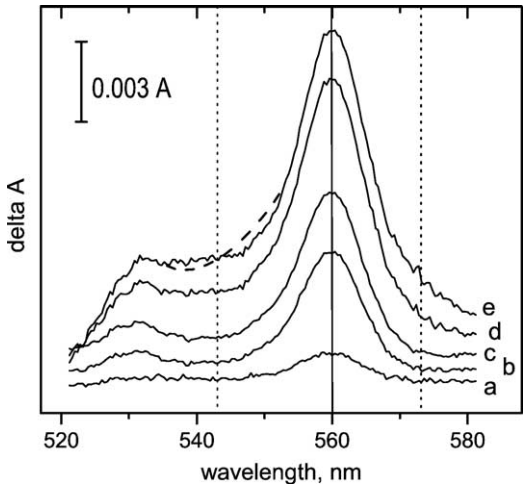

Fig. 1. Reduced minus oxidized difference spectra of Cyt b559 in suspension of untreated PS II membrane fragments from sugar beet. The spectra were recorded at redox potentials of +435 mV (trace a), +378 mV (trace b), +346 mV (trace c), +123 mV (trace d) and +41 mV (trace e) using the absolute absorbance spectrum of the sample at +480 mV as baseline. The decrease of the redox potential was achieved by progressive addition of sodium dithionite. Chlorophyll concentration was 61 μg/ml. Vertical lines are drawn at 543 nm, 560 nm and 573 nm. The bold dashed line in curve e represents the contribution of the "C550" that emerges at low redox potentials (see text for detail).

an irreversible absorbance increase in the broad range of 520–590 nm (data not shown, see Ref. [46]). The derivative shaped signal arising at low redox potentials is ascribed to the so-called C550 signal, that originates from the electrochromic bandshift of pheophytin due to the negatively charged semiquinone $Q_A^{-\cdot}$ of the reduced primary plastoquinone acceptor of PSII [47–49]. It was shown that in PS II membrane fragments incubated at low redox potentials in the presence of redox dyes the primary acceptor Q_A is slowly transformed to a high potential form [50]. Accordingly, the Q_A reduction occurs at higher redox potentials and the "C550 signal" will interfere with the reduction of LP Cyt b559. This phenomenon is illustrated in curves d and e of Fig. 1. To minimise a possible interference of the "artefact" signals with the measured amplitude of the difference spectrum due to Cyt b559 reduction, we choose a pair of reference points at 543 nm and 573 nm for constructing the baseline. Similar reference points at 543 nm and 575 nm were used in former determinations of $\Delta\varepsilon$ of Cyt b559 bound to the PS II complex [36].

In addition to spectral artefacts that interfere with the difference spectrum of PS II-bound Cyt b559, another reason for a marked variation of data published for the Cyt b559/PS II stoichiometry has to be taken into consideration. The different redox forms of Cyt b559 (essentially three forms in PSII membrane fragments) could significantly differ in their $\Delta\varepsilon$ values of the α-band. In order to address this important problem, experiments were performed with samples that markedly differ in their content of the various redox forms of Cyt b559.

Fig. 2 presents a comparison of the amplitudes obtained for full reduction of Cyt b559 in two types of PS II membrane fragments, one type of preparation with a high content of the HP Cyt b559 form (curve a) and the other completely deprived of this redox form by heat pretreatment at 55 °C (curve b). In a good correspondence with published data [18,21,22], curve a is fitted by a sum of the three one-electron transitions characterized by E_m values of +391 mV, +241 mV and +82 mV and relative contributions of 75%, 18% and 7%, respectively. Curve b shows data of the analogous redox titration of PS II membrane fragments pretreated at 55 °C. In a perfect agreement with previous reports [51,52], we found that this treatment leads to a complete transformation of HP Cyt b559 into lower potential forms. The fitting of the curve b reveals that the data are described by the sum of the two one-electron transitions with E_m values of +240 mV (IP form) and +129 mV (LP form) and relative contents of 38% and 62%, respectively. A comparison of curves a and b of Fig. 2 reveals that the maximum amplitudes attained are almost identical.

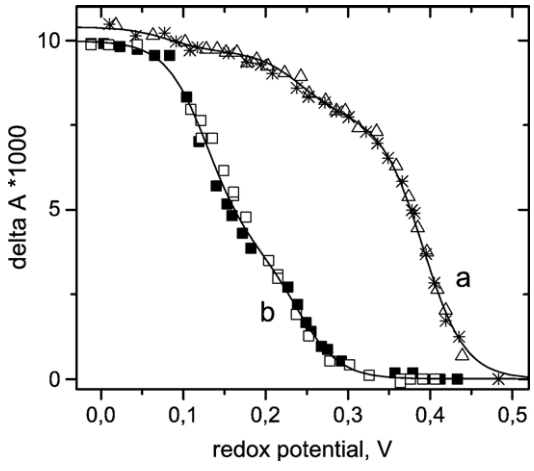

Fig. 2. Amplitude in the maximum at 560 nm of the Cyt b559 difference spectrum as a function of the ambient redox potential in suspensions of untreated (a) and heat treated (b) PS II membrane fragments from sugar beet. The absorbance spectrum of the samples at a redox potential of +480 mV was taken as baseline. Stars and filled squares represent data points for reductive titrations and open symbols for oxidative titrations. For the full-lined fit curves of the data, see text. Details of heat treatment are outlined in Materials and methods. Chlorophyll concentration was 75 μg/ml.

Table 1 summarizes the statistics of the data shown in Fig. 2 and presents in addition the results of similar measurements with PS II membrane fragments that are lacking the HP form of Cyt b559 due to a pretreatment at pH 9.2 (see Materials and methods for details). These samples closely resemble heat treated samples in the population of the redox forms of Cyt b559. Based on the data of Table 1, the $\Delta\varepsilon$ values of the different redox forms of Cyt b559 are inferred to be virtually identical (within the limits of the experimental error).

The PSII E and PSII F subunits of Cyt b559 from cyanobacteria and higher plants exhibit striking sequence homologies [10] and the heme group similar redox and spectral properties [8,18,21–23,25,44]. Furthermore, the experiments of the present study show that $\Delta\varepsilon$ of Cyt b559 in higher plant PS II complexes is virtually invariant to the change in the E_m value of the heme group by as much as 150–250 mV. Taking into account these findings it seems reasonable to assume that the $\Delta\varepsilon$ values are very similar for Cyts b559 from cyanobacteria and higher plants. This conclusion offers the possibility to address the problem of the Cyt b559/PSII stoichiometry in plants on the basis of the unambiguous $\Delta\varepsilon$ values gathered from thermophilic cyanobacteria.

Isolated PS II core complexes from *elongatus* provide the most appropriate sample material for a straightforward determination of the value of $\Delta\varepsilon$ of Cyt b559. This sample type is used for growing 3D crystals and presents the currently most purified and structurally best-characterized preparation of PS II [4,6,7,39]. As, in contrast to higher plants, the PS II complexes of cyanobacteria contain two types of bound cytochromes – Cyt b559 and Cyt c550 – the spectral and redox properties of both heme proteins were characterized and their $\Delta\varepsilon$ values determined.

Fig. 3 shows typical redox titration curves of Cyt b559 in solubilized PS II core complexes from *T. elongatus*. An inspection of the figure reveals a single one-electron

Table 1
Amplitude of full reduction of Cyt b559 in samples of PS II membrane fragments with different Cyt b559 redox composition

PS II membrane sample	Relative content of redox forms of Cyt b559, %[a]			ΔA_{559}, optical units *1000[a]
	HP	IP	LP	
Untreated	74±3	17±3	9±4	10.4±0.6
Heat-treated[b]	0	41±6	59±6	10.1±0.3
High pH-treated[b]	0	35±10	65±10	10.2±0.5

[a] The values of relative amplitudes of HP redox form and of the total redox absorbance difference of Cyt b559 represent the average of six experiments.
[b] For the details of the treatment procedures, see Materials and methods. Chlorophyll concentration of the samples was adjusted to 75 μg/ml.

Nernstian redox wave characterized by a midpoint potential E_m of +240 mV. The average value for E_m obtained from five different redox titration experiments was +244±11 mV. This value corresponds to the IP (intermediate potential) redox form of Cyt b559 identified in redox titrations of PSII preparations from higher plants (see Fig. 2 of the present work and Refs. [18,21,22]). No other redox forms of Cyt b559 could be detected in the wide range of redox potentials from −250 mV up to +450 mV.

At redox potentials below 0 mV, the reduction of Cyt c550 takes place. In the spectral region close to its α-band maximum, there exist small spectral contributions owing to the electrochromic C550 signal. Recently the E_m value of the redox couple $Q_A/Q_A^{-\cdot}$ in *T. elongatus* PS II core complexes was determined to be −20 mV at pH 6 [25]. Therefore, redox changes of Q_A are expected to occur in the range of the titration of Cyt c550. In our preparation of *T. elongatus*, the derivative-like electrochromic signal induced by $Q_A^{-\cdot}$ formation is characterized by a small trough at 548 nm and an insignificant absorbance increase at 541 nm (not shown). Accordingly, this feature leads to a distortion of the Cyt c550 "reduced minus oxidized" difference absorbance spectrum and may slightly decrease its α-band absorption. To eliminate this effect, we used an approach described for spinach PS II membranes [50]. Anaerobic samples were incubated in presence of mediators for 15 min at redox potentials below −100 mV before starting the redox titration of Cyt c550. This procedure induced a shift in the E_m of Q_A to higher potentials, i.e., in the treated samples the redox transition of Q_A took place at redox potentials above +100 mV (not shown) and therefore the overlapping with the electrochromic signal is eliminated within the region of Cyt c550 redox titration. Fig. 4 presents a typical redox titration curve for Cyt c550. An average value of −94±21 mV for the E_m was gathered from three different titration experiments.

The determination of extinction coefficients of specific absorbing species requires the knowledge of both the amplitude of absorbance of an individual chromophore and the value of its concentration in the absorbing sample. The amplitudes of redox difference absorbance changes in the α-bands of Cyt b559 and Cyt c550 of *T. elongatus* samples were determined from the full redox titration curves. The cofactor concentration of heme containing proteins is usually derived from measurements of the corresponding pyridine hemochrome spectra [53]. The PS II core complex from *T. elongatus* contains two different types of bound cytochromes. The "reduced minus oxidized" difference spectra of the pyridine hemochromes for b- and c-type hemes strongly overlap thus complicating the use of the method for simultaneous concentration determination of both hemes (see Ref. [53]). On the other hand, the PSII core complexes from *T. elongatus* provide a most appropriate sample material for straightforward determination of the Δε of both cytochromes because the Chl/Cyt b559 and Chl/Cyt c550 stoichiometries are precisely

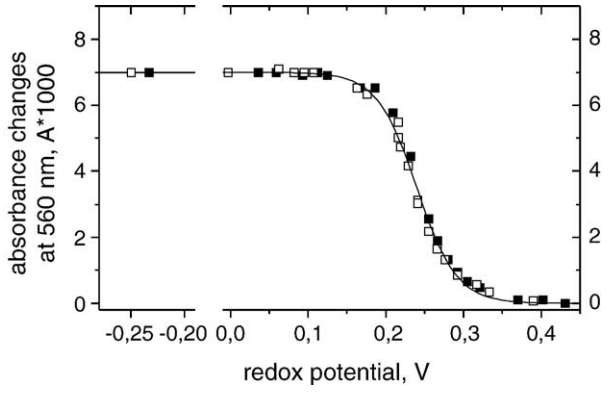

Fig. 3. Amplitude in the maximum at 560 nm of the Cyt b559 difference spectrum as a function of the ambient redox potential in suspensions of PS II core complexes from *T. elongatus*. The absorbance spectrum of the sample in the region 520–590 nm at redox potential of +450 mV was taken as baseline. Open and filled symbols represent data points obtained in oxidative and reductive titrations, respectively. The full line is the best fit for a singe-electron redox step with an E_m value of +240 mV. Chlorophyll *a* concentration was 9.0 μg/ml.

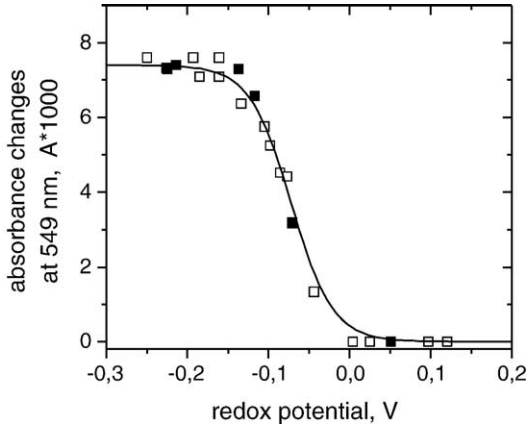

Fig. 4. Amplitude in the maximum at 549 nm of the Cyt c550 difference spectrum as a function of the ambient redox potential in suspension of PS II core complexes from *T. elongatus*. The absorbance spectrum of the sample in the region 500–590 nm at redox potential of 105 mV was taken as a baseline. Open and filled symbols represent data points obtained in oxidative and reductive titrations, respectively. The full line is the best fit for a single-electron redox step with E_m value of −72 mV. Chlorophyll *a* concentration was 9.0 μg/ml.

known from the crystal structure and thorough biochemical analyses [4,6,7,39].

Fig. 5 shows the "reduced minus oxidized" difference absorbance spectra of Cyt b559 and Cyt c550 recorded in suspensions of PS II core complexes from *T. elongatus* (panel A). For further characterization, analogous experiments were performed with the same type of complexes but obtained by dissolving of 3D protein crystals of this material in buffer solution. The results of these measurements are shown in panel B. Curves a and b in both panels were obtained after successive additions of $K_3[Fe(CN)_6]$ and sodium ascorbate, respectively, while curves c represent the differences between the spectra recorded at redox potentials of +50 mV and −195 mV. An inspection of the results in panels A and B reveals that the corresponding difference spectra are virtually identical for the two sample types. This finding indicates that Cyt b559 and Cyt b550 are not suffering from any significant structural modification during crystal formation and subsequent dissolution.

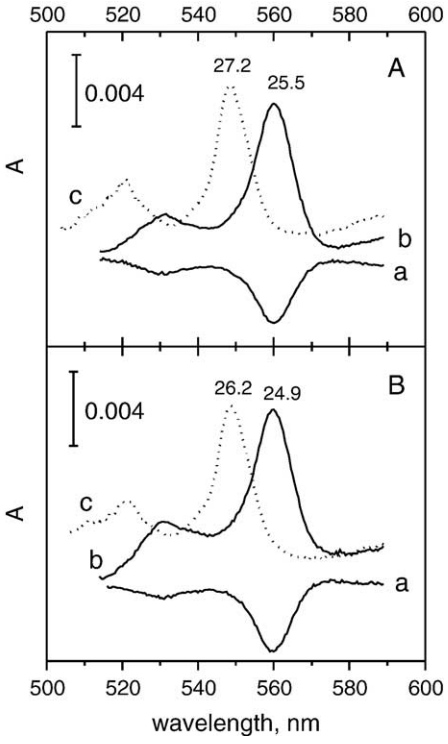

Fig. 5. Reduced minus oxidized difference absorbance spectra of cytochromes c550 and b559 in preparations of PS II core complexes from *T. elongatus* (panel A) or of the same sample type obtained after dissolving of 3D crystals (panel B). Curves a represent difference spectra monitored after addition of 100 μM $K_3[Fe(CN)_6]$ to dark adapted samples. Curves b were obtained as differences after addition of 2 mM sodium ascorbate to samples containing 100 μM $K_3[Fe(CN)_6]$. Curves c were the difference spectra recorded at −195 mV when the correspondent absolute spectra at +50 mV were taken as baselines. Spectral contributions from the electrochromic signal of Q_A/Q_A^- to curves c were eliminated by the procedure described in Materials and methods. The numbers at the peaks are the Δε values of "reduced minus oxidized" difference extinction coefficients in the correspondent bands. Chlorophyll *a* concentration for the samples in panels A and B was 9.4 μg/ml and 9.1 μg/ml, respectively.

The "reduced minus oxidized" difference spectrum of Cyt b559 in our PS II core complexes from *T. elongatus* is characterized by α- and β-band maxima at 560 and 531 nm, respectively, and a FWHM of 10.7 nm of the α-band. A comparison of the amplitudes of the α-band maxima of Cyt b559 in curves a and b of panels A and B indicates that about 45–50% of Cyt b559 is initially reduced in both sample types. Partial reduction of Cyt b559 in the dark is in agreement with its relatively positive E_m value. It is important to note that the α-band maxima of Cyt b559 in the difference spectra observed after addition of 2 mM ascorbate (curves b in Fig. 5) are characterized by the same amplitudes as those obtained in the course of the redox titration of Cyt b559. These virtually identical amplitudes are indicative of full reduction of Cyt b559 by ascorbate addition. On the basis of a Chl/PS II ratio of 36 [6,7,39] and the Cyt b559/PS II stoichiometry of 1:1 in PS II core preparations from *T. elongatus* [4,6,7,39], the value of "reduced-minus-oxidized" difference extinction coefficient of Cyt b559 in the α-band maximum can be determined. The results of five independent measurements lead to an average value of 25.1 ± 0.5 mM^{-1} cm^{-1} for Δε.

Curves c in panels A and B of Fig. 5 represent the "reduced minus oxidized" difference spectra of Cyt c550 in the two types of *T. elongatus* preparations. The spectrum of Cyt c550 is characterized by α- and β-bands at 549 and 521 nm, respectively, and FWHM of 9.3 nm of the α-band. Based on the 1:1 stoichiometry of Cyt c550/PS II, the "reduced-minus-oxidized" difference extinction coefficient of Cyt c550 in the α-maximum is determined to be 27.0 ± 0.5 mM^{-1} cm^{-1} (average of three different measurements). To the best of our knowledge, this is the first report on Δε of Cyt c550 bound to the PSII core complex of thermophilic cyanobacteria. This Δε value closely resembles the number of 25 mM^{-1} cm^{-1} reported for purified Cyt c550 from the mesophilic cyanobacterium *Synechocystis sp*. PCC 6803 [54].

The experiments described above have characterized the properties of the two types of cytochromes bound in the PS II complex from *T. elongatus*. In addition, the present studies on PS II membrane fragments from higher plants reveal that the amplitude of the α-band difference extinction coefficient of Cyt b559 does not depend on the redox form of the heme group. On the basis of this finding and using the Δε value of 25.1 mM^{-1} cm^{-1} determined for PSII core complexes from *T. elongatus*, we obtain a value of 1.1 ± 0.1 Cyt b559 per 230 Chl for our PS II membrane fragments from sugar beet.

4. Discussion

The present study describes a thorough analysis of the Cyt b559/PSII stoichiometry in higher plants and discusses the possible origin of a significant divergence in the published numbers for different PS II preparations. An

important prerequisite for obtaining reliable values is the appropriate choice of the reference points in order to achieve a correct evaluation of the "reduced minus oxidized" difference spectra of Cyt b559 in samples with a high Chl content. The present analysis reveals that the variations among the data reported on the Cyt b559/PSII stoichiometry in different preparations are not due to a possible dependence of the $\Delta\varepsilon$ value on the redox form of Cyt b559, but rather arise from uncertainties in the $\Delta\varepsilon$ value of Cyt b559 used. All previous $\Delta\varepsilon$ determinations of this heme protein were performed on preparations where Cyt b559 attained ill-characterized low potential redox form(s) that was (were) reducible only by dithionite. As a consequence, some variations of the reported $\Delta\varepsilon$ values could originate from incomplete Cyt b559 reduction under uncontrolled redox conditions, i.e., the extent of reduction could be underestimated. Therefore, for a correct determination of the $\Delta\varepsilon$ value, it is most important to gather the amplitude of the absorbance difference between reduced and oxidized Cyt b559 from the data of a complete redox titration curve.

In the present study, the $\Delta\varepsilon$ value of Cyt b559 was determined by using highly purified and well characterized preparations of PS II core complexes from the thermophilic cyanobacterium *T. elongatus*. We have analysed the redox and spectral properties of the two cytochromes – Cyt b559 and Cyt c550 – bound to the PS II core complex of *T. elongatus*. Both cytochromes were found to exhibit homogenous redox properties in this type of preparation. The value of -94 ± 21 mV for E_m of Cyt c550 at pH 6.5 is in close correspondence to that of -80 mV recently reported for another *T. elongatus* PS II core complex preparation [25]. In contrast to the perfect agreement for Cyt c550, a markedly different feature is obtained for Cyt b559. While the study of Roncel et al. reported an equimolar population of the HP and IP[2] forms of Cyt b559 [25], we found only the IP (intermediate potential) redox form of Cyt b559 in our samples, characterized by an average E_m value of +244 mV at pH 6.5. This number is slightly below the pH independent E_m value of +260 mV reported for the Cyt b559 component with the lower redox potential in the *T. elongatus* sample of Ref. [25]. Values of +240 to +260 mV closely resemble the E_m of +230 to +240 mV that is typical for the IP form of Cyt b559 in PS II membrane fragments from spinach and sugar beet (present work and Refs. [18,21,22]).

At a first glance, the absence of HP Cyt b559 in our purified preparations of *T. elongatus* PS II core complexes appears to be surprising because in this thermophilic bacterium the HP form of Cyt b559 was inferred to be rather stable, based on the finding that an analogous preparation from this organism contained up to 50% of Cyt b559 in the HP form, and about 30% of this initial HP population was still present in the preparation after 15 min of sample heating at 55 °C [25].

A closer inspection of the properties of both *T. elongatus* sample types reveals a further striking difference. The type of PSII core complexes from *T. elongatus* used in the former study [25] was reported to contain a functionally competent Q_B site [55], while in our sample preparation, it was recently reported to be changed [39] and no Q_B electron density could be resolved in the crystal structure at 3.2 Å resolution [7]. These findings suggest the existence of a structural correlation between the Q_B site and the microenvironment of the heme group of Cyt b559 with respect to the sensitivity towards the procedure of sample isolation and purification. This feature appears to be not only a specific property of thermophilic cyanobacteria but a general phenomenon of PSII because also in PSII core complexes from higher plants the Q_B site is seriously affected [56] concomitant with the complete transformation of Cyt b559 into a LP (low potential) form [22]. Based on these findings and taking into account recent information on the PSII structure [4–7], the Q_B site and the microenvironment of the heme group of Cyt b559 are inferred to be interdependent via structural interactions between the protein matrices of these cofactors. This idea gains support by an independent line of evidence from earlier studies on the effect of a mild trypsin treatment of PSII membrane fragments from spinach. It was found that this procedure which severely distorts the Q_B site [57] simultaneously transforms the HP form of Cyt b559 into the LP form without significant affecting the oxygen evolution capacity [38,57,58].

As a consequence of these considerations, we conclude that the preparation of highly purified PSII core complexes is accompanied by structural modifications that affect both the sensitive Q_B site and the microenvironment of the Cyt b559 heme group. It is attractive to speculate that an intimate interrelationship between the properties of the Q_B site and Cyt b559 is of a functional relevance for the presumed role in protection to photoinhibition [10,11,29–31].

Apart from the very interesting information on the redox properties of the heme groups in PSII of thermophilic cyanobacteria the present study provides a straightforward determination of the difference extinction coefficients of cyanobacterial Cyt b559 and Cyt c550 bound to PSII of *T. elongatus*. The value of 25.1 mM^{-1} cm^{-1} for $\Delta\varepsilon$ of IP Cyt b559 in *T. elongatus* complexes is substantially larger than that previously determined for Cyt b559 isolated from spinach [8,35] and slightly exceeds $\Delta\varepsilon$ values reported in Ref. [36] for isolated and PS II-bound spinach Cyt b559. It has to be emphasized that, in a marked contrast to previous studies, in the present work, $\Delta\varepsilon$ was determined for the first time for a specific redox form (in this case the IP) of Cyt b559 where the redox transition was completely controlled by a full titration curve. Furthermore, the number of the chlorophyll molecules of the core complexes from *T. elongatus* was determined by both X-ray crystallographic

[2] Designated by the authors as LP form.

structure analysis [4–7] and thorough biochemical investigations [39] of the same sample material. In both cases, virtually, the same ratio of 36 Chl/PSII was obtained. The maximum uncertainty of this content is one Chl. Therefore, the total error of the $\Delta\varepsilon$ value determined in this study can be limited at ± 1 mM^{-1} cm^{-1}.

Using a $\Delta\varepsilon$ of Cyt b559 of 25.1 mM^{-1} cm^{-1} determined for PSII core complexes from *T. elongatus*, we obtained a ratio very close to 1 for Cyt b559/PS II in PS II membrane fragment preparations from higher plants. Therefore, it seems justified to say that the long lasting controversial discussion on this subject is coming to an end with the conclusion that all PSII complexes contain only one copy of this heme protein.

Acknowledgement

We thank Drs. A. Zouni and K.-D. Irrgang for valuable advises in isolation of PS II core complexes, helpful suggestions in carrying out the experiments and stimulating discussion on the PS II structure. The support by grants from DFG (436 Rus 113/635/0-1, Sfb 498, TP C7) and Russian Fund of Basic Research (05-04-49488) are gratefully acknowledged.

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