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Functional implications of pigments bound to a cyanobacterial cytochrome b_6f complex

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A highly purified cytochrome b_6f complex from the cyanobacterium *Synechocystis* sp. PCC 6803 selectively binds one chlorophyll a and one carotenoid in analogy to the recent published structure from two other b_6f complexes. The unknown function of these pigments was elucidated by spectroscopy and site-directed mutagenesis. Low-temperature redox difference spectroscopy showed red shifts in the chlorophyll and carotenoid spectra upon reduction of cytochrome b_6, which indicates coupling of these pigments with the heme groups and thereby with the electron transport. This is supported by the correlated kinetics of these redox reactions and also by the distinct orientation of the chlorophyll molecule with respect to the heme cofactors as shown by linear dichroism spectroscopy. The specific role of the carotenoid echinenone for the cytochrome b_6f complex of *Synechocystis* 6803 was elucidated by a mutant lacking the last step of echinenone biosynthesis. The isolated mutant complex preferentially contained a carotenoid with 0, 1 or 2 hydroxyl groups (most likely 9-*cis* isomers of β-carotene, a monohydroxy carotenoid and zeaxanthin, respectively) instead. This indicates a substantial role of the carotenoid – possibly for strucure and assembly – and a specificity of its binding site which is different from those in most other oxygenic photosynthetic organisms. In summary, both pigments are probably involved in the structure, but may also contribute to the dynamics of the cytochrome b_6f complex.

The cytochrome b_6f (cyt b_6f) complex is one of the three integral membrane protein complexes in the photosynthetic electron transport chain. It functions as a plastoquinol-plastocyanin oxidoreductase and mediates the electron flow between photosystem II and photosystem I [1,2], thereby contributing to building up a proton gradient across the thylakoid membrane that is used for the generation of ATP [3]. In cyanobacteria, this complex is involved both in the photosynthetic and in the respiratory electron transport chain and is therefore indispensable for growth [4].

The cyt b_6f complex consists of four main subunits, cyt f (apparent moleculare mass of 29 kDa), cyt b_6 (24 kDa), the Rieske iron sulfur protein (22 kDa), and subunit IV (18 kDa), encoded by the genes *petA*, *petB*, *petC*, and *petD*, respectively [4]. With exception of subunit IV, all subunits bind redox-active cofactors: cyt f contains one *c*-type heme, cyt b_6 two *b*-type hemes and

Abbreviations
Chl, chlorophyll; cyt, cytochrome; β-DM, β-dodecyl maltoside; LD, linear dichroism; PS1, photosystem I.

one recently discovered new heme named 'heme x' [5], and the Rieske protein one [2Fe-2S]-cluster. For higher plants and green algae, up to five additional smaller subunits of the cyt b_6f complex have been identified (PetG, L, M, N, O). The deletion of *petG* [6] or *petL* [7] in *Chlamydomonas reinhardtii* resulted in a greatly decreased content of the cyt b_6f complex in the thylakoid membrane. PetN is essential for the chloroplast cyt b_6f complex [8], and PetL was suggested to stabilize the complex [7]. PetO apparently is involved in state transitions [9]. In cyanobacterial cyt b_6f complex, the small-subunit composition seems to be different: while the *petO* gene is missing, the *petN* gene is present in the *Synechocystis* genome [8], but the corresponding protein has not yet been detected in this organism. Subunits PetG, PetL and PetM have been shown to be part of the cyanobacterial cyt b_6f complex [10,11], of which at least PetM does not seem to be essential [12].

In cyt b_6f preparations of both pro- and eukaryotic origin [13–16], one chlorophyll a (Chl a) molecule per monomeric unit was shown to bind to the complex. In addition, the cyt b_6f complex appeared to bind a carotenoid as well [14,16]. The existence of both pigments in a 1 : 1 stoichiometry per monomeric complex could recently be confirmed by X-ray structural analysis of a prokaryotic [5] and an eukaryotic [17] cyt b_6f complex: both in the case of the cyanobacterial complex (*Mastigocladus laminosus*) and the green algal complex (*Chlamydomonas reinhardtii*) the carotene was assigned as 9-*cis* β-carotene. This is in agreement with the carotene reported before for the cyt b_6f complex from spinach. In contrast, the carotene in *Synechocystis* sp. PCC 6803 was shown to be echinenone [18].

Despite the structural data that are now available, the function of both the chlorophyll and the carotenoid in the cyt b_6f complex remains unclear. These pigments conceivably could have a structural role as has been shown for the formation of thylakoids [19,20] and for the stable assembly of pigment–protein complexes in photosynthetic organisms [21–25]. Besides the presence of the carotenoid echinenone, *Synechocystis* offers the well-established possibility to manipulate biochemical pathways and individual proteins by directed mutagenesis [26].

In this report we present an in-depth characterization of the chlorophyll and echinenone pigments that are bound to the isolated cyt b_6f complex of *Synechocystis* sp. PCC 6803. Chemical and physical comparison of the wild type complex with that of targeted mutants has provided new information on their potential role within the cyt b_6f complex beyond the information that has been derived from the X-ray analysis of another cyanobacterium with a different carotene [5].

Results

Spectroscopic characterization of the cyt b_6f complex

Hemes and chlorophyll

Figure 1 shows the 4 K absorbance spectrum of the dithionite-reduced, purified cyt b_6f complex from the *Synechocystis* sp. PCC 6803 strain lacking photosystem I (PS1-less) (solid line). The two main peaks at 422 nm and 430 nm correspond to the Soret bands of cyt *f* and cyt b_6, respectively. The β-bands of cyt *f* and cyt b_6 are observed at 530 and 531 nm, respectively, while the X- and Y-transitions of the α-band of cyt *f* occur at 548 and 555 nm, respectively, and those of cyt b_6 at 556 and 562 nm, respectively ([27] and references therein for definitions and orientations of the various transitions). An additional peak in the 4 K absorption spectrum at 671 nm in combination with a shoulder at about 437 nm suggested the presence of Chl a [15], which was confirmed by reversed-phase HPLC. Integration of the chlorophyll peak area and comparison with defined chlorophyll standard amounts yielded the chlorophyll content of the samples. These chlorophyll amounts were related to the cyt *f* content determined at room temperature of the respective samples, and a ratio of about one chlorophyll molecule (1.0 ± 0.06) per cyt b_6f was calculated. In addition, the 4 K absorption spectrum revealed a shoulder between 450 and 520 nm, suggesting the presence of a carotenoid (see below).

The reduction of the cyt b_6f complex with dithionite caused a 1 nm shift in the absorbance spectrum of the

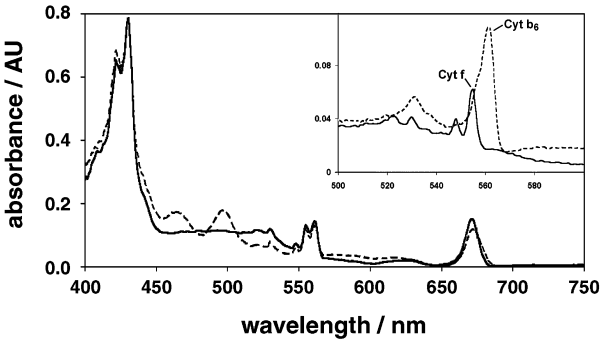

Fig. 1. Absorbance spectra of cyt b_6f complexes isolated from various *Synechocystis* 6803 mutant strains. Absorbance spectra of cyt b_6f from the PS1-less strain (solid line) and the PS1-less/CrtO-less mutant (dashed line) at 4 K. Both samples were reduced with Na-dithionite. Inset: difference spectra of cyt *f* (ascorbate-reduced minus ferricyanide-oxidized, solid line) and cyt b_6 (dithionite-reduced minus ascorbate-reduced, dashed line) recorded at 4 K using the complex isolated from the PS1-less mutant.

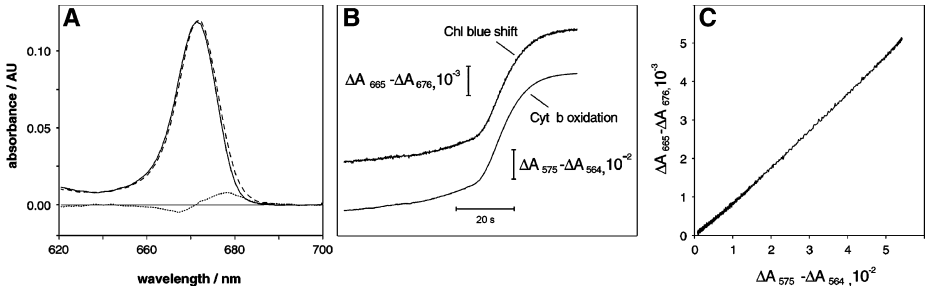

Fig. 2. Spectroscopic characterization of cyt b_6f isolated from the PS1-less mutant strain. (A) 4 K absorbance spectrum of chlorophyll associated with the isolated cyt b_6f complex. Solid line, recorded after oxidation by 100 μM ferricyanide, followed by reduction of cyt *f* with 2 mM ascorbate. Dashed line, chlorophyll peak after the reduction of cyt b_6 by dithionite. Dotted line, difference spectrum of the solid and dashed lines. (B) Kinetics of the reoxidation of cytochrome b_6 by air and of the absorbance shift of chlorophyll after reduction of the sample with 0.5 mM dithionite at room temperature (buffer: 20 mM Mes, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 0.02% β-DM). Cytochrome and chlorophyll absorbance differences were recorded simultaneously at their respective maxima of absorbance change with a time resolution of 80 ms. (C) Plot of the kinetics of the cyt *b* redox changes vs. the Chl a absorbance shift using the data shown in Fig. 4B.

chlorophyll molecule to longer wavelengths (Fig. 2A). This shift was not observed upon reduction with ascorbate, which reduces cyt *f* but not cyt b_6 ([13] for redox potentials). This strongly suggests a position of chlorophyll within the range of a possible charge interaction with one or both of the *b* hemes. As both available cyt b_6f structures [5,17] show that the Chl a and the heme b_n planes are parallel and about 1.6 nm apart, it is very likely that the shift is caused by heme b_n. Figure 2B shows the kinetics of the chlorophyll absorbance shift in comparison with the kinetics of the cyt b_6 redox change. Both kinetics were recorded at the wavelength of maximal difference of absorbance changes (665 nm minus 676 nm for chlorophyll and 575 nm minus 564 nm for cyt *b*) and start after full reduction of the sample with dithionite, followed by reoxidation by air. Cyt *b* oxidation and the Chl a bandshift occur in parallel, yielding a linear relationship when plotted against each other (Fig. 2C). This supports a direct correlation between the absorption spectrum of chlorophyll and the redox state of a *b*-type cytochrome.

To determine the orientations of the various cofactors with respect to the long axis of the cyt b_6f particle, linear dichroism (LD) spectroscopy was performed. Figure 3 (solid line) shows the 77 K LD spectrum of the ascorbate-reduced cyt b_6f complex obtained from the echinenone-deficient mutant. The spectrum obtained from the wild type cytochrome b_6f complex was virtually identical (data not shown). The spectrum showed a distinct negative signal at 671 nm with a very similar spectral shape and peak wavelength as the Q_y (0–0) peak of the absorption spectrum (dashed line). In addition, the LD spectrum shows small positive and negative features around 630 and 620 nm, respectively, as well as a sharp negative feature at 555 nm and positive features near 548 and 530 nm. These data indicate

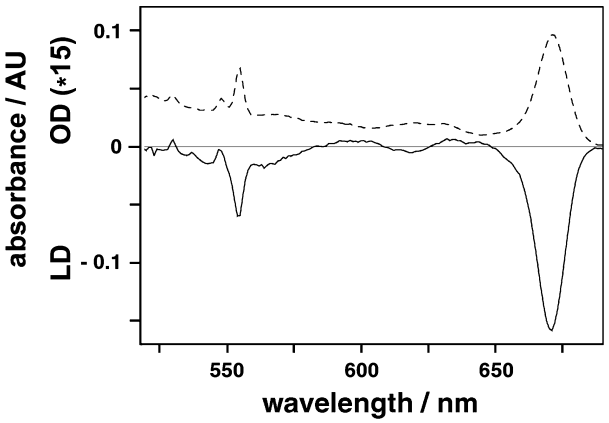

Fig. 3. Comparison of absorbance and LD spectrum. The absorbance spectrum in the chlorophyll region (upper half, dashed line) and the LD spectrum (lower half, solid line) of the ascorbate-reduced, isolated b_6f complex from the CrtO-less mutant at 77 K are compared. The LD spectrum was recorded using b_6f complexes oriented in a two-dimensionally squeezed gelatin gel. The values on the *y*-axis represent the absolute absorbance and LD values.

negative LD values for the Q_y transitions of chlorophyll (around 670 and 620 nm) and the Y transition of the α-band of cyt *f*, as well as positive LD values for the Q_x transition of chlorophyll (which dominates the Chl absorption around 630 nm and between about 570 and 600 nm [28]), the X transition of the α-band of cyt *f* and of the β-band of cyt *f*.

Apart from the cyt b_6 contribution, the spectrum is virtually identical to that of the complex from *Chlamydomonas reinhardtii* recorded by Schoepp *et al.* [27]. The dithionite-reduced and ferricyanide-oxidized LD spectra of our *Synechocystis* preparation appeared very similar to those reported in *Chlamydomonas* (not shown, [27]). This indicates that the chlorophyll and

cyt *f* molecules adopt very similar orientations in *Chlamydomonas* and *Synechocystis* and suggests that the chlorophyll molecule binds at a very similar position in the cyt b_6f complex from the two organisms.

Carotenoids

Reversed-phase HPLC pigment analysis of the purified cyt b_6f confirmed the presence of both Chl a and a carotenoid (Fig. 4A); the carotenoid was identified as the ketocarotenoid echinenone (Fig. 4B), one of the four common carotenoids in *Synechocystis* sp. PCC 6803 that makes up 15–20% of the total carotenoid content of the cell [29]. The absence of other carotenoids in the preparation suggested the selective binding of echinenone to the complex. To analyze whether echinenone had a specific role in the cyt b_6f complex, we deleted *crtO*, the gene coding for β-carotene ketolase, from the PS1-less mutant. CrtO is required for echinenone synthesis [30]. Introduction of this mutation did not affect growth kinetics, and the cyt b_6f complex purified from this mutant was normal in terms of heme content and redox properties, indicating the absence of major structural or functional changes in the complex.

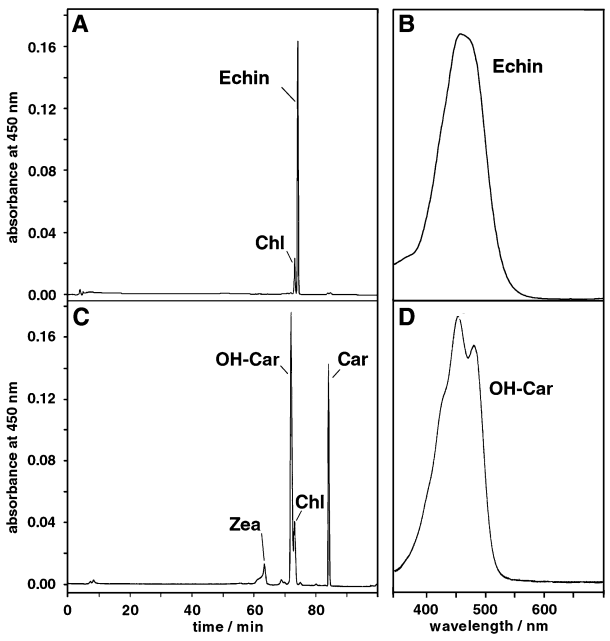

Fig. 4. Pigment analysis by reversed phase chromatography (Spherisorb ODS 2). The pigments were eluted by three successive linear gradients, with increasing hydrophobicity (increased ethylacetate percentage: 0 → 20%, 20 → 50%, 50 → 100%), at room temperature and at an average flow rate of 0.7 mL·min^{-1}. (A) Acetone extract of purified cyt b_6f of the PS1-less mutant. (B) Absorbance spectrum of echinenone. (C) Acetone extract of purified cyt b_6f of the PS1-less/CrtO-less mutant. (D0 Absorbance spectrum of the mono-hydroxy-β-carotene observed in the cyt b_6f complex.

Pigment analysis of the cyt b_6f complex from the CrtO-less mutant showed that echinenone had been replaced by three other carotenoids (Fig. 4C). Two of these carotenoids appear to be β-carotene and zeaxanthin, two other major carotenoids in *Synechocystis* sp. PCC 6803. However, the HPLC properties of the third and major carotenoid in the cyt b_6f complex of the echinenone-less mutant does not correspond to one of the four major carotenoids in *Synechocystis*, and appears to be a mono-hydroxy-β-carotene instead. All three carotenoids in the echinenone-minus mutant are 9-*cis* isomers, showing a characteristic 4–5 nm blue shift of the main absorption bands, increased absorption at 340 nm and decreased absorption at 280 nm in a very similar way to that shown for the 9-*cis* isomer of β-carotene [31]. In whole cell extracts the content of 9-*cis* isomers is less than 1% of the total carotenoid content (data not shown). All-*trans* forms prevail. Based on the absorption characteristics at 340 and 280 nm of echinenone in the cyt b_6f complex isolated from strains retaining CrtO, this carotene appears to be in the all-*trans* form.

A characteristic difference in the carotenoid content of the PS1-less mutant and the derived strain lacking echinenone was also suggested by the 4 K absorbance spectrum of the cyt b_6f complex isolated from this mutant (Fig. 1, dotted curve): while there is no difference in the cyt *f* and cyt b_6 peaks, the mutant lacking echinenone shows two peaks at about 462 nm and 496 nm. At room temperature, the red-most transition displayed a well-resolved peak at 490 nm, while the second transition revealed a shoulder near 460 nm (not shown). Both maxima are about 5 nm red-shifted compared to those of β-carotene in the cyt b_6f complexes from spinach [16] and *Chlamydomonas reinhardtii* [14]. The red shift of the red-most transition of the carotenoid in the *Synechocystis* cyt b_6f complex upon cooling to 4 K (about 6 nm or 250 cm^{-1}) is similar to that of β-carotene in CP47 and considerably larger than that of β-carotene in polymer matrices [32]. The large temperature effect in CP47 was explained by a phase transition of the protein [32]. The similarly large temperature effect of the carotenoid in cyt b_6f from *Synechocystis* is compatible with this view and confirms the notion that this molecule is buried in the protein.

Figure 5 shows the absorption spectrum of the cyt b_6f complex from the CrtO-less strain in the region of the main absorption bands of the hemes and carotenoids; reduction of cyt b_6 was found to induce a red shift of about 1.5 nm of the carotenoid absorption bands at 496 and 462 nm, whereas reduction of cyt *f*

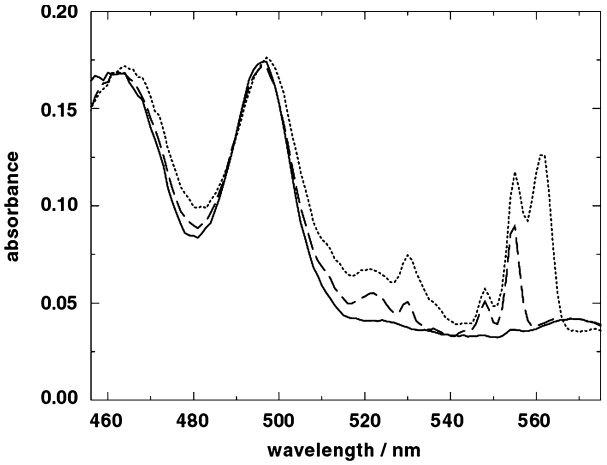

Fig. 5. Absorbance spectra of the cyt b_6f complex from the CrtO-less mutant at 4 K. The spectra were recorded in the presence of 100 μM ferricyanide (solid line), 20 mM ascorbate (dashed line), or after addition of a few grains of dithionite (dotted line). The carotenoid absorption bands peaking near 496 and 462 nm shift to the red upon reduction of cyt b_6.

does not induce a carotenoid bandshift. A 1.5 nm shift upon cyt b_6 reduction was also observed in the second-derivative spectra and at room temperature (not shown). Carotenoid bandshifts could not be observed in the cyt b_6f complex prepared from the PS1-less strain retaining echinenone, probably due to the structureless absorption spectrum of echinenone (Fig. 1, solid line). The occurrence and extent of the carotenoid bandshift resembles that of the chlorophyll molecule (Fig. 2A) and strongly suggests a charge interaction between the carotenoid molecule and the b_6 subunit.

In our cyt b_6f complex preparation, the molecular stoichiometry of carotenoids appears to be less than that of chlorophyll. Because pure echinenone was not available as pigment standard, its relative content in the purified cyt b_6f complex was estimated by comparing with the respective peak area of β-carotene. The integration of the respective peak areas yields 0.6 ± 0.15 echinenone per cyt b_6f complex in the PS1-less strain and 0.65 ± 0.15 carotenoids per cyt b_6f complex (sum of all three species of Fig. 4C) in the CrtO-minus strain. As the published X-ray data suggest a fixed position of one carotenoid per complex, our quantification implies that some carotenoid may be washed out during preparation in part of the centers.

Discussion

Two recently published cyt b_6f complex structures – of the cyanobacterium *Mastigocladus laminosus* [5] and of the green algae *Chlamydomonas* [17] – showed the presence of one chlorophyll molecule and one carotenoid per monomeric complex, confirming previous reports on the presence of pigments in pro- and eukaryotic cyt b_6f complexes [13,15,16,33]. In both cases the carotenoid was assigned as 9-*cis* β-carotene. By comparison with the X-ray structure of cyt bc_1 complexes [17], a structural role of these pigments in cyt b_6f is apparent from a different packing and a modified architecture of subunits involved in their binding. By analogy, a similar arrangement of both pigments can be expected in the cyanobacterium *Synechocystis* sp. PCC 6803. However, in this case the carotenoid is echinenone, which is suggested to be an efficient UV-B photoprotector in various cyanobacteria [34]. As the specific function of these pigments in cyt b_6f complexes in general and of echinenone in *Synechocystis* cyt b_6f in particular is still unknown, we applied a targeted mutagenesis approach to probe for the exclusiveness of echinenone and for potential functional implications of both pigments with their environment.

Apart from the presence of echinenone, the isolated cyt b_6f complex from *Synechocystis* sp. PCC 6803 had several interesting spectroscopic properties: the peak wavelengths of the α-bands of cyt *f* occur at considerably longer wavelength than those in *Chlamydomonas reinhardtii* (about 551 and 547 nm [27]), whereas those of cyt b_6 occur at about the same position in both organisms. Ponamarev *et al.* [35] showed that if position 4 of PetA is occupied by a Trp residue (as in *Synechocystis* sp. PCC 6803 and other cyanobacteria), the α-band of cyt *f* at room temperature is shifted 1–2 nm to the red than if position 4 is occupied by Phe or Tyr (as in most eukaryotic organisms). The red-shift of the peak maximum of the α-band may be related to an increased splitting between the X and Y transitions at 4 K, which is probably caused by asymmetry in the heme pocket of the protein [36]. This splitting is relatively large (7 nm, or 230 cm^{-1}) in cyt *f* of *Synechocystis* PCC 6803 compared to most other *c*-type cytochromes [36].

The LD-signals from the two types of cyt b_6f complexes – i.e. from *Chlamydomonas* and *Synechocystis* – orient in a similar way. In the case of disc-shaped particles (as is usually assumed for membrane-bound particles [37]) and two-dimensional squeezing, a positive LD implies a larger angle between the transition dipole and the normal of the disc than the magic angle (55 degrees), whereas a negative LD implies a shorter angle than the magic angle [38]. If the plane of the disc equals the plane of the particle in the membrane, positive and negative LD values indicate a small and large angle, respectively, between the transition dipole and

the plane of the membrane. Thus, the X transitions of chlorophyll and cyt *f* are probably at smaller angles with the plane of the membrane than the magic angle (35 degrees), whereas the Y transitions are at larger angles. For the chlorophyll molecule this orientation differs from most antenna chlorophylls, for which the average Q_y transitions are at small angles relative to the plane of the membrane [37]. In conclusion, our LD-data indicate a similar orientation of the chlorophyll in the *Synechocystis* b_6f complex as the chlorophyll in the crystal structure of *Mastigocladus laminosus* [5], with the X-axis approximately parallel and the Y-axis about perpendicular to the membrane plane.

The spectroscopic data presented in this paper indicate an interaction of chlorophyll with the cyt b_6 subunit and/or its redox components, i.e. the heme groups. This is in line with earlier observations that indicated a structural proximity of chlorophyll and the native cyt b_6 subunit by copurification, with the chlorophyll being retained even upon partial denaturation [18]; a binding of chlorophyll to cyt b_6 was also suggested by Poggese using native polyacrylamide gel electrophoresis [39]. Both crystal structures show that the tetrapyrrole ring of chlorophyll is bound primarily by subunit IV, while the phytol chain extends towards the third transmembrane helix of the b_6 subunit and may be the main reason for the copurification with this subunit due to hydrophobic interactions (Fig. 6).

On the other hand, our report provides several indications for a functional proximity of chlorophyll and at least one heme in the cytochrome b_6 subunit: (a) the red-shift of the chlorophyll peak at 671 nm simultaneously with the reduction of the *b*-type heme suggests a short distance between these two components; (b) the previously observed extremely short fluorescence lifetime of this chlorophyll [15] suggests a binding to a specific pocket in the cyt b_6f complex where a heme or an amino acid side chain is able to quench its excitated state; this may protect the protein from oxidative damage.

If we assume a very similar orientation of the *Synechocystis* chlorophyll as in the crystal structure, which is supported by our LD-data, the *b*-type heme closest to the tetrapyrrole ring of the chlorophyll is cyt b_h (Fig. 6). According to the crystal structure, the center-to-center distance of the tetrapyrrole ring of the chlorophyll to the heme is approximately 16.7 Å, which is sufficiently small to enable charge transfer between both ring systems.

Besides chlorophyll, a carotenoid is associated with the isolated cyt b_6f complex of *Synechocystis* [40] in substoichiometric amounts. The ratio of about 0.55–0.77 carotenoids per monomeric cyt b_6f complex determined in this report is in agreement with values

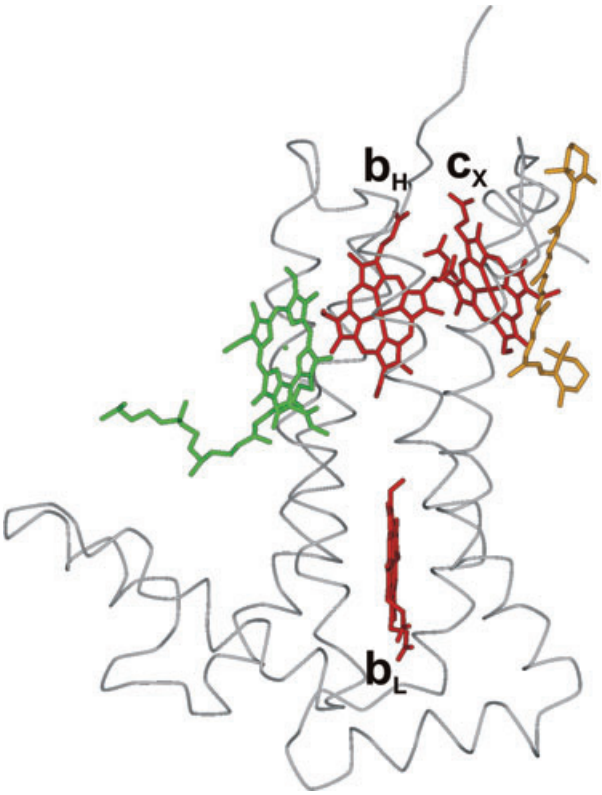

Fig. 6. Structure of the isolated cytochrome b_6 subunit from *Mastigocladus laminosus* [5] with bound cofactors. Red, heme; green, chlorophyll; orange, carotenoid. The chlorophyll molecule is in close proximity to the heme b_H, while the carotenoid is close in space to the covalently bound heme c_x.

reported for other mesophilic organisms like spinach [16] and *Chlamydomonas reinhardtii* [16,33], which tend to loose some pigment upon isolation and purification. The presence of a carotenoid within the cyt b_6f complex has been confirmed by the X-ray structure: in the case of the cyanobacterium *Mastigocladus laminosus*, a β-carotene is sandwiched between the α-helix of PetL and PetM [5] with one hexameric ring extending towards helix A of the PetB (subunit b_6) (Fig. 6). Although helices of the small subunits have been assigned differently in the *Chlamydomonas* structure, the localization of the carotene is identical in both structures.

Similar to chlorophyll, after a mild dissociation of the cyt b_6f complex from *Synechocystis* sp. PCC 6803, the carotenoid was found to be exclusively associated with the cyt b_6 subunit [18]. A short distance between the carotenoid and *b*-hemes of the cyt b_6 subunit is also suggested by the red-shift of the carotene peaks in the CrtO-less mutant simultaneously with the reduction of the *b*-hemes. Considering the cyt b_6f structural model and assuming again a similar location in *Synechocystis* as in *Chlamydomonas* and *Mastigocladus*, the most

probable functional interaction occurs between one ring of the carotenoid and the stroma-exposed heme cyt c_x with an approximate ring center-to-center distance of 11.2 Å.

The carotenoid in *Synechocystis* is echinenone, the content of which in the cells is smaller compared with β-carotene, zeaxanthin and myxoxanthophyll. This indicates that the binding of echinenone to the complex is rather specific.

Results obtained with the CrtO-less strain suggest that the carotenoid binding site in the cyt b_6f complex of *Synechocystis* prefers a carotenoid with a polar =O (echinenone) or –OH (monohydroxy-β-carotene) group on one side of the carotenoid (the other ring of these two carotenoids is identical to that of β-carotene). This is in contrast to cyt b_6f complexes from spinach, *Chlamydomonas reinhardtii* and *Mastigocladus laminosus*, which prefer β-carotene [16], a carotenoid that lacks polar =O or –OH groups on both sides of the molecule. However, these three cyt b_6f complexes and the CrtO-less mutant of *Synechocystis* seem to prefer 9-*cis* isomers, which points to significant similarities of the carotene binding pocket in all organisms. This 9-*cis* conformation is also in line with a recent HPLC and Raman characterization of β-carotene in the cyt b_6f complex from spinach [41], but is in contrast to the interpretation of another Raman characterization [42]. In the latter, however, the choice of the Raman frequency used to distinguish both types of conformations was questioned [41].

Probably due to sterical constraints of the binding pocket, echinenone apparently cannot easily be replaced by other carotenoids. This suggests a structural role of carotenoid(s) in the cyt b_6f complex, perhaps similar to the situation in the light harvesting complex of higher plants [21] or the D1 protein of photosystem II [24]. Such a plant-specific function is also suggested by the high resolution 3D structure of the cyt b_6f complex. A possible function for the carotenoid has not yet been firmly established. While it was suggested that it prevents the generation of singlet oxygen by photoexcited Chl a [16], a triplet energy transfer from chlorophyll to carotenoid did not occur at 77 K in cyt b_6f from *Synechocystis* [15]. Also, no singlet energy transfer from the carotenoid to chlorophyll has been observed by fluorescence measurements [15]. These observations are in line with the structural model showing an approximate distance of 14 Å between both pigments, which is too far for triplet and singlet energy transfer. However, as the edge of the chlorophyll is exposed to the lipid phase, the presence of additional carotenoids interacting with the chlorophyll *in situ* cannot be ruled out [5].

In combination with the structural data, the effects observed in this communication could be interpreted in two different scenarios. (a) Indication for a signal transduction chain: as these pigments are not found in the closely related cyt bc_1 complex of the respiratory chain, they may represent a plant-specific, structure-dominated principle. Due to their localization and orientation, they could interact with other components of the photosynthetic apparatus such as PS1 or a kinase. In this case, chlorophyll would act as a sensor that connects the interacting partner with the Q_o-site, while the carotenoid might have a similar role at the Q_i-site [5]. For the chlorophyll, an absorption bandshift was observed upon binding of inhibitors (stigmatellin or 2,5-dibromo-6-methyl-3-isopropyl-1,4-benzochinon) to the Q_o-site in a cyt b_6f complex isolated from spinach, which supports this hypothesis from the reverse direction (C. Klughammer, unpublished result). (b) Indication for protein reorganization: during electron transfer, the strong local electric field around the *b*-type cytochrome causes an electrochromic shift of the nearby pigments which in turn could indicate a protein reorganization of the complex, i.e. the observed shift is caused by protein relaxation. Such an effect has been reported for other proteins [43].

Irrespective of the physiological role of both pigments, these observations also indicate their potential usefulness as 'natural' indicators for redox-induced changes in the cyt b_6f complex.

In summary, this report shows that the two pigments found in the cyt b_6f-complex, chlorophyll and echinenone, have a specific structural and possibly also a functional impact. While the results obtained with the echinenone-less mutant indicate a high selectivity of the carotene binding pocket due to specific sterical constraints, the correlation of both pigments with redox changes of the *b*-type cytochrome on the cytoplasmic/stromal side suggests the possibility of functional interaction. These results should stimulate further experiments, for which the available 3D structure of the cyt b_6f complex in combination with site-directed mutagenesis of pigment-stabilizing residues is an excellent basis.

Experimental procedures

Synechocystis sp. PCC 6803 strains and growth conditions

For the isolation of cyt b_6f complexes from *Synechocystis* sp. PCC 6803, a PS1-less mutant strain was used, in which an internal deletion in the *psaAB* operon inactivates both genes [44]. Cells of this strain were grown photoheterotrophically

at 30 °C in standard BG-11 medium enriched with 30 mM glucose and at an incident light intensity of 5 μmol photons·m^{-2}·s^{-1} in a 25 L foil photobioreactor (Bioengineering, AG, Wald, Switzerland). Cultures were harvested after 3 days [at an attenuance (*D*) at 730 nm of about 1.0] and concentrated by a hollow fiber concentration device (Amicon DC-10 L, Millipore GmbH, Schwalbach, Germany) to 1 L, followed by centrifugation at 6000 ***g*** for 10 min. Thylakoid membranes were prepared according to [45], with the exception that the cells were disrupted in a glass bead mill (model KDLA, Dyno-Mill, Bachofen AG, Basel, Switzerland) at 0 °C for 30 s, using 0.5 mm glass beads. After centrifugation at 200 000 ***g*** and 4 °C for 40 min the thylakoid membrane pellet was resuspended in a buffer containing 20 mM Mes/NaOH (pH 6.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 20% (v/v) glycerol and protease inhibitors (10 μM tosyllysyl chloromethylketone, 100 μM phenylmethylsulfonylfluoride) yielding a final chlorophyll concentration of 0.2–0.4 mg·mL^{-1}. Thylakoids were frozen in liquid N_2 and subsequently stored at −70 °C.

Generation of a PS1-less/CrtO-less mutant of *Synechocystis*

The PS1-less/CrtO-less mutant was generated by transformation of the PS1-less mutant with the plasmid pTRCRT-O kindly provided by G. Sandmann (Johann Wolfgang von Goethe University, Frankfurt/Main Germany). This plasmid contained a copy of the *Synechocystis crtO* gene that was interrupted by a kanamycin cassette [30]. For transformation, the PS1-less strain of *Synechocystis* was grown to $D_{730} = 0.5$, pelleted (5000 ***g***, 5 min, room temperature), and resuspended in BG-11 medium to a $D_{730} = 2.5$. This suspension (400 μL) was incubated with 0.3–3 μg plasmid DNA for six hours at 30 °C under illumination at 5 μmol photons·m^{-2}·s^{-1}. Of these cells, 200 μL were plated on nitrocellulose filters on top of BG-11 plates containing 30 mM glucose; after 18 h they were transferred to BG-11 plates containing 5 μg·mL^{-1} kanamycin. Colonies emerged after two weeks; they were transferred to new plates every 2–4 days, increasing the kanamycin concentration by 5–10 μg·mL^{-1} each time. The maximal kanamycin concentration used was 50 μg·mL^{-1}. One of the transformants was checked by PCR for complete segregation and this segregated strain was used for further analysis. As expected, this strain lacked echinenone according to pigment analysis using reversed-phase HPLC [30].

Purification of the cyt b_6f complex from the PS1-less strain of *Synechocystis*

Unless specified otherwise, all following steps were performed under dim light and at 6–8 °C. The isolated membranes were first incubated with 0.1 mg·mL^{-1} RNase and DNase (Boehringer, Ingleheim, Germany) at 20 °C for 18 min; upon addition of 0.05% (w/v) β-dodecyl maltoside (β-DM), the mixture was incubated for another 2 min. After centrifugation (200 000 ***g***, 4 °C, 40 min) the pelleted membranes were resuspended in buffer [20 mM Mes/NaOH (pH 6.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.5 M mannitol, 20% (v/v) glycerol], and diluted to a chlorophyll concentration of 150 μg·mL^{-1}.

Membrane proteins were extracted by incubation with 1% (w/v) β-DM for 30 min at 20 °C. After centrifugation (200 000 ***g***, 4 °C, 40 min) and 1.5-fold dilution with a high-salt buffer [20 mM Mes/NaOH, pH 6.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 3 M ammonium sulfate, 0.02% (w/v) β-DM] the supernatant was loaded onto a hydrophobic interaction column (POROS 20 BU; Applied Biosystems, Foster City, CA, USA) that was run at a flow rate of 7 mL·min^{-1} at 10 °C. Upon applying a decreasing ammonium sulfate gradient, the cyt b_6f complex eluted at a concentration of about 1 M ammonium sulfate. The cyt b_6f containing fractions were concentrated and dialyzed against a low salt buffer [20 mM Mes/NaOH (pH 6.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.02% (w/v) β-DM] before purifying them further on an anion exchange column (Uno Q6, Bio-Rad Laboratories, Munich, Germany). Applying a $MgSO_4$ gradient at a flow rate of 4 mL·min^{-1}, the cyt b_6f complex eluted at about 15 mM $MgSO_4$ and was stored at −70 °C.

The presence of all expected subunits was confirmed by SDS/PAGE, immunoblotting (using antibodies against PetA, PetB, PetC, and PetD) and EPR-measurements (to demonstrate the Rieske protein).

Pigment analysis

Pigment analysis of thylakoid membranes preparations and purified cyt b_6f complexes was carried out by reversed-phase HPLC. Samples were diluted 10-fold with ice-cold acetone, vortexed briefly and centrifuged (12 000 ***g***, 4 °C, 5 min). The supernatant containing the pigments was filtered through a membrane (Spartan, 0.45 μm, Schleicher und Schuell GmbH, Dassel, Germany) and injected onto a RP HPLC column (Spherisorb ODS 2, Crom); this column had been equilibrated using a hydrophilic solution RP-A [38.5% (v/v) acetone, 46.5% (v/v) methanol, 5% (v/v) water and 10% (v/v) PIC A (5 mM tetrabutylammonium sulfate, Waters, Milford, MA, USA)]. Pigments were eluted by three linear gradients with increasing hydrophobicity: 0 → 20%, 20 → 50%, 50 → 100% solution RP-B [100% (v/v) ethylacetate], at an average flow rate of 0.7 mL·min^{-1}. Pigments were analyzed online by a Photodiode Array Detector 966 (Waters) from 350 nm to 700 nm and identified/quantified by comparison with standards [46].

Alternatively, pigment analysis was performed according to [47]. For this procedure, pigments were extracted with 80% acetone, centrifuged and filtered, and loaded on a RP HPLC-column (Spherisorb C18), which was equilibrated in

buffer A [85% acetonitrile, 13.5% methanol, 1.5% 0.2 M Tris/HCl (pH 8.0)]. The column was run for 30 min at 1 mL·min^{-1} in buffer A, after which a 5 min linear gradient (0–100%) was applied using buffer B (83.3% methanol, 16.7% *n*-hexane); subsequently the column was run for another 30 min in buffer B. The HPLC system was equipped with a diode-array optical absorption spectrophotometer, which allowed identification of the peaks in the chromatogram by their absorption spectra.

Spectroscopic methods

All spectroscopic measurements of the cyt b_6f complex were carried out in 20 mM Mes/HCl (pH 6.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.03% β-DM. UV-Vis absorbance spectra at room temperature were recorded on a Beckman DU 7400 spectrophotometer (Beckman Coulter GmbH, Krefeld, Germany), with a spectral bandwidth of 1.2 nm. For redox measurements of the cytochromes, the air-oxidized cyt b_6f samples were oxidized with 100 μM ferricyanide or reduced with 20 mM ascorbate (for cyt *f*) or dithionite (for cyt b_6). Absorbance and fluorescence spectroscopy at 4 K and 77 K were performed according to [15]. For these measurements, the β-DM concentration was increased to 0.07% and glycerol was added to a final concentration of 75% (v/v). LD spectroscopy was performed at 77 K as described in [37], using a two-dimensionally squeezed gelatin gel. The samples were diluted in molten 6.4% (w/v) gelatin at 32 °C and oriented by squeezing the 12.5 × 12.5 mm polymerized gel in two perpendicular directions to the 10 × 10 mm dimensions of the cuvette.

EPR spectra were recorded at the Séction de Bioénergétique, CEA-Saclay, France, on a Bruker EPR200 machine equipped with a helium cryostat from Oxford Instruments GmbH (Wiesbaden, Germany).

Chemically induced spectral changes at room temperature were recorded with a time resolving multichannel spectrophotometer based on a Zeiss spectral sensor module (MCS-VIS; Carl Zeiss AG, Oberkochen, Germany) equipped with a photo diode array for the wavelength region 360–780 nm and a spectral resolution of 3 nm (tec5 Sensorik und Systemtechnik GmbH, Oberursel, Germany). The continuous measuring light was guided by a single optical fiber from a halogen lamp to a sample compartment with a glass cuvette with 1 cm optical path length and with stirring. The transmitted light was focussed on a second fiber, which was connected to the spectral sensor module. Spectra were recorded by computer with a time resolution of 80 ms. Transmission changes ΔT were calculated by dividing the spectra by a reference spectrum recorded immediately before the experiment and ΔA was calculated by the equation:

$$\Delta A = -\log\{(\Delta T/T_1) + 1\}$$

In order to selectively observe redox changes of *b*-type cytochromes, a sample was fully prereduced by 0.5 mM ascorbate and 0.5 mM dithionite, and rapidly stirred in an open cuvette. After consumption of the dithionite by oxygen a slow reoxidation of the *b* cytochromes occurred and the absorption changes were recorded. A further oxidation of cytochrome *f* was prevented by the presence of ascorbate. Therefore, the differential absorption change ΔA(575 nm) − ΔA(564 nm) can be directly taken as a measure of cytochrome *b* oxidation. This signal was compared to the differential absorption change ΔA(665 nm) − ΔA(676 nm), representing the absorption changes at the maximum and minimum of the spectrum of the chlorophyll bandshift spectrum, respectively.

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