Characterization of two cytochrome b_6 proteins from the cyanobacterium *Gloeobacter violaceus* PCC 7421

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Abstract In the genome of the untypical cyanobacterium *Gloeobacter violaceus* PCC 7421 two potential cytochrome b_6 proteins PetB1 and PetB2 are encoded. Such a situation has not been observed in cyanobacteria, algae and higher plants before, and both proteins are not characterized at all yet. Here, we show that both apo-proteins bind heme with high affinity and the spectroscopic characteristics of both holo-proteins are distinctive for cytochrome b_6 proteins. However, while in PetB2 one histidine residue, which corresponds to H100 and serves as an axial ligand for heme b_H in PetB1, is mutated, both PetB proteins bind two heme molecules with different midpoint potentials. To recreate the canonical heme b_H binding cavity in PetB2 we introduced a histidine residue at the position corresponding to H100 in PetB1 and subsequently characterized the generated protein variant. The presented data indicate that two *bona fide* cytochrome b_6 proteins are encoded in *Gloeobacter violaceus*. Furthermore, the two *petB* genes of *Gloeobacter violaceus* are each organized in an operon together with a *petD* gene. Potential causes and consequences of the *petB* and *petD* gene heterogeneity are discussed.

Abbreviations

DDM	*n*-dodecyl-β-D-maltoside
IPTG	Isopropyl-β-D-thiogalactopyranosid
MalE	Maltose binding protein
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecylsulfate
TM	Transmembrane

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Introduction

The cytochrome $b_6 f$ complex is a central component of the photosynthetic electron transport chain in chloroplasts and cyanobacteria (Cramer et al. 2006). It is located in thylakoid membranes where it mediates electron transport from photosystem 2 to photosystem 1 by oxidizing plastoquinol and subsequently transferring the electrons to the soluble electron carrier cytochrome c_6 or plastocyanine. In cyanobacteria, the photosynthetic and respiratory electron transport chain are highly interconnected and

the cytochrome b_6f complex is involved in electron transport from photosystem 2, NAD(P)H, or the succinate dehydrogenase to photosystem 1 as well as to a terminal oxidase, respectively (Hart et al. 2005; Scherer 1990; Schmetterer 1994).

Structurally, the cytochrome b_6f complex consists of four major subunits: cytochrome f (PetA), cytochrome b_6 (PetB), the Rieske iron-sulfur protein (PetC), and subunit IV (PetD). Cytochrome f spans the membrane with a single α-helix located at the proteins' C-terminus, and the large N-terminal soluble domain contains a covalently bound c-type heme. The Rieske subunit contains a single transmembrane α-helix at its N-terminus and a large soluble domain with the Rieske [Fe$_2$S$_2$] cluster. Cytochrome b_6 consists of four transmembrane α-helices (A–D). A pair of two histidines coordinates a heme in between transmembrane helices B and D on both sides of the membrane. Based on their different midpoint potentials these two non-covalently bound hemes have been named high-potential heme (b_H) and low-potential heme (b_L). The midpoint potentials of these hemes are typically in the range of +25 to −50 mV and −50 to −150 mV for heme b_H and heme b_L, respectively (Kallas 1994). Together with cytochrome b_6, the cytochrome b_6f complex core subunit, subunit IV (PetD), is involved in quinol binding and oxidation. According to the "Q-cycle" model (Crofts et al. 1983; Mitchell 1975), the two electrons provided by the quinol branch at the quinol oxidation site of the cytochrome b_6f complex. One electron of the bound quinol is transferred via the Rieske subunit (PetC) and cytochrome f (PetA) to the soluble one-electron carrier cytochrome c_6 or plastocyanine and subsequently to photosystem 1 or a terminal oxidase. The other electron is transferred from heme b_L via heme b_H to a quinone bound at the quinone reduction site of the complex, and after complete reduction the quinol is released into the quinol pool, from where it can be oxidized again by the cytochrome b_6f complex. Two turnovers of the cytochrome b_6f complex are needed to reduce both heme b_L and b_H and to generate one plastoquinol molecule.

In the recently solved crystal structures of the cytochrome b_6f complexes from the green alga *Chlamydomonas reinhardtii* and from the cyanobacterium *Mastigocladus laminosus* a third heme molecule (heme c_i) has been identified, which is also bound to the cytochrome b_6 subunit (Kurisu et al. 2003; Stroebel et al. 2003). This heme is covalently attached via a single cysteine residue to the transmembrane helix A of cytochrome b_6 in close contact to heme b_H. Although the exact physiological function of this heme is still elusive it has been suggested that heme c_i is involved in a cyclic electron transport around the cytochrome b_6f complex and photosystem 1 (Zhang et al. 2004) and/or is directly involved in plastoquinone

binding at the cytochrome b_6f complex (Yamashita et al. 2007). Heme c_i is, however, not important for proper assembly of cytochrome b_6 and for binding of the hemes b_L and b_H (Dreher et al. 2008).

Besides the four major subunits, for which homologous proteins can be found in cytochrome bc_1 complexes (Soriano et al. 1999), additional four small polypeptides are subunits of the cytochrome b_6f complex. PetG, PetL, PetM, and PetN have molecular masses of less than 10 kDa, and each polypeptide spans the membrane once in an α-helical conformation. While the exact function of these accessory subunits is still elusive it has been shown that in cyanobacteria the subunits PetL and PetM can be deleted without a dramatic effect on the cytochrome b_6f complex activity (Schneider et al. 2001; Schneider et al. 2007). In contrast, the other two small subunits appear to be of structural and/or functional importance (Schneider et al. 2007).

While in chloroplasts the cytochrome b_6f complex subunits are typically encoded by a single gene, recently it has been noted that in cyanobacteria the Rieske protein is encoded by multiple genes (Schneider et al. 2004; Schneider et al. 2002; Tsunoyama et al. 2009). Although the physiological function of this Rieske protein family is not completely understood, some family members appear to share similar functions while others differ and probably have different physiological roles (Schneider and Schmidt 2005). Besides the Rieske protein, in cyanobacteria all other cytochrome b_6f complex subunits are typically encoded by a single gene as in algae and higher plants. However, analysis of the genome sequence of the cyanobacterium *Gloeobacter violaceus* has revealed that two proteins with homology to cytochrome b_6 proteins of various organisms are encoded in the genome (Nakamura et al. 2003). While this unusual feature has been recognized, these proteins have not been characterized in detail yet. *G. violaceus* is an unusual cyanobacterium since it lacks thylakoid membranes, and the proteins involved in photosynthetic and respiratory electron transport are all located in its cytoplasmic membrane (Rippka et al. 1974).

We have recently shown that the cytochrome b_6 protein of spinach can be heterologously expressed in *E. coli* to obtain large protein quantities for subsequent analyses (Dreher et al. 2007; Prodöhl et al. 2007a). After heterologous expression the spinach cytochrome b_6 protein incorporates into the *E. coli* inner membrane and binds heme from the *E. coli* endogenous heme pool (Dreher et al. 2007). Furthermore, the protein can be purified from inclusion bodies for subsequent in vitro analyses (Prodöhl et al. 2007a).

Here we show that fusion of the two potential *G. violaceus* cytochrome b_6 proteins to the *E. coli* maltose binding protein (MalE) also allows a high level production of these proteins

in *E. coli*. The expressed proteins highly accumulated within the *E. coli* inner membranes as well as in inclusion bodies. Both heterologously expressed proteins bind heme and display spectroscopic characteristics typical for cytochrome b_6 proteins. However, while PetB2 has a not strictly conserved heme b_H binding cavity, both proteins bind two heme molecules with different midpoint potentials. Since in the *G. violaceus* PetB2 sequence one histidine residue, which serves as a heme b_H axial ligand, is mutated, we introduced a histidine residue at the corresponding position and characterized the generated PetB2 variant. All the presented data indicate that two *bona fide* cytochrome b_6 proteins are encoded in *G. violaceus*, potential functions of which are discussed.

Material and methods

Expression and purification of PetB fusion proteins

The *petB1* and *petB2* genes of *Gloeobacter violaceus* have been amplified by PCR using genomic *G. violaceus* DNA as a template. At the 5′ end of the gene a *Nde*I restriction site has been introduced by the used primers and at the 3′ end a *Bam*HI site. After restriction digestion of the PCR fragments with *Nde*I and *Bam*HI the *petB* genes were ligated to the equally restriction digested plasmid pRHMb6 (Prodöhl et al. 2007a), in which a protein of interest is genetically fused to the *E. coli* MalE protein containing a N-terminal deca-histidine tag.

The amino acid F105 of PetB2 was mutated by using the QuickChange site directed mutagenesis kit from Stratagene according to the manufacturer's instructions.

For protein expression, the generated expression plasmids were transferred into *E. coli* HMS174 (DE3) cells. 10 mL LB medium with 100 µg/mL ampicillin was inoculated with a single colony and grown over night at 37 °C. 1 L LB medium with 100 µg/mL ampicillin was inoculated with the over night culture, at $OD_{600} = 0.6$ protein expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG), and the cells were harvested after 3 h. For protein purification cells were broken by sonication in buffer (50 mM HEPES pH 7.5, 10 mM EDTA), and inclusion bodies were sedimented by centrifugation (10,000 g, 15 min, 4 °C). The supernatant was further separated by ultracentrifugation (190,000 g, 1 h, 4 °C) finally resulting in a soluble protein fraction and membranes.

For purification of the PetB proteins, inclusion bodies were solubilized in 10 mM Tris (pH 8.0), 100 mM phosphate buffer containing 50 mM sodium dodecylsulfate (SDS) and incubated for 10 min at 90 °C. After incubation, non-solubilized material was removed by centrifugation

and the supernatant was further purified. 45 mM KCl was added to the protein solution to precipitate the bulk of SDS and to reduce the SDS concentration to 5 mM. The solution was incubated for 10 min on ice and precipitated potassium dodecylsulfate was removed by centrifugation. The supernatant was incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) for 1 h at 4 °C. Afterwards, the affinity matrix was washed three times with Tris/phosphate buffer (pH 8.0) containing 2 mM SDS, and apo-cytochrome b_6 was finally eluted with Tris/phosphate buffer (pH 4.5) containing 2 mM SDS. *n*-dodecyl-β-D-maltoside (DDM) was added to a final concentration of 5 mM, and the rest of SDS was removed by addition of KCl as described above. The proteins were finally dialyzed against 50 mM Tris (pH 8.0), 50 mM NaCl.

For measuring absorbance spectra of *E. coli* membranes after expression of the PetB1 and PetB2 fusion proteins, *E. coli* membrane proteins (200 µg protein) were extracted with 50 mM DDM and measured either air oxidized or after reduction with 5 mM dithionite.

In vitro reconstitution of PetB proteins

The concentration of the purified PetB proteins was determined using the BCA assay kit from Pierce (Rockford, USA) following the manufacturer's instructions. For reconstitution, commercially available hemin chloride (Sigma, Taufkirchen, Germany) was suspended in 50% ethanol and the heme was solubilized by addition of NaOH to a final concentration of 2 mM. This heme solution was diluted fourfold into 50 mM Tris (pH 8.0), 50 mM NaCl, 5 mM DDM buffer. The heme concentration was determined spectroscopically using an extinction coefficient of $\lambda_{385\ nm}=56\ mM^{-1}\ cm^{-1}$ (Ozols and Strittmatter 1964).

For reconstitution, the proteins were mixed with heme in defined ratios (see also Results & Discussion). For heme reduction, 5 mM sodium dithionite was added to the solution from a freshly prepared 1 M stock solution. Incorporation of heme and assembly of the holo-cytochrome was followed by UV/Vis spectroscopy using a Perkin Elmer Lambda 35 instrument. Spectra were taken from 350 nm to 700 nm with a resolution of 0.5 nm.

For the heme titration experiments, increasing amounts of a solution containing 50 µM cytochrome b_6 apo-protein were individually mixed with a solution of a constant concentration of 5 µM reduced heme and the increase in absorption at 428 nm was measured for various protein/ heme ratios (at a constant heme concentration).

Determination of the heme midpoint potentials

For redox titrations the in vitro reconstituted proteins were again purified via a Ni-NTA agarose column to remove

unbound heme. Cytochromes were finally eluted from the column with buffer containing 5 mM DDM and 500 mM imidazole. The samples were extensively dialyzed afterwards to remove imidazole. Samples for the electrochemical approach contained 50 mM Tris (pH 8.0), 100–200 mM NaCl and 5 mM DDM. Redox titrations have been performed with a spectroelectrochemical thin layer cell (Moss et al. 1990). The gold grid was modified with 2 mM cysteamine and mercaptopropionic acid in a ratio of 1:1. For better equilibration, redox mediators were added as previously reported (Hellwig et al. 1998). Data were collected at 7 °C on a CARY 300 UV/Vis spectrometer. After an equilibration time of 15–20 min for each step, three scans were averaged. Data were fitted with the help of the program Origin 6.0 (Microcal, Northhampton, MD) on the basis of the Nernst equation. The n-value (number of transferred electrons) was varied between 0.7 and 1. The value leading to the best fit was then used.

Results and discussion

The genome of Gloeobacter violaceus contains two open reading frames coding for potential cytochrome b_6 proteins

In the completely sequenced genome of the cyanobacterium *Gloeobacter violaceus* PCC 7421 genes encoding the cytochrome b_6f complex subunits PetA (*glr3039*), PetB (*gll1919*), PetC (*glr3038*), PetD (*gll1918*), PetG (*gsl0511*), and PetN (*gsl3700*) have been identified, while genes coding for the two small subunits PetM and PetL have not been assigned yet (Nakamura et al. 2003). Although these subunits appear not to be absolutely essential components of the cyanobacterial cytochrome b_6f complex, the PetL and PetM sequences are not conserved well and most likely, due to this, these subunits could not be properly assigned yet. Besides the typical *petB* gene *gll1919* (*petB1*) a second gene (*gll1870, petB2*) codes for a protein having some sequence similarity with the cytochrome b_6 protein of other organisms. While the open reading frame (ORF) *gll1919* encodes a 215 amino acid long polypeptide and the protein displays 77% sequence identity (90% similarity) to the cytochrome b_6 protein of the cyanobacterium *Synechocystis* sp. PCC 6803, the ORF *gll1870* codes for a 217 amino acid long protein having only 23% sequence identity with the *Synechocystis* protein (53% similarity). The two *G. violaceus* proteins share 26% sequence identity and 56% similarity with each other. Because of significant sequence similarities with PetB proteins from various sources and due to the characteristics described in more detail below we suggest to name the cytochrome b_6 protein encoded by the ORF *gll1919* PetB1 and the protein encoded by the ORF *gll1870* PetB2.

In Fig. 1 a sequence alignment of the two *G. violaceus* PetB sequences is shown. Both sequences have four hydrophobic regions, which are supposed to form transmembrane α-helices. In helix B and D of the PetB1 protein four conserved histidine residues are located which form the fifth and sixth axial ligands of heme b_L (H86 and H187) and heme b_H (H100 and H202), respectively. However, as can be seen in Fig. 1, the sequence of PetB2 does not contain all four typically conserved histidine residues in the transmembrane regions. While PetB1 contains a histidine at amino acid position 100, at the corresponding region of PetB2, a phenylalanine residue is found instead. Based on the available cytochrome b_6 structures, the propionate groups of heme b_H would interact with R103 in PetB1. Since this residue is also mutated in the PetB2 sequence the available sequence information might indicate that PetB2 binds only heme b_L. Furthermore, heme c_i, which is bound via a single cysteine residue to helix A of cytochrome b_6 (Kurisu et al. 2003; Stroebel et al. 2003), will be attached via C35 to PetB1. In the corresponding region of PetB2 this amino acid is replaced by a tyrosine residue, and, therefore, PetB2 is probably not able to bind heme c_i covalently. While mutations of heme b_H axial ligands can have severe effects in vivo (Kuras et al. 1997) it has to be noted that a spinach cytochrome b_6 protein having a mutation at a position, which corresponds to H100 in PetB1, still assembles and binds both heme b_L and heme b_H in vitro (Dreher et al. 2008).

Heterologous expression of PetB1 and PetB2

Fusion of transmembrane apo-cytochromes to the *E. coli* MalE protein enables a high-level production of these proteins in *E. coli*. After fusion of PsbF from the cyanobacterium *Synechocystis* PCC 6803, which forms together with the PsbE protein the cytochrome b_{559} subunit of photosystem 2, as well as of apo-cytochrome b_6 of spinach to MalE, the heterologously expressed proteins accumulated to a high extent in the *E. coli* inner membrane. The membrane integrated apo-proteins bound heme from the endogenous *E. coli* heme pool resulting in assembly of the respective holo-cytochromes (Dreher et al. 2007; Prodöhl et al. 2005). Furthermore, the chimeric proteins accumulated in inclusion bodies, which allowed large scale purification of the proteins for subsequent in vitro analyses (Prodöhl et al. 2007a, b; Volkmer et al. 2006). Importantly, an influence of the large fusion domain on heme binding can be neglected (Dreher et al. 2008).

To characterize the proteins encoded by the ORFs *gll1919* (*petB1*) and *gll1870* (*petB2*) in more detail, we genetically fused the encoded proteins to the MalE protein of *E. coli*. During protein expression the *E. coli* cells became brownish, which already indicated that the two

Fig. 1 Alignment of the *Gloeobacter violaceus* PetB1 and PetB2 protein sequences. The regions predicted to span a biological membrane in an α-helical conformation are *boxed*. Histidine residues in the transmembrane (TM) helices B and D are involved in heme binding to apo-cytochrome b_6 and are *highlighted*. The cysteine residue in TM A (C35 in PetB1), which is involved in covalent binding of heme c_i to cytochrome b_6, is marked with an *arrow*. TM regions were identified based on a sequence alignment of the *Gloeobacter violaceus* PetB sequences with proteins from *Chlamydomonas reinhardtii*, *Mastigocladus laminosus*, and *Nostoc* sp. PCC 7120 for which crystal structures are available (Baniulis et al. 2009; Kurisu et al. 2003; Stroebel et al. 2003)
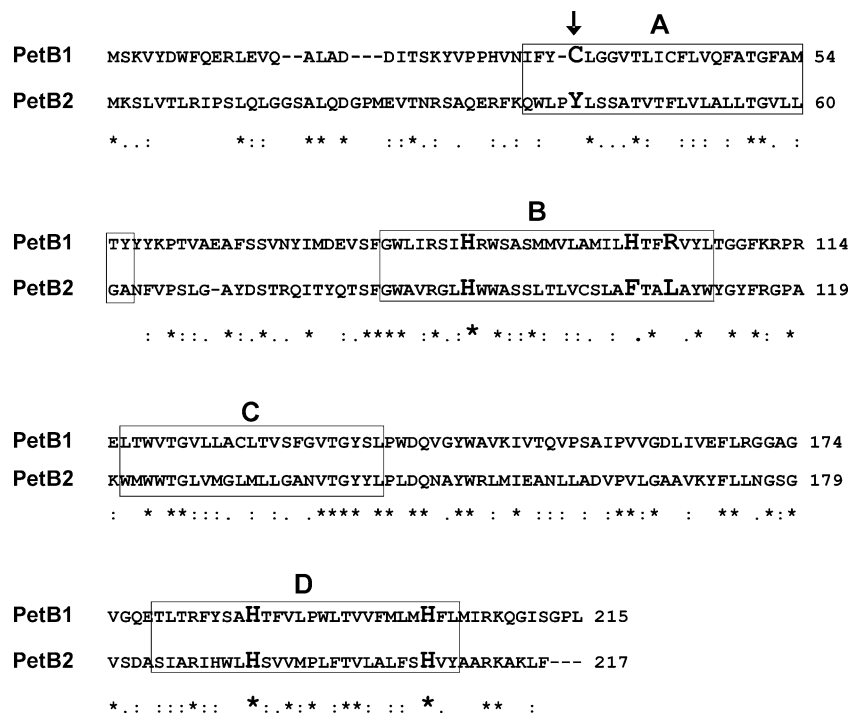
proteins incorporated and assembled into the *E. coli* inner membrane similar to the situation observed after expression of the spinach cytochrome b_6 protein (Dreher et al. 2007). As can be seen in Fig. 2, both proteins integrated to a significant degree into the *E. coli* inner membrane, and absorption spectra indicated the presence of new cytochromes in these membranes. These new cytochromes have α-band redox difference absorption maxima at about 561 nm, which is characteristic for cytochrome b_6 proteins. Besides the membrane incorporated fraction, a fraction of the chimeric proteins was deposited in inclusion bodies after expression, which allowed purification of large quantities of the heme free apo-proteins for subsequent characterizations (Fig. 2). Addition of free heme to the purified apo-proteins resulted in incorporation of the heme cofactor and in formation of holo-cytochrome b_6 proteins (Fig. 3). The redox difference spectra of the in vitro reconstituted PetB1 and PetB2 proteins showed α-band maxima at 561 nm, and β-band maxima at 531 nm. These maxima correspond well to α- and β-band maxima reported for other b-type cytochromes and suggest that both *petB* ORFs encode *bona fide* cytochrome b_6 proteins. Furthermore, the spectra are indicative of heme bound in a low-spin state (Harbury et al. 1965; Schünemann et al. 1999). The spectroscopic properties of PetB1 and PetB2 are summarized in Table 1.

However, the spectroscopic characterization of the membrane embedded proteins did not allow determining how many heme molecules are bound per apo-cytochrome. Since in the PetB2 sequence one histidine residue is

mutated, it appears possible that PetB2 only binds heme b_L (as discussed above).

Heme binding properties of cytochrome b_6 proteins

To determine the amount of heme molecules bound by PetB1 and PetB2, respectively, we isolated the heterologously expressed proteins (Fig. 2), and assembly of holo-cytochrome b_6 was followed in vitro by titrating a solution of reduced heme with small quantities of the cytochrome b_6 apo-proteins (Fig. 4). After addition of increasing amounts of apo-cytochrome b_6 to free heme, the increase in the absorption at 428 nm was followed. The absorption increased as long as free heme was bound by the added protein, whereas no further increase was observed when all free heme was bound by the added protein. Interestingly, the heme titration experiment indicated that there is no difference in heme binding between PetB1 and PetB2 of *G. violaceus*, regardless of the fact that in the PetB2 protein the heme ligating residue H105 is mutated. Analysis of the titration data indicates that two heme molecules bind to both PetB1 and PetB2 (grey line, turning point). To further analyze heme binding to PetB2 in more detail we replaced residue F105 of PetB2 with histidine, and thereby restored the "typical" situation in cytochrome b_6 proteins. Heme titration experiments with PetB2 F105H revealed that the titration curve of this protein did not alter when compared to the PetB1 and PetB2 wt proteins, and this protein variant does most likely also bind two heme molecules (grey line, turning
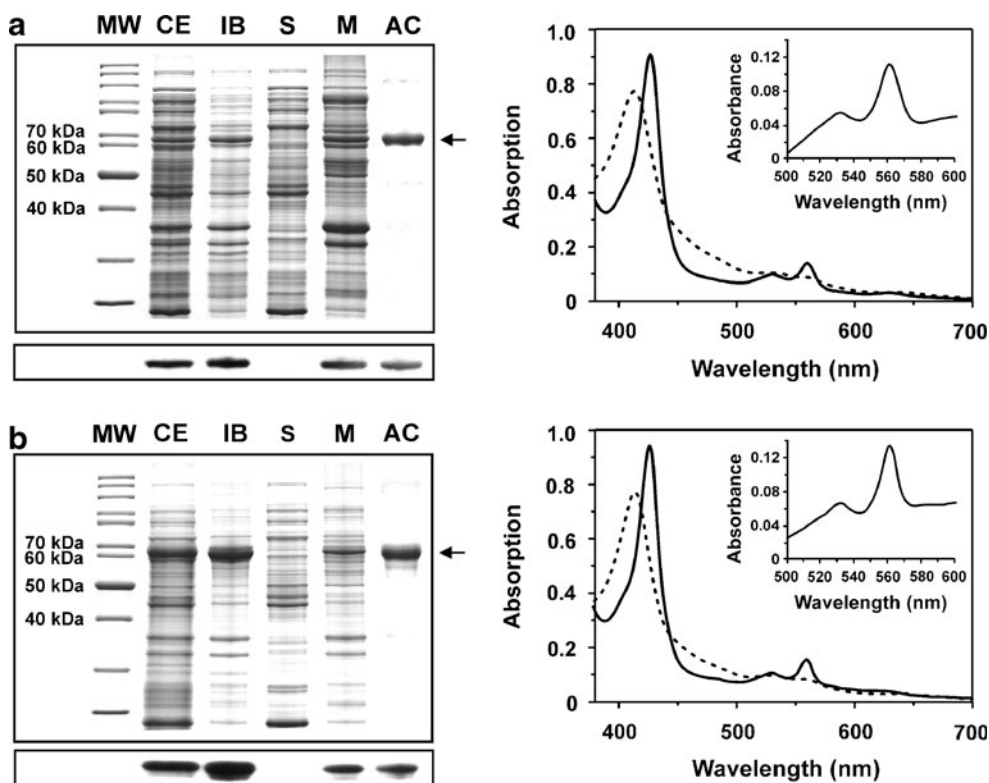
Fig. 2 Expression of the PetB1 and PetB2 fusion proteins in *E. coli*. In (**a**) the SDS gel and the absorption spectra of PetB1 and in (**b**) the SDS gel and spectra of PetB2 are shown. Cell fractions equivalent to 50 μL cells with an OD$_{600}$ = 0.6 or 1 μg of the purified proteins were separated on a 14% SDS gel. *MW* molecular weight marker; *CE* total cellular extract; *IB* inclusion body fraction; *S* soluble proteins; *M* membranes; *AC* protein purified by affinity chromatography. An antibody directed against the histidine-tag (from Sigma) cross-reacted with a protein of about 60 kDa and thus recognized the histidine-tagged PetB fusion proteins (panel below the SDS gels). Vis spectra of *E. coli* membranes after expression of the PetB1 (**a**) and PetB2 (**b**) fusion proteins are shown under air oxidizing conditions (*dashed line*) or after reduction with 5 mM sodium dithionite (*solid line*). The redox difference spectrum (*inlet*) shows an absorption maximum (α-band maximum) at 561 nm for both proteins

point) (Fig. 4). The observation that PetB2 and PetB2 F105H appear to have identical heme binding properties highly suggests that already the wt PetB2 protein can bind two heme molecules non-covalently. Since the absorption spectra of both the wt and the F105H mutated PetB2 proteins are identical, substitution of F105 appears to not alter the heme binding properties (as further discussed below).

To characterize the heme binding properties of the *G. violaceus* PetB proteins in more detail, redox potentials of the PetB proteins were determined by means of a spectro-electrochemical approach (Fig. 5). In line with the above described measurements, two potentials with values of −246 and −96 mV were determined for PetB1, corresponding to the low-potential and high-potential hemes b_L and b_H, respectively (Table 1). Furthermore, also for the wt PetB2
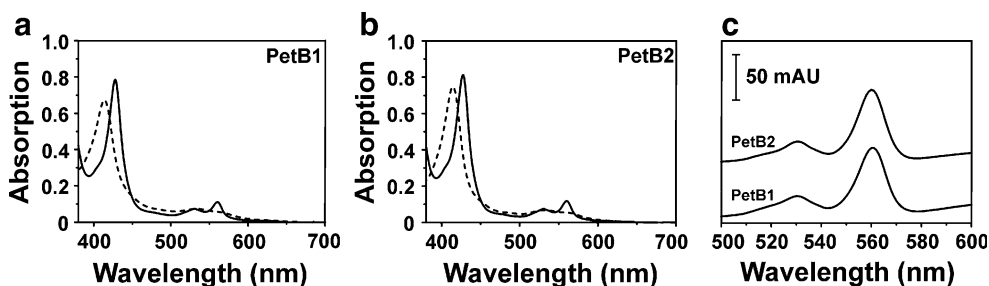

Fig. 3 Absorption spectra of oxidized and reduced cytochrome b_6 proteins PetB1 (**a**) and PetB2 (**b**). The apo-cytochromes were refolded in vitro in 5 mM DDM and free heme was added. Spectra of 150 μg protein were measured either air oxidized (*dashed line*) or after reduction with 5 mM sodium dithionite (*solid line*). Absorption maxima are listed in Table 1. **c** shows the redox difference spectra with α-band absorption maxima at 561 nm for both PetB proteins

Table 1 Absorption maxima and electrochemical properties of the *Gloeobacter violaceus* wt PetB proteins and the PetB2 F105H mutant

	Soret-band		β-band		α-band		E_m of heme (± 15mV)	
							b_L	b_H
PetB1							−246 mV	−96 mV
Ox	414	nm	528	nm	558	nm		
Red	428	nm	531	nm	561	nm		
PetB2							−239 mV	−73 mV
Ox	415	nm	529	nm	558	nm		
Red	428	nm	531	nm	561	nm		
PetB2 F105H							−204 mV	−100 mV
Ox	415	nm	529	nm	559	nm		
Red	428	nm	531	nm	561	nm		

protein two midpoint potentials were determined having E_m values of −239 and −73 mV, respectively. These two potentials also correspond to the heme b_L and heme b_H and are perfectly in line with the above mentioned observation that PetB2 also binds two heme molecules. As expected, characterization of the PetB2 F105H variant clearly indicated the presence of two heme molecules having E_m values of −204 (heme b_L) and −100 mV (heme b_H). Thus, for both PetB2 and PetB2 F105H the determined E_m values are very similar, indicating that H105 is essentially not required for heme b_H binding and functioning. It has to be mentioned that the E_m values determined for hemes b_L and b_H are more negative than the E_m values previously reported for other cytochrome b_6 proteins. E_m values of less than −200 mV for heme b_L would not allow efficient electron transfer from plastiquinol to heme b_L. The E_m values of the cytochrome b_6 hemes are most likely more

Fig. 4 Heme binding curve for PetB1, PetB2 and PetB2 F105H. Heme titration experiments were performed as described in Material and Methods. Each data point represents an independent measurement. For all three proteins binding of about two hemes per protein was determined

positive when the protein is incorporated in the cytochrome b_6f complex, and it is likely that the detergent environment affects the midpoint potentials.

Conclusions and physiological impact

The findings of this study suggest that two cytochrome b_6 proteins are encoded in the genome of the cyanobacterium *Gloeobacter violaceus*. PetB1 shows a high sequence identity to cytochrome b_6 proteins of other organisms and it binds two heme molecules non-covalently, which is typical for cytochrome b_6 proteins and a prerequisite for the protein function. Furthermore, the PetB1 sequence contains a cysteine residue at amino acid position 35, which is critical for binding of heme c_i in vivo (Kurisu et al. 2003; Stroebel et al. 2003). Because of these features we propose that PetB1 is the favoured and prominent cytochrome b_6 protein in *G. violaceus* and is a functional core subunit of the *G. violaceus* cytochrome b_6f complex.

But what about PetB2? PetB2 displays only a weak sequence identity to different cytochrome b_6 proteins of various sources. However, the protein appears to have retained a structure typical for cytochrome b_6 proteins. The protein has about the same length as PetB1 and four hydrophobic regions are preserved, which form transmembrane α-helices. The results shown in Fig. 2b demonstrate that PetB2 can properly integrate into a biological membrane, and the wt protein is capable of binding two heme molecules non-covalently. While the amino acids critical for heme b_L binding are conserved in PetB2, one histidine residue, which serves as an axial ligand of heme b_H, is replaced by a phenylalanine in the PetB2 sequence. Furthermore, in PetB2 not only two residues involved in heme b_H binding (H100 and R103 in the PetB1 sequence) have been mutated but also numerous other residues. However, although many amino acids are substituted in PetB2 when compared to PetB1, the wt PetB2 protein binds two heme molecules with two defined midpoint
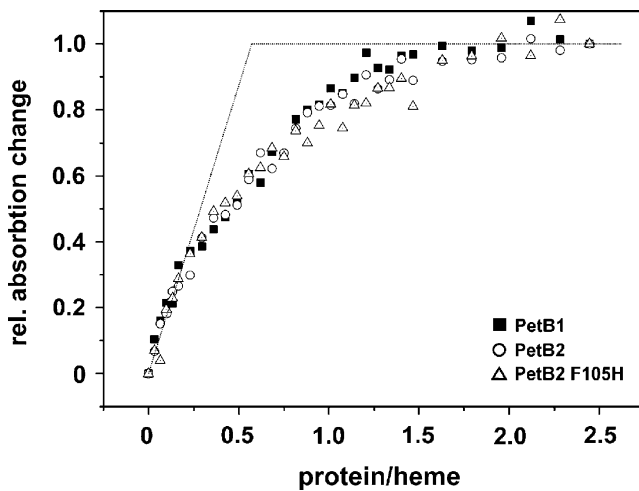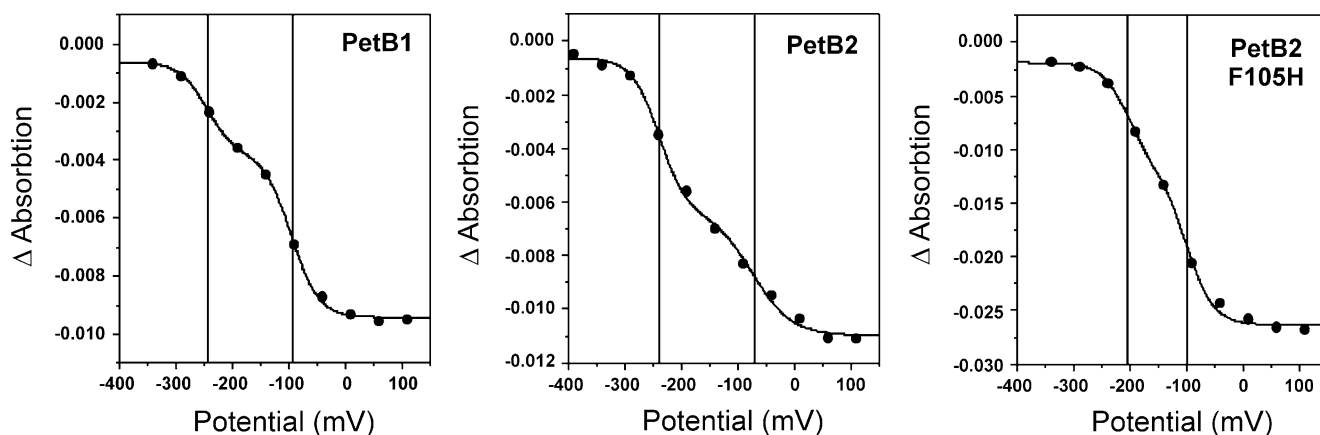
Fig. 5 Redox dependent spectral changes of the cytochrome b_6 proteins (PetB1, PetB2 and PetB2 F105H) as monitored in the visible spectral range. For each protein two redox potentials were found for a best fit at 561 nm (compare Table 1) with a n-value of 1 for each transition (number of transferred electrons) except for the high-potential transition of PetB2, that was fitted with a n-value of 0.7 (see Material and Methods)

potentials. Replacement of F105 with histidine did not enhance heme binding *per se*, which indicates that the PetB2 structure has evolutionary adopted to bind heme b_H with only a single histidine residue. Since the overall geometry of a heme binding cavity can be preserved, replacement of one axial heme b_H histidine ligand does not necessarily result in a general loss of the heme binding capacity. Mutants can be generated in which axial heme ligands are replaced but the heme binding niche is preserved, resulting in proper heme binding to the apo-protein (Barrick 1994; Kamiya et al. 2001; Newmyer and de Montellano 1996; Pond et al. 2000). However, replacement of an axial ligand could result in a penta-coordinated high-spin heme, which is not observed, and all spectra are indicative for a hexa-coordinated low-spin heme. For the cytochrome b_6 protein from spinach it has recently been shown that replacement of the axial ligand H100, which corresponds to residue 105 in the PetB2 sequence, by alanine does not abolish heme b_H binding and the spectra were characteristic for a heme in its low-spin

state (Dreher et al. 2008). Thus, a histidine at position 105 appears to not be essential for binding of heme b_H. While neither alanine nor phenylalanine is expected to act as an axial heme b_H ligand, it appears likely that water, OH^-, Cl^-, or other anions act as a sixth ligand to this heme, which finally results in a hexa-coordinated low-spin heme. Furthermore, we can also not exclude that an amino acid side chain at a different position than amino acid 105 serves as an axial ligand for heme b_H in PetB2. The exact nature of the sixth axial ligand of heme b_H will be elucidated in future experiments.

But what could the physiological function of PetB2 be? We cannot answer this question at this stage and it appears possible that PetB2 is not involved in the traditional functioning of the cytochrome b_6f complex since it might lack heme c_i. It is therefore possible, that PetB2 is a result of a spontaneous *petB* gene duplication and that the protein has no preserved function in the organism. If PetB2 is of no importance and has no physiological function this would

Fig. 6 Alignment of the *Gloeobacter violaceus* PetD1 and PetD2 protein sequences. The regions predicted to span a biological membrane in an α-helical conformation (TM helices E-G) are *boxed*
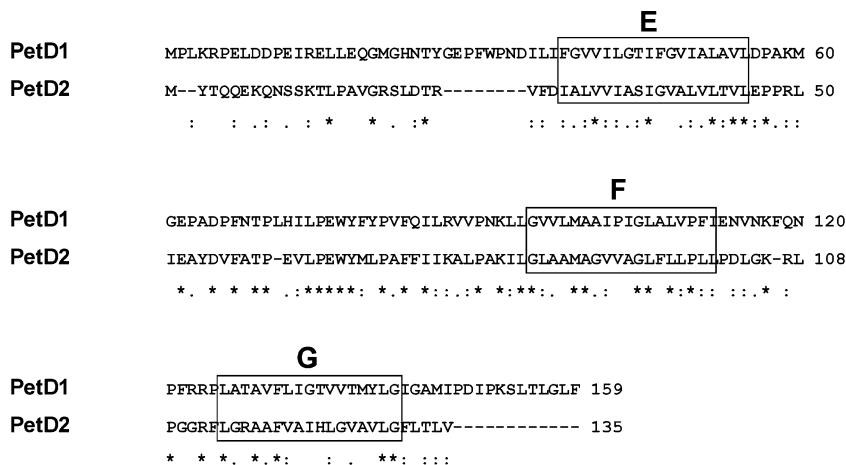
explain why so many mutations have accumulated in the *petB2* sequence. Consequently, the *petB2* gene would then have to be defined as a pseudogene in *G. violaceus*. However, it is interesting that, while many mutations have been accumulated in the *petB2* gene, resulting in potential expression of a PetB protein, which just shares 26% sequence identity to PetB1, none of the *petB2* codons has been mutated to a stop codon. If expression of *petB2* is of no physiological importance or advantage, the gene would eventually have been interrupted and/or highly degraded in time. Therefore, even PetB2 could still have a yet unknown physiological function in vivo. A conserved cysteine residue critical for heme c_i binding has only been identified in cytochrome b_6f-like complexes, in which a "split" cytochrome b subunit is present and these complexes thus possess the four-helix cytochrome b_6 and subunit IV proteins (Cramer and Zhang 2006). Cytochrome bc_1 complexes of other bacteria and of mitochondria contain a single cytochrome b subunit, the N-terminus of which is highly homologous to cytochrome b_6 and the C-terminus to subunit IV (Widger et al. 1984). Cytochrome bc_1 complexes fulfil about the same function as cytochrome b_6f complexes by mediating the transfer of electrons from a quinol to a soluble electron carrier. Thus, binding of a third heme molecule to the cytochrome b subunit is not essential for the complex function *per se*, and it is possible that other mutations have accumulated in the PetB2 and PetD2 proteins (see below), which makes binding of heme c_i to the cytochrome b_6 subunit dispensable. Furthermore, it appears to be possible that a functional cytochrome b_6f complex assembles even in the absence of covalent heme c_i attachment to the cytochrome b_6 subunit (Saint-Marcoux et al. 2009).

However, while the presented data indicate that *petB2* encodes a cytochrome b_6 protein, at this stage we cannot completely rule out the possibility that the gene encodes a transmembrane b-type cytochrome distinct from cytochrome b_6.

In all cyanobacterial genomes completely sequenced so far the *petB* gene is organized in an operon together with the *petD* gene, which encodes the subunit IV (PetD) of the cytochrome b_6f complex (Kallas et al. 1988). Interestingly, besides the two *petB* genes *G. violaceus* also contains two *petD* genes, and these genes are both organized in an operon together with the corresponding *petB* gene, which further supports the assumption that also *petB2* encodes a *bona fide* cytochrome b_6 protein. The protein encoded by the gene *gll1918* (*petD1*) shows a high degree of identity (>70%) to subunit IV proteins of other organisms. In contrast, the protein encoded by the gene *gll1869* (*petD2*) has only limited sequence conservation when compared to other subunit IV proteins. The protein has deletions at its C-terminus as well as within the N-terminal region (Fig. 6). However, both proteins have three hydrophobic

regions, which most likely span the membrane in an α-helical conformation and *e.g.* the PEWY region in the loop connecting helices E and F is also conserved in both proteins (residues 75–78 in PetD1). The highly conserved PEWY sequence has been shown to be involved in formation and function of the quinol oxidation pocket in the cytochrome b_6f complex and has a key function in cytochrome b_6f turnover (Finazzi 2002; Zito et al. 1999; Zito et al. 1998).

It is remarkable that also the *petD2* gene encodes a protein with similar length and structural properties as the canonical PetD protein, and while many mutations have accumulated in the *petD2* gene, the gene does also not contain any internal stop codon, and thus still encodes a full length PetD protein. It is likely that PetB2 and PetD2 have coevolved and fulfil together a yet undefined physiological function.

While this physiological function would eventually be best characterized by detailed biochemical analyses of the purified *G. violaceus* cytochrome b_6f complex combined with mutational studies, such studies would be an unpromising and demanding task due to the non-established genetic tools for *G. violaceus* and the extremely slow growth rate of the organism, which makes this organism an unsuitable source for the production of high protein amounts. Thus, studies with heterologously expressed proteins—as described here—are a potent and promising strategy to study structure-function relationships of individual proteins of the cyanobacterium *Gloeobacter violaceus*.

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