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Cytochrome c_{6B} of *Synechococcus* sp. WH 8102 – Crystal structure and basic properties of novel c_6-like family representative ☆

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ABSTRACT

Cytochromes *c* are soluble electron carriers of relatively low molecular weight, containing single heme moiety. In cyanobacteria cytochrome c_6 participates in electron transfer from cytochrome b_6f complex to photosystem I. Recent phylogenetic analysis revealed the existence of a few families of proteins homologous to the previously mentioned. Cytochrome c_{6A} from *Arabidopsis thaliana* was identified as a protein responsible for disulfide bond formation in response to intracellular redox state changes and c_{550} is well known element of photosystem II. However, function of cytochromes marked as c_{6B}, c_{6C} and c_M as well as the physiological process in which they take a part still remain unidentified. Here we present the first structural and biophysical analysis of cytochrome from the c_{6B} family from mesophilic cyanobacteria *Synechococcus* sp. WH 8102. Purified protein was crystallized and its structure was refined at 1.4 Å resolution. Overall architecture of this polypeptide resembles typical I-class cytochromes *c*. The main features, that distinguish described protein from cytochrome c_6, are slightly red-shifted α band of UV–Vis spectrum as well as relatively low midpoint potential (113.2 ± 2.2 mV). Although, physiological function of cytochrome c_{6B} has yet to be determined its properties probably exclude the participation of this protein in electron trafficking between b_6f complex and photosystem I.

1. Introduction

In cyanobacterial cells, the interaction between different photosynthetic membrane complexes is possible due to the presence of mobile electrons' carriers. Plastoquinone molecules transport electrons between PSII and cytochrome b_6f complex within phospholipid bilayer. Metalloproteins: cytochrome c_6 and/or plastocyanine are responsible for electron transport between cytochrome b_6f complex and PSI in thylakoid lumen. The oxidized form of these metalloproteins (Cu^{2+}-carrying plastocyanine as well as Fe^{3+}-containing cytochrome c_6) adopts one electron from cytochrome *f*, being a part of the b_6f complex [1]. As a result of this reaction the reduced electron carrier is formed which moves within the lumen towards PSI. For specific targeted traffic of mentioned metalloproteins electrostatic and/or hydrophobic interactions are responsible [2]. After docking to PSI, metalloprotein passes an electron onto photooxidized dimer of chlorophyll $P700^+$ molecules. This event closes the electron transfer from b_6f to PSI [1]. Photosynthetic electron transport is not the only function of cyanobacterial cytochrome c_6 and plastocyanin. In cyanobacteria, photosynthetic and respiratory electron transport chains physically overlap in thylakoid membranes. In opposition, analogous protein complexes localized in the cytoplasmic membranes participate only in respiration process. In cyanobacteria typical cytochromes of type *c* which would pass electrons to cytochrome *c* oxidase (in cyanobacteria - oxidase type aa_3) are absent. Nevertheless, a long time ago hypothesis was formed, that b_6f complex, quinone pool or cytochrome c_6 are elements common to the processes of photosynthesis and respiration in cyanobacteria. Subsequent data seem to confirm the validity of such supposition over the years [3]. The third process, which involves the cyanobacterial cytochrome c_6 is the process of anoxygenic photosynthesis, where hydrogen sulfide is a source of electrons. In this process, cytochrome c_6 can transport electrons between quinones and iron-sulfur centers during the anaerobic oxidation of sulphides [4].

Abbreviations: Cyt, cytochrome; Wat, water.

☆ Database: Atomic coordinates and structure factors for Cytochrome c_{6B} of *Synechococcus* sp. WH 8102 are available from the Protein Data Bank under the accession code 4KMG.

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Bialek and co-workers have identified two new groups of c_6-like cytochromes. Due to the earlier discovery of c_{6A} type cytochromes, these groups are named analogously as c_{6B} and c_{6C} [5]. Family of cyanobacterial c_{6B} cytochromes is quite closely related to chloroplastic c_{6A} cytochromes. However, cytochromes of group c_{6B} and c_{6C} do not include in their primary structure 12-residues-long loop, characteristic for c_{6A} family. Another difference is conserved tyrosine residue at position 61 of cytochrome c_{6B} and c_{6C}, which is analogous to phenylalanine or tryptophan occupying corresponding position in c_6 and c_{6A} cytochromes. In the c_{6B} group sequences are mainly derived from non-binding nitrogen marine cyanobacteria species–*Prochlorococcus* and *Synechococcus*. These species are characterized by about 95% identity to the 16S rRNA sequence, but consists of a number of physiologically and genetically different ecotypes [6–9].

The presence of genes encoding c_6-like cytochromes in genomes of many cyanobacteria provides a basis for extending hypotheses about the role of these proteins in the proper functioning of cells. The diversity of cyanobacteria, in which these genes are present (single-celled green algae, filamentous or nitrogen-fixing) indicate that c_6-like cytochromes appeared relatively early in the history of the evolution of these microorganisms [5]. Compared to enormity of research projects concerning cytochromes in general, c_6-like cytochrome are rarely mentioned in the literature. This fact may be primarily due to their relatively recent discovery. Data presented in this paper extend current knowledge about c_6-like cytochromes. Unfortunately, biological function of these proteins still remains unclear.

2. Materials and methods

2.1. Protein expression and purification

Escherichia coli strain DH5α was co-transformed with pUC_c6B and pEC86 plasmids. The former harbors a gene encoding mature cyt c_{6B} from *Synechococcus* sp. WH8102, whereas the latter the heme maturation gene [10]. 5 mL of overnight culture were used to inoculate 1.75 L of TB medium in a 2 L flask. Cultures were grown for 8 h at 37 °C with vigorous agitation and then IPTG was added to final concentration of 0.75 mM. Subsequently, cultures were grown for 72–96 h at 30 °C with agitation at 80 rpm. Cells were harvested at 5000*g*, 4 °C, washed in 30 mM Tris, pH 8.0, 0.1 M NaCl, 20% sucrose, 1 mM EDTA. After centrifugation and resuspension in the same buffer the periplasmic proteins were released by lysozyme treatment (0.2 mg/mL) at RT for 20 min with shaking and then centrifuged at 25,000*g* for 25 min at 4 °C. The supernatant, which contains the periplasmic protein fraction, was incubated with ammonium sulfate (45% saturation, 30 min, 4 °C) and centrifuged at 20,000*g* for 20 min at 4 °C, and the pellet was discarded. Ammonium sulfate was added to the supernatant to 95% saturation and treated as described above. The red pellets were resuspended in 20 mM phosphate buffer pH 6.2, 1 mM PMSF and dialyzed against the same buffer, overnight at 4 °C. Solution after dialysis was loaded onto a HiTrap SP HP column (GE Healthcare) connected to AKTA Purifier system and equilibrated with the same buffer. Proteins were eluted with linear 0–200 mM NaCl gradient. Fractions collected from the first column were dialyzed against 50 mM ethanolamine pH 9.0. After overnight dialysis the sample was applied to a HiTrap Q HP column (GE Healthcare) equilibrated with the same buffer. Proteins were eluted with linear 100–500 mM NaCl gradients. Purified cyt c_{6B} was characterized by SDS–PAGE and heme staining as described in [5]. The A_{557}/A_{280} ratio of purified cyt c_{6C} was 0.623. The proteins were aliquoted and stored at −20 °C.

2.2. Cytochromes absorption spectroscopy and redox titrations

All spectroscopic measurements were carried out using a Beckman DU800 spectrophotometer. Measurements were conducted at room temperature using a 1 cm path-length cuvette. Cytochrome was diluted to final concentration of 5 μM in 10 mM Tris pH 7.5 containing 1 mM potassium ferricyanide (spectra of oxidized cytochromes) or 1 mM sodium dithionate (spectra of reduced cytochromes). Redox titrations were performed as described in [11]. Procedure was repeated three times in both directions in a custom-made anaerobic cuvette, containing platinum electrode and calomel reference electrode, under argon flow, in 50 mM MOPS pH 7.0 and 100 mM KCl in the presence of redox mediators: tetrachlorohydroquinone (TCHQ, $E_{m,7}$ = 350 mV), 2,3,5,6 tetramethyl-p-phenylenediamine (DAD, 260 mV), 1,2-naphthoquinone-4-sulfonate (NQS, $E_{m,7}$ = 210 mV), 1,2-naphthoquinone (NQ, 130 mV), phenazine methosulfate (PMS, 80 mV), phenazine ethosulfate (PES, 55 mV), duroquinone (DQ, 5 mV). All redox mediators were at concentration of 45 μM and cytochrome at 5 μM. 50 mM Potassium ferricyanide was used as an oxidant and 50 mM sodium dithionate as a reductant. Spectra (range 400–600 nm) were recorded in intervals of 10–30 mV. Midpoint potentials were obtained from cytochromes α-band absorbance plotted against corresponding voltage.

2.3. Crystallization

Initial screening for cytochrome c_{6B} crystallization conditions was performed at the HTX facility, EMBL-Hamburg using the Classic, Classic 2, PACT and AmSO4 Qiagen suites [12]. The sitting-drop vapour-diffusion technique was used at 292 K, by mixing 0.3 μl protein (7.5 mg/mL in 20 mM sodium citrate, pH 3.2) and 0.3 μl reservoir solution. The optimization of crystallization conditions was carried out using the hanging-drop vapor-diffusion method at 292 K, by mixing 1 μl protein solution and 1 μl reservoir solution. Red crystals suitable for X-ray analysis were obtained from

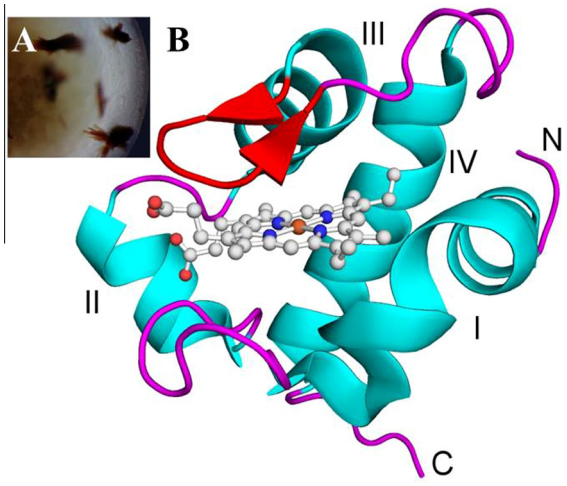

Fig. 1. Crystal structure of cytochrome c_{6B} from *Synechococcus* sp. WH 8102. (A) Crystals of cytochrome c_{6B} from *Synechococcus* WH 8102 obtained in 2.2 M sodium malonate, pH 7.0, 19 °C, over a period of 10 days. Average dimension of 200 μm. (B) Overall structure of cytochrome c_{6B} from *Synechococcus* sp. WH 8102 (PDB:4KMG). I–α-helix I; II–α-helix II; III–α-helix III; IV–α-helix IV. The structure resembles characteristic features of cytochromes of class c_6. Polypeptide chain wraps around the heme moiety (grey). Secondary structure is described by four α-helices (cyan), several loops (magenta) and one β-hairpin (red). The N- and C-terminus are indicated.

Table 1
Data-collection and refinement statistics.

Data collection	
Space group	$P2_1$
Unit cell parameters (Å, °)	a = 106.11, b = 28.98, c = 24.68 β = 93.30
X-ray source	BL 14.2 BESSY, Berlin
Wavelength (Å)	0.91841
Temperature (K)	100
Mosaicity (°)	0.28
Resolution range (Å)[a]	30.00–1.40 (1.49–1.40)
R_{int}[a,b] (%)	5.9 (55.9)
$<I/\sigma(I)>$[a]	13.8 (2.0)
Reflections	
Measured	52939
Unique	14664
Redundancy[a]	3.6 (3.1)
Completeness (%)[a]	97.9 (90.1)
Wilson B-factor (Å^2)	20.1
Refinement	
Resolution range (Å)	27.97–1.40
No. of reflections	
Working set	13712
Test set	950
R_{work}/R_{free}[c] (%)	12.0/18.1
No. of atoms	
Protein	687
Solvent	95
$<B>$(Å^2)	
Protein	15.1
Solvent	22.3
R.m.s. deviations from ideal	
Bond lengths (Å)	0.016
Bond angles (°)	1.769
Ramachandran statistics (%)	
Favored	96.5
Additional	3.5

[a] Values in parentheses are for the highest resolution shell.
[b] $R_{int} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - <I(hkl)>| / \Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of the intensity of reflection hkl and $<I(hkl)>$ is the mean intensity of reflection hkl.
[c] $R = \Sigma||F_o|-|F_c|| / \Sigma|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively. R_{free} is calculated analogously for the test reflections, which were randomly selected and excluded from the refinement.

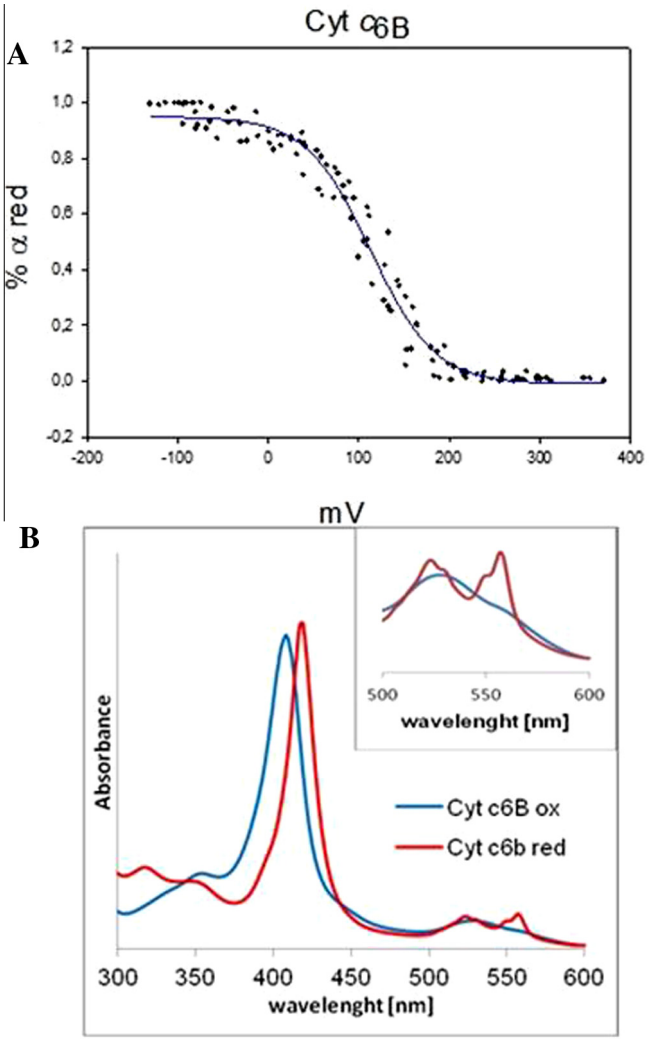

Fig. 2. Biophysical properties of cytochrome c_{6B} from *Synechococcus* sp. WH 8102. (A) Redox titration of cytochrome c_{6B} from *Synechococcus* sp. WH 8102. Absorbance of fully reduced cytochrome was taken as 100%. Absorbance recorded during step-by-step oxidation was taken as corresponding percent. Midpoint potential was obtained from cytochrome α-band absorbance plotted against corresponding voltage and fitted to one-component Nernst equation with aid of SigmaPlot. Cytochrome c_{6B} midpoint potential is $E_{m,7}$ = 113.2 ± 2.2 mV. (B) UV–Vis spectra of cytochrome c_{6B} from *Synechococcus* sp. WH 8102. UV–Vis spectra of reduced cyt c_{6B} is characteristic for c_6-like cytochromes. α-band is red-shifted in comparison to c_6 cyts (maximum absorbance at 557.0 nm). Maximum absorbances of β-, γ-, δ-bands are at 523.5, 418.3 and 316.9 nm, respectively. Cyt c_{6B} ox–spectra of oxidized cyt c_{6B}; Cyt c_{6B} red–spectra of reduced cyt c_{6B}.

2.2 M sodium malonate, pH 7.0 over a period of 10 days (see Fig. 1A).

2.4. Data collection and processing

The mother liquor was amenable to cryo-cooling. A single crystal was scooped in a nylon loop and flash-frozen in a nitrogen gas stream at 100 K. Diffraction data were measured on a Rayonics MX-225 CCD detector on beamline BL 14.2 at the BESSY II synchrotron facility, Berlin. Integration, scaling and merging of the intensity data was accomplished using the XDS package [13]. An overall B-factor of 20.1 Å^2 was estimated from the Wilson plot using the CORRECT program from the XDS package [13]. The data were checked for diffraction anisotropy [14]. A very low spread in values of the three principal components (4.42 Å^2) indicated almost no anisotropy. Space group, unit-cell and data-collection parameters are given in Table 1.

2.5. X-ray structure solution and refinement

The structure was solved by molecular replacement using the MOLREP program from the CCP4 suite [15,16] and the structure of c_6-like cytochrome from *Synechococcus* sp. PCC 7002 (PDB code 4EIE) as the search model. The amino-acid sequence of the model shows 47.7% identity and 75% similarity (LALIGN; [17]) with cyt c_{6B}. Maximum-likelihood structure-factor refinement was carried out in REFMAC [18] using all intensity data, with the exception of 950 reflections (6.5%) flagged for cross-validation purposes. No σ cutoff was applied. To account for diffuse solvent effects, a correction according to the Babinet principle was applied. Manual rebuilding of the model was performed in COOT [19]. The main steps of the refinement included (1) isotropic refinement with manually added water molecules, (2) anisotropic refinement, (3) addition of H atoms according to geometrical criteria as implemented in REFMAC. Full refinement statistics are given in Table 1.

3. Results and discussion

3.1. Structure description

Overall, the structure of *Synechococcus* sp. WH 8102 cyt c_{6B} reveals the characteristic properties of other class I cytochromes *c*. In the electron density map, the first amino acid residue (Gly)

is missing. Cytochrome c_{6B} consists of a single polypeptide chain of 88 amino acid residues (Fig. 1B). The heme ring is covalently bound to the polypeptide chain as in other cyts of c_6 family–by two thioether bonds formed by sidechains of Cys14 and Cys17. The central iron cation of the heme moiety is axially coordinated by the imidazole ring of His18 and the side chain of Met58. Secondary structures found in the cyt c_{6B} molecule (four α-helices, one 3_{10}-helix, several β-turns and β-hairpin motif) are separated by more or less organized loops. α-Helix I is formed by amino acid residues Glu5-His18. This long N-terminal structural element, like in the other cyts c_6, possesses a characteristic kink at position Cys14. A part of this helix is the heme-binding motif –CXXCH-. Subsequent 3_{10}-helix (Val19-Gly21) is localized right next to it. The second α-helix is formed by residues Leu33-Arg38. The succeeding α-helix III consists of residues Thr44-Lys53. The last, fourth α-helix is formed by residues Gly69-Gln82. The relatively long regions between helices are well described by the different β- and γ- type turns. The N-terminus of this cytochrome is arranged in a β-turn of type II (residues Thr2-Glu5). Further β-turns are present between helix I and II–within a well-defined in the cyt c_6 family, conservative Ω-loop. In this region β-turns of type IV (Asn23-Arg26), type VIII (Ile24-Arg27), type I (Arg26-Lys29) as well as type IV (Arg27-Asn30) are localized. Inside the loop separating the second and third α-helices, reversed γ-turn is located, formed by residues Leu41-Ser43. In the context of comparison of cyts c_6 structures, the most interesting motifs are located in the long loop separating helices III and IV (region between residues Gly54 and Gly68). In this region, structure of a β-hairpin of class 2:2 IIP is present. The first arm of this β-hairpin is formed by residues Gly54-Ile55, subsequent bend is formed by Gly56-Gln57 and the second arm by Met58-Ser59. The side chain of residue Met58, part of this β-hairpin, is also one of the heme iron axial ligands. Furthermore, within the discussed loop, residues Gly62-Leu65 are arranged in the pattern of an α-helix. The last β-turn in this region is a β-turn type II formed by residues Gly66-Gly69. The C-terminal fragment of the molecule is arranged in a β-turn formed by residues Asn83-Thr86.

3.2. Heme binding pocket environment

In contrast to typical cyts *c*, the environment of the heme binding pocket in cyt c_{6B} is hydrophobic, as in other cytochromes referred to c_6-like, e.g. c_{6A} from *Arabidopsis thaliana* [20] or c_{6C} from *Synechococcus* sp. PCC 7002 [Krzywda & Zatwarnicki *et al.* – in preparation]. This feature results from the presence of a hydrophobic amino acid side chain (i.e. leucine, isoleucine, valine) in position occupied by a conservative glutamine in typical cyts c_6 [5]. Furthermore, presence of such polar residue inside heme binding pocket seems to have crucial influence on midpoint redox potential as well as for spectral properties, which is in agreement with previous literature reports [21]. The present cyt c_{6B} possesses hydrophobic side chain of isoleucine-50 in the heme binding pocket, which probably is the major factor affecting its midpoint potential ($E_{m,7}$ = 113.2 ± 2.2 mV; Fig. 2A) which is very low in comparison to other cyts c_6. Additionally, maximum absorption of α-band occurs at 557.0 nm, which is approx. 4 nm red-shifted in comparison to classical c_6 cytochromes (Fig. 2B). As previously mentioned the heme ring of cyt c_{6B} is covalently bound via two thioether bonds with side chains of Cys14 and Cys17. Heme iron is coordinated by a nitrogen atom $N^{\varepsilon 2}$ of His18 and sulfur atom S^{δ} of Met58. The bond angle is 175.4°. The distances between the atoms are as follows: $N^{\varepsilon 2}$ His18–Fe^{3+} = 2.0 Å, S^{δ} Met58–Fe^{3+} = 2.3 Å. His18 imidazole ring is stabilized by a hydrogen bond with the carbonyl oxygen atom of Gly22. The network of hydrogen bonds in cyt c_{6B} heme binding pocket (Fig. 3A) is completely different when compared to other structures of cyanobacterial cyts c_6 (Fig. 3B) [22]. In close proximity to the heme ring, only one water molecule (wat-218) is present. It occupies two positions (A and B) with a 0.5:0.5 ratio. Additionally, the side chain of glutamine Gln39 may also adopt two different conformations-interaction in between this side chain and heme ring is possible only with the adoption of a conformation designated as "A". As a result of such conformational change the hydrogen bonds are formed between propionate hp-7 and N^{ε} atoms and O^{ε} of Gln39 side chain. Moreover, wat-218 in position "A218" forms hydrogen bond with O^{ε} atom of Gln39. Furthermore, like in other cyanobacterial cyts c_6 and c_6-like [Krzywda & Zatwarnicki *et al.* – in preparation] heme propionate hp-7 is stabilized by a hydrogen bond with the carbonyl oxygen of Asn30. The heme propionate hp-6 forms hydrogen bond network "insulated" from the two bonds between the hp-7 propionate oxygen atoms and atom $N^{\eta 1}$ of Arg26 side chain and in addition, hydrogen bond between oxygen atom of hp-6 and carbonyl nitrogen of Gln57. The water molecule wat-218, when manning the "B218" position, changes slightly hydrogen bonds network in the heme binding pocket. Compared to network discussed above, bond

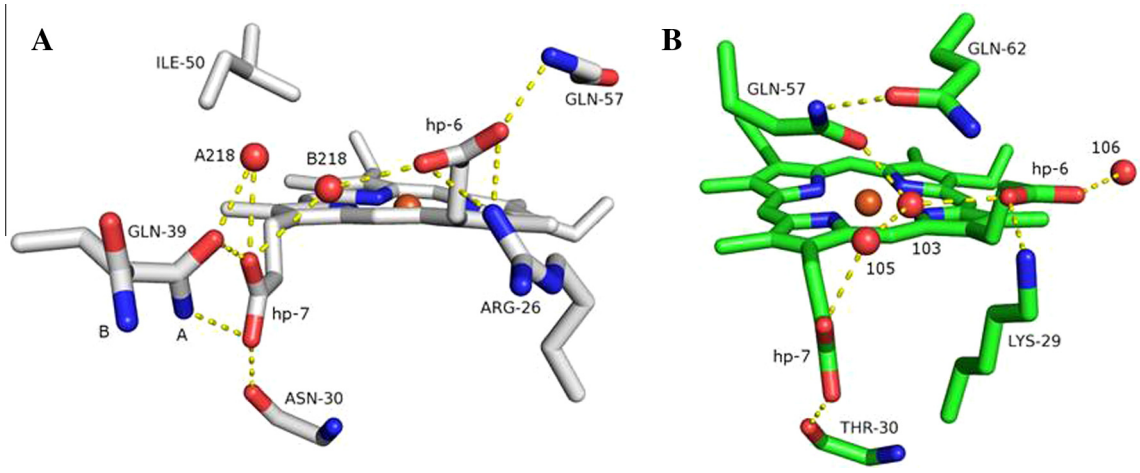

Fig. 3. Comparison of heme-binding pockets of cytochrome c_{6B} from *Synechococcus* sp. WH 8102 and cytochrome c_6 from *Synechococcus* sp. PCC 7002. (A) Heme binding pocket of cytochrome c_{6B}. In the closest proximity of the heme moiety, only one water molecule is present: wat-218. This molecule partly occupies positions A218 and B218 (ratio 0.5:0.5). Hydrogen bonds formed by side chains of Arg_{26}, Gln_{37} (in position A), Gln_{57} and carbonyl oxygen of Asn_{30} stabilize heme moiety. hp-6,7–heme propionate 6 and 7, respectively. (B) Heme binding pocket of cytochrome c_6 of *Synechococcus* sp. PCC 7002; PDB:3DR0 [22]. In the closest proximity to heme ring, 3 water molecules are present (marked as 103, 105 and 106). These water molecules create hydrogen bond network, together with side chains of glutamine-57, glutamine-62, lysine-29 and carbonyl oxygen of threonine-30.

between wat-218 and Gln39 are no more present. On the other hand, a bond between wat-218 and heme propionate hp-6 appears, in result creating interaction chain hp-6–wat-218–hp-7.

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