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The cyanobacterial protoporphyrinogen oxidase HemJ is a new *b*-type heme protein functionally coupled with coproporphyrinogen III oxidase

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Running title: *Characterization of Synechocystis protoporphyrinogen oxidase*

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ABSTRACT

Protoporphyrinogen IX oxidase (PPO), the last enzyme that is common to both chlorophyll and heme biosynthesis, catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX. There are several isoforms of PPO including the well-characterized oxygen-dependent HemY and an oxygen-independent enzyme HemG. However, cyanobacteria encode HemJ, the least characterized form of PPO. Previously, a gene essential for PPO activity in the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*) was identified and annotated as *hemJ*. Herein, we report the expression of FLAG-tagged *Synechocystis* HemJ protein (HemJ.f) and affinity isolation of HemJ.f under native conditions, which revealed that the protein binds heme *b*. The most stable form of HemJ.f was a dimer with higher oligomeric forms also present. The estimated heme *b* content was 0.85 per monomer. No enzymatic activity was detected in the purified HemJ.f using both oxygen and artificial electron acceptors. This was consistent with the hypothesis that the enzymatic mechanism for HemJ is distinct from other isoforms of PPO. The heme absorption spectra and distant HemJ homology to several membrane oxidases indicates that the heme in HemJ is redox-active and is involved in electron

transfer. HemJ was conditionally complemented by another PPO HemG from *Escherichia coli*. If grown photoautotrophically the complemented strain accumulated tripropionic tetrapyrrole harderoporphyrin, which suggests a defect in enzymatic conversion of coproporphyrinogen III to protoporphyrinogen IX, catalyzed by coproporphyrinogen III oxidase (CPO). This novel observation supports the hypothesis that HemJ is functionally coupled with CPO and that this coupling is disrupted after replacement of HemJ by HemG.

INTRODUCTION

The last common step for heme and chlorophyll biosynthesis (1), conversion of protoporphyrinogen IX (Proto) into protoporphyrin IX (Proto), is catalyzed by the enzyme protoporphyrinogen IX oxidase. Although the six electron oxidation of Proto occurs also spontaneously, its enzymatic conversion is apparently necessary for the correct channeling of Proto to chelatases for metal insertion. Three analogous enzymes HemY, HemG, and lastly HemJ (recently reannotated as PgoX, PgdH1 and PgdH2 (2)), exhibiting no mutual homology, have been found to serve as a protoporphyrinogen

oxidase (3-5). The HemY is an oxygen-dependent enzyme with size of approximately 55 kDa, creating membrane bound dimers and using flavin-adenine dinucleotide as a cofactor (6). HemY occurs in most of bacteria phyla and in almost all eukaryotes and it seems that it might be the ancestral type of PPO (7).

HemG (21 kDa) is mostly found in γ -proteobacteria (7). The protein forms membrane-associated oligomers (8,9) and uses noncovalently bound flavin mononucleotide as a cofactor (9). HemG is functional in aerobic as well as anaerobic conditions (9). Electrons from the Proto oxidation catalyzed by HemG are withdrawn via ubiquinone, cytochrome *bo'* and cytochrome *bd* oxidases to oxygen, or under anaerobic conditions to the terminal electron acceptors fumarate and nitrate by corresponding reductases (8).

Most recently, the *slr1790* gene from the cyanobacterium *Synechocystis* has been described as the third PPO and named *hemJ* (3). HemJ is predicted to have evolved within α -proteobacteria and spread to cyanobacteria and various proteobacteria through multiple horizontal gene transfers (7). As with other PPOs, *hemJ* appears to be essential and could not be inactivated in *Synechocystis* (3). However, a partially segregated *Synechocystis* strain accumulated Proto, originating from nonenzymatic oxidation of Protogen, and contained less than half of the chlorophyll content compared to wild type (WT). Accordingly, the *hemJ* deletion strain from *Acinetobacter baylyi* (*A. baylyi*) exhibited auxotrophy for hemin and displayed accumulated porphyrins when supplemented with 5-aminolevulinic acid (10).

To demonstrate that HemJ is a bona fide PPO, HemJ mutants were complemented with other PPO isoforms: several *hemY* variants and *hemG* rescued hemin auxotrophy in *A. baylyi* (10), and expression of *Arabidopsis thaliana hemY* in *Synechocystis* enabled the construction of a *hemJ* mutant strain that accumulated Proto (3). This accumulation of intermediates is consistent with substrate channeling between the terminal enzymes of heme synthesis. Formation of multienzyme heme biosynthetic complex, containing the three terminal enzymes CPO, PPO and ferrochelatase (FeCH) is predicted (11) and partly documented (12,13). For prokaryotes, identification of such complexes is further

complicated by existence of multiple enzyme isoforms and different heme biosynthetic pathways (2). In addition to PPOs there are two forms of CPOs in *Synechocystis* as in most organisms performing oxygenic photosynthesis: HemN and HemF (14). Under micro-oxic conditions, the conversion of coproporphyrinogen III (Coprogen) to Protogen is catalyzed by an oxygen-independent CPO encoded by *hemN*, while oxidative decarboxylation of the substrate requires oxygen and is catalyzed by HemF, an enzyme structurally and functionally unrelated to HemN (14,15). HemN is a monomeric, iron-sulfur cluster containing protein (16), HemF is dimeric protein with two independent active sites (17). Herein, we report the biochemical characterization of HemJ homologously expressed in *Synechocystis* and the results of complementation of *hemJ* mutant by expression of *hemG* from *Escherichia coli* (*E. coli*). We also show that there is a functional coupling between *Synechocystis* CPO and HemJ.

RESULTS

Synechocystis HemJ forms a heme-binding oligomer

As heterologous expression of HemJ proteins from various organisms frequently resulted in no or poor expression (3,10), we expressed the HemJ enzyme fused on its C-terminus with 3xFLAG-tag (HemJ.f) homologously in *Synechocystis* under *psbAII* promoter. After full segregation of the *hemJ.f* strain, it was possible to delete WT copy of the *hemJ* gene, demonstrating that the HemJ.f protein is functional (Fig. S1). However, for the HemJ isolation a strain containing both WT and tagged variants of HemJ was used to potentially co-isolate HemJ.f together with the native form of the protein (see below): oligomeric states have previously been described for other PPO variants (8). After affinity chromatography, the purified HemJ.f eluate was markedly reddish. Native separation of the eluate on clear native gel (CN-PAGE) resulted in two reddish bands - CN1, and only slightly visible CN2 (Fig. 1). Photosystem I (PSI) was shown to bind nonspecifically to the anti-FLAG affinity resin (18). Hence, HemJ.f was also isolated from strain lacking PSI (Δ PSI) to achieve maximum purity (Fig. 1). Absorption spectra of the eluted HemJ.f

protein showed an absorption maximum at 412 nm (Fig. 2A), which was presumed to be the Soret band of a bound tetrapyrrole. After deletion of PSI only a small amount of chlorophyll (absorbing 671 nm) and carotenoids (absorbance 450 - 520 nm) were co-eluted with HemJ.f (Fig. 2A). The bound tetrapyrrole was extracted by acetone and was identified as protoheme by HPLC (Fig. S2). To further spectroscopically characterize this bound heme, absorption spectra of the HemJ.f eluate from the Δ PSI strain were measured after oxidation by air or after reduction by dithionite (Fig. 2B). The oxidized spectrum indicates a high spin heme *b* ($\lambda_{\text{max}} = 411$ nm), and the reduced spectrum ($\lambda_{\text{max}} = 424$ nm) is typical for a ferrous six-coordinate *b*-type heme. To assess the stoichiometry of heme binding to HemJ.f, protein isolated by affinity chromatography was further purified by gel filtration (Fig. 3A). Fractions with the highest absorption at 415 nm were collected and used for heme and protein quantification. The protein concentration was estimated from the absorbance at 280 nm using calculated extinction coefficient for HemJ.f. The estimated molar ratio of heme to HemJ.f monomer was 0.85. Eluted fractions did not display any sign of chlorophyll or carotenoids (Fig. 3B). Gel filtration was also used to estimate the size of the native HemJ complex (Fig. 3A). The main elution peak corresponds to a protein of ~150 kDa, indicating higher oligomeric state of HemJ.f (27 kDa monomer) even when taking into account its association with detergent. Analysis of the HemJ.f eluate by SDS-electrophoresis (SDS-PAGE) revealed that several proteins specifically co-elute from anti-FLAG resin with HemJ.f (Fig. 4A). These protein bands were identified by mass-spectrometry as Sll1106, FtsH proteases and ATP synthase subunits (Tab. S1). Sll1106, together with HemJ, was also identified by mass spectrometry in the CN1 band from CN-PAGE (Fig. 1). Nevertheless, the reddish CN1 band was present even when HemJ.f was purified from a Δ sll1106 background (Fig. S3), indicating that heme is associated with HemJ.f. Furthermore, when a gel strip from CN-PAGE (Fig. 1) was separated in a second dimension by SDS-PAGE (Fig. 4B), colored CN1 and CN2 bands dissociated into two spots, apparently representing monomer and dimer of the HemJ.f (27 kDa). Presence of the HemJ.f dimer even on the denaturing SDS gel suggests its remarkable

stability. The upper CN2 band contains the HemJ.f oligomer, most probably tetramer, which is in agreement with the size of the complex determined by the gel filtration (Fig. 3A).

In vitro PPO activity of the purified HemJ.f

As previous studies provided conflicting results regarding the ability of the isolated HemJ to perform Protoporphyrinogen oxidation/dehydrogenation, we have measured PPO activity of the eluted HemJ.f. However, no measurable PPO activity in the eluate was detected with or without artificial electron acceptors menadione or benzoquinone. Marginal activity comparable with the spontaneous oxidation of Protoporphyrinogen was detected in *Synechocystis* crude extract and in the membrane fraction, although this activity was probably an artefact as it was present also in these fractions after heat treatment (90 °C, 3 min.). The recombinant human HemY (19) was used as a positive control for the PPO activity measurement.

The HemJ is not fully replaceable by HemG

As we were not able to detect HemJ activity, we decided to confirm the function of HemJ via complementation with the homolog HemG. Two strains were engineered, one with the *Synechocystis hemJ* gene placed under a copper-regulated promoter (*petJ*) and the second expressing *hemG* from *E. coli* under *psbAII* promoter. In both strains it was possible to delete the original *hemJ* gene, indicating that both constructs were functional. In the *petJ:hemJ* strain, it was possible to decrease amount of *hemJ* by adding copper to the growth medium. This led to lower amounts of enzymatically-produced Proto, followed by decrease in the levels of phycobilins and chlorophyll and lower levels of major chlorophyll-binding photosynthetic complex, especially trimeric PSI (Fig. S4A, B). To identify how the repression of HemJ expression affects tetrapyrrole biosynthesis, the accumulation of chlorophyll/heme intermediates was analyzed in the *petJ:hemJ* strain suppressed by copper (Fig. 5). The suppressed strain exhibited significantly decreased levels of the chlorophyll precursors Mg-protoporphyrin IX (MgP) and monovinyl protochlorophyllide (Pchl_{id}) along with accumulation of coproporphyrin III (Copro) and

Proto; the last tetrapyrrole was also visibly excreted into the medium. Detection of both Copro and Proto was likely to result from the accumulation and non-enzymatic oxidation of their reduced precursors in the cell (3,20).

The HemG complementation strain $\Delta hemJ/hemG$ grew on glucose slightly slower than WT (Fig. S5) and accumulated lower amounts of chlorophyll and phycobilins (Fig. S4C). However, the strain did not grow autotrophically (Fig. S5). When the $\Delta hemJ/hemG$ cells were transferred to glucose free medium, the cellular level of chlorophyll-binding photosynthetic complexes gradually decreased (Fig. S4D). Also chlorophyll precursors were almost undetectable in the cells, except for monovinyl chlorophyllide (MV Chlide) (Fig. 6), most probably originating from chlorophyll recycling by dephytylation of matured chlorophyll molecules from pigment-protein complexes (21). In addition, a large quantity of an unidentified tetrapyrrole with absorption spectrum resembling Copro eluted at 13.4 min. on the HPLC profile of the extract from the $\Delta hemJ/hemG$ strain incubated without glucose (Fig. 6). This compound was completely missing in the WT strain (Fig. 6A) as well as in the $\Delta hemJ/hemG$ mutant grown on glucose (Fig. S6).

Harderoporphyrin accumulation in the $\Delta hemJ/hemG$ strain

This unknown tetrapyrrole observed in $\Delta hemJ/hemG$ mutant was isolated and analyzed using HPLC coupled to high resolution tandem mass spectrometry (HPLC-HRMS/MS). The compound exhibited a m/z value of 609.2708 corresponding to the elemental composition $C_{35}H_{37}N_4O_6$ (Tab. S2). Based on the similarity of this elemental composition to Copro ($C_{36}H_{39}N_4O_8$), the fragmentation spectra of these two molecules were compared. Fragmentation of the Copro molecular ion led to successive benzylic cleavages with the loss of a $\cdot CH_2COOH$ radical ($\Delta 59$ Da) from all four propionic acid residues (m/z 655 \rightarrow 596 \rightarrow 537 \rightarrow 478 \rightarrow 419) as described previously (22) (Fig. 7a, Tab. S2). The queried compound exhibited only three consecutive losses of $\cdot CH_2COOH$ radicals suggesting presence of only three propionic groups. From the difference of the MW of both compounds, it can be assumed that the fourth propionic acid group of the tetrapyrrole ring is substituted by a vinyl group.

Such substitution of the tetrapyrrole ring is consistent with that described for harderoporphyrin (Hardero) (23), although the exact position of the vinyl group on the tetrapyrrole ring cannot be determined via MS/MS experiments. Hence, the compound accumulating in the $\Delta hemJ/hemG$ strain with MW 609 is likely to be Hardero, a spontaneously oxidized intermediate of the CPO reaction, which is normally generating Protogen.

DISCUSSION

HemJ is the most common form of PPO in prokaryotes and is found in most proteobacteria and cyanobacteria (2). However, from the three completely different types of enzyme possessing PPO activity, it was the last one to be described and there is very little structural and functional information on this protein. We have isolated tagged HemJ protein from solubilized *Synechocystis* membranes and found that it forms an oligomeric, most likely tetrameric complex (Figs. 3A, 4B). Due to hydrophobic nature of this enzyme, it is unclear if the tetramer is the native form or results from non-physiological aggregation. However, the HemJ dimer is very stable even after SDS electrophoresis (Fig. 4B). Also the remote HemJ homologs with known 3D structure (see below) have previously been shown to form dimers (24,25).

The natively isolated HemJ complex was reddish in color (Fig. 1) due to the presence of heme *b* (Fig. 2). Interestingly, Gomelsky and Kaplan (26) previously overexpressed a *Rhodobacter sphaeroides* (*R. sphaeroides*) *hemJ* gene (*orf1*) located upstream of their gene of interest and noted that the *E. coli* strain overproducing this protein turned pink. Our assessment of heme stoichiometry indicated a ratio of 0.85 heme *b* per subunit. It seems plausible that HemJ binds a single heme *b* with partial heme loss occurring during isolation.

To predict localization of the observed heme within HemJ, we have modeled the 3D structure of HemJ protein (Fig. 8). The modeling was performed on HemJ peptide from *R. sphaeroides* (WP_023003745) containing only 4 helices as is found for most of the proteins of the HemJ family. The *Synechocystis* additional domain, which is present only in cyanobacteria and forms the 5th

helix, did not provide enough coverage for co-evolutionary modeling and also the overall homology in this domain was much lower. The HemJ protein forms a very common structural motif in membrane proteins, a four-alpha-helix bundle (See Supplementary results for details). Advanced alignment and secondary structure prediction methods (27) revealed remote homology of HemJ to other heme-binding membrane redox proteins. In several of our models, the invariant His16 of HemJ (His12 in Fig. S7) was aligned with heme-binding His residues of the remote templates. As His is the most common residue providing at least one of usual two heme ligands (28) and as His16 is the only invariant His in the PF03653 Pfam family, we believe that His16 of HemJ also ligates heme *b*. This would locate the observed heme *b* buried in the middle of membrane, perpendicularly to the membrane surface (Fig. 8). Absorbance spectra of reduced HemJ (Fig. 2B) indicate the presence of a ferrous six-coordinate heme *b* with the second ligand probably provided by another amino acid sidechain. Interestingly, from five amino acids (His, Met, Cys, Tyr and Lys) that can act as axial heme ligands of hemoproteins (28), there is an invariable Lys94 (Lys91 in Fig. S7) located on the distal side of the proposed heme location. This Lys could provide the sixth ligand of HemJ. Moreover, conserved Trp90 (Trp87 in Fig. S7), rarely substituted by Phe in other members of the Pfam family, may stabilize the heme pocket, as aromatic residues (phenylalanine, tyrosine, and tryptophan) play important roles in protein-heme interactions through stacking interactions with the porphyrin (28). The distant homology to several membrane oxidases together with heme absorption spectra indicates that the heme *b* in HemJ is redox active and is involved in electron transfer.

This is in agreement with the expected oxidase/dehydrogenase activity of HemJ which is still poorly understood. Before the enzyme identity was known, PPO activity belonging to HemJ was characterized in the membrane fraction of *R. sphaeroides* (29). This activity was inhibited by respiratory inhibitors and also by extraction of quinones from membranes with pentane and no enzyme activity was detected after membrane solubilization (29). This indicated that PPO activity in *R. sphaeroides* is closely linked with components of the respiratory electron transport

chain. In *A. baylyi*, both membrane and soluble fractions were necessary to detect HemJ activity suggesting that a soluble factor may be required. In another study, authors documented oxygen-dependent PPO activity of purified HemJ from *R. sphaeroides* (3). However, detected specific PPO activity was much lower than that for combined fractions in *A. baylyi* mentioned above (10) and the authors also did not note the presence of a colored cofactor in the purified enzyme. We were not able to detect any oxygen-dependent PPO activity of the HemJ protein isolated from solubilized membranes. No activity was detected even in the presence of menadione, the electron acceptor for HemG (9). This is in line with the report that HemJ does not complement an *E. coli* $\Delta hemG$ mutant, suggesting that the connection of HemJ to the respiratory chain is distinct from that of HemG (8).

Even though we were not able to measure PPO activity in our eluate, data presented herein (Figs. 5, S4A,B) further document that HemJ is indeed a bona fide PPO as indicated previously (3,10). As would be expected for PPO, HemJ downregulation resulted in accumulation of tetrapyrrole precursors Copro and Proto and at the same time in depletion of chlorophyll precursors (Fig. 5). The HemJ protein is indispensable, and complete inactivation of the encoding gene was possible only after expression of another copy of HemJ or HemG. While we were able to complement missing HemJ with HemG as shown before (10), this replacement was not without consequences. The resulting $\Delta hemJ/hemG$ strain did not grow autotrophically (Fig. S5) and the tetrapyrrole biosynthetic pathway was disturbed (Fig. 6A).

Reactive protoporphyrin intermediates generally do not accumulate in strains with undisturbed tetrapyrrole biosynthetic pathway. Furthermore, mutation of genes encoding tetrapyrrole biosynthetic enzymes or treatment of cells with enzyme inhibitors frequently results in accumulation of abnormally high levels of such intermediates (4). PPO-deficient mutants commonly accumulate earlier heme intermediates - uroporphyrin, Copro and also Proto (30). Accumulation of oxidized forms is caused by non-specific oxidation of Coprogen and Protogen in the cell or during isolation (3,20). Repression of HemJ expression in the *petJ:hemJ* strain also led

to Copro and Proto accumulation (Fig. 5). The complemented $\Delta hemJ/hemG$ strain expressing HemG accumulated less Copro and Proto when grown on glucose compared to the WT (not shown), indicating that the pathway is functional and provides enough Proto for normal chlorophyll accumulation and almost normal growth (Figs. S4C, D; S5). After transfer to medium without glucose the cells of the $\Delta hemJ/hemG$ strain stopped growing. Chlorophyll levels also quickly decreased, suggesting that not enough Proto accessible to magnesium chelatase is made (Fig. S4C). Interestingly, at the same moment cells started to accumulate an unusual porphyrin identified as Hardero (Figs. 6, 7), a probable auto-oxidation product of harderoporphyrinogen (Harderogen). Harderogen is a tricarboxylic intermediate in a two-step decarboxylation of the heme precursor Coprogen (31), catalyzed by CPO. This indicates problems with the $\Delta hemJ/hemG$ mutant at this stage of tetrapyrrole biosynthesis. Even though there are two CPO enzymes (HemN and HemF) in *Synechocystis*, HemF is expected to serve as the sole CPO under prevailing aerobic conditions (15). We speculate that HemF is functionally coupled with HemJ and that this coupling is disturbed in the $\Delta hemJ/hemG$ strain. Surprisingly, the improper coupling was not demonstrated by accumulation of Protogen (Proto) but by accumulation of Harderogen. This indicates a reduced ability of *Synechocystis* HemF to convert Harderogen to Protogen after replacement of downstream PPO of HemJ-type by that of the HemG-type. The only other examples of Hardero accumulation are for specific mutants of human CPO, which is an oxygen-dependent HemF (32). The reduced ability to convert Harderogen to Protogen in the abnormal CPO was explained by reduced affinity for Harderogen, which may leave the enzyme more easily (33). This would suggest that replacement of HemJ by HemG also destabilizes the active site of HemF, implying that a physical contact between HemJ and HemF leads to conformational changes of the HemF active site. As both Coprogen and Harderogen are substrates for CPO, the release of Harderogen could also occur when an excess of Coprogen is accumulated in the cell (34). However, Coprogen also accumulated in the *petJ::hemJ* strain (Fig. 5) without concomitant accumulation of Hardero. We do not know exactly why we see Hardero

accumulation particularly under autotrophic growth. It is possible, that glucose, similarly to oxygen regulates the accumulation of both CPO forms HemF and HemN. The red microalga *Galdieria partita*, also containing both forms of CPO, excretes Coprogen in the medium when growing mixotrophically on glucose (35). This was explained by inactivation of the oxygen-dependent CPO in microaerobic conditions when grown on glucose (36). In *Synechocystis*, the two-component regulatory histidine kinase Hik31 is involved in the response to glucose and in switching between photoautotrophic and photoheterotrophic growth (37). At the same time, Hik31 has an additional role in the transition between aerobic and low-oxygen growth (38).

Substrate channeling and the presence of stable or transient multienzyme complexes is expected to be an advantageous mechanism for pathways with reactive intermediates to protect the cell from oxidative damage. In tetrapyrrole biosynthesis, most information supporting the presence of multienzyme complexes for at least some enzymes is coming from immunoprecipitation followed by immunoblotting and/or mass spectrometry (12,13). In erythroids, the presence of a mitochondrial heme metabolism complex minimally consisting of FeCH, PPO and aminolevulinic acid synthase was documented (12). In bacteria, co-immunoprecipitation and immunogold labeling supported a complex involving HemY type of PPO and FeCH in the thermophilic cyanobacterium *T. elongatus* (13).

In pull-down experiments we aimed to co-purify HemJ with its partners in tetrapyrrole biosynthesis. The most prominent protein co-purified with HemJ was Sll1106 (Fig. 4A), a protein with unknown function containing extended glycine zipper motif, which can be found in a number of channel forming proteins (39). However, it is unlikely that Sll1106 is required for PPO function as in the set of 40 cyanobacterial genomes, HemJ was coded in 32 genomes whereas Sll1106 homologs were present in only 20 (data not shown). A Sll1106 homolog was found also in some cyanobacteria coding for HemY-type of PPO. Thus, we do not expect Sll1106 to be directly involved in HemJ activity and its function remains to be elucidated. Interestingly, all four FtsH protease homologs encoded in *Synechocystis* were present in

significant quantities in the HemJ.f eluate (Fig. 4A). FtsHs are universally conserved trans-membrane metallo-proteases responsible for quality control of membrane- and membrane-associated proteins. They form hetero-oligomeric complexes with distinct functions (40-42). For example, the FtsH2/3 hetero-oligomeric complex responsible for PSII repair and biogenesis was the most abundant in the eluate. This may reflect a functional connection to PSII or just reflect the membrane region in the cell where the PPO reaction is performed. This is also consistent with a hypothetical model for a chlorophyll biosynthesis multienzyme complex being in close proximity to PSII assembly (43).

EXPERIMENTAL PROCEDURES

Construction of *Synechocystis* strains

The strains used in this study were derived from non-motile, glucose-tolerant *Synechocystis* strain obtained from the laboratory of Peter J. Nixon (Imperial College, London, UK). To prepare a strain expressing HemJ (WP_020861394.1) with a 3xFLAG tag at the C-terminus (HemJ.f), the *hemJ* gene (*slr1790*) was cloned into the pPD-CFLAG plasmid containing the *Synechocystis psbAII* promoter, a sequence encoding the tag, kanamycin resistance cassette and flanking sequences for homologous recombination that allow insertion of *hemJ* tagged on its C-terminus into the *Synechocystis* genome in place of the *psbAII* gene (44). As pPD-CFLAG plasmid containing *hemJ.f* (pPD-*hemJ.f*) isolated from *E. coli* frequently contained frameshift mutations within *hemJ*, the fragment for transformation was obtained by PCR using pPD-*hemJ.f* ligation mixture as a template. The resultant PCR fragment was used for transformation of WT and Δ PSI strains (45). The vector used for disruption of *hemJ* by a chloramphenicol resistance cassette was constructed using the megaprimer PCR method (46). Firstly, upstream and downstream regions of *hemJ* were amplified from WT genomic DNA with flanking sequences of chloramphenicol resistance cassette from pACYC184 vector. Then the chloramphenicol resistance cassette and regions upstream and downstream of *hemJ* were mixed in one PCR reaction with the primer

annealing temperature at 50 °C. 5 μ l of PCR reaction mixture was used for transformation into the *hemJ.f* *Synechocystis* strain. To construct the *petJ:hemJ* strain expressing HemJ under the control of copper-repressed promoter, p*PsbAIIpetJ*-FLAG plasmid (47) was used with the *hemJ* gene inserted into specific restriction sites, leaving out the 3xFLAG tag. The resulting plasmid was used for WT transformation, and the WT copy of *hemJ* was deleted afterwards. To prepare *Synechocystis* strain expressing HemG from *E. coli* under the *Synechocystis psbAII* promoter, the *hemG* gene (48) was again cloned into the pPD-CFLAG plasmid, leaving out the 3xFLAG tag. The WT strain was transformed with the resultant plasmid, and the WT copy was subsequently deleted. In all cases, *Synechocystis* transformants were selected on BG11 agar plates with increasing levels of corresponding antibiotic. Full segregation was confirmed by PCR.

Growth conditions

Synechocystis strains were grown photoautotrophically in BG11 medium in shaken conical flasks at 29 °C and irradiance of 40 μ mol photons m⁻² s⁻¹. Δ *hemJ/hemG* strain was grown in the medium supplemented with 5 mM glucose. *PetJ:hemJ* strain was grown in the medium without copper and addition of 1 μ M CuSO₄ was used for repression of *hemJ* expression. For the purification of HemJ.f protein, 4 liters of cells were grown mixotrophically supplemented with 5 mM glucose to an OD₇₃₀ of 0.5 to 0.7.

Analysis of cellular tetrapyrroles

Whole cells absorption spectra were measured with Shimadzu UV-3000 spectrophotometer. Heme/chlorophyll precursors were measured from 2 ml of culture OD₇₃₀ ~ 0.4 using HPLC according to procedure described in Pilný et al. (49). For the detection of porphyrins accumulated in the Δ *hemJ/hemG* strain, 50 ml of cells were grown without glucose for three days and harvested at OD₇₃₀ ~ 0.4. Pigments were extracted by an excess of 70 % methanol, separated by a HPLC method described in Pilný et al. (49) and their absorbance was detected by a diode array detector.

Preparation of thylakoid membranes and protein complexes purification

Harvested cells were washed and resuspended in thylakoid buffer containing 25 mM MES/NaOH, pH 6.5, 25 % glycerol, 10 mM MgCl₂, 10 mM CaCl₂ and then broken in a Mini-Beadbeater-16 (Biospec, USA). For protein purification, EDTA-free Protease Inhibitor (Roche) was added into the buffer prior to breaking the cells. Membranes were pelleted by centrifugation (55,000 x g, 20 min., 4 °C), resuspended in excess of the thylakoid buffer and the centrifugation step was repeated.

For the isolation of HemJ.f protein, the membrane fraction was resuspended in the thylakoid buffer and solubilized for 1 hour at 10 °C with 1.5 % n-Dodecyl-β-D-maltoside, (DDM - Enzo Life Sciences). After centrifugation (55,000 x g, 20 min., 4 °C), the solubilized proteins were purified using anti-FLAG-M2 agarose resin (Sigma-Aldrich). The resin was intensively washed with 20 resin volumes of the thylakoid buffer containing 0.04 % DDM and the HemJ.f was finally eluted with two resin volumes of the same buffer containing in addition 300 µg/ml of 3xFLAG peptide (Sigma-Aldrich).

Electrophoresis and gel filtration

The protein composition of purified complexes was analyzed by SDS-PAGE in a denaturing 12-20 % polyacrylamide gel containing 7 M urea (50). For native electrophoresis, solubilized membrane proteins or isolated complexes were separated on 4 to 14 % CN-PAGE (51). To resolve individual components of protein complexes, the gel strip from the CN-PAGE was first incubated in 2 % SDS and 1 % dithiothreitol for 30 min. at room temperature, and then proteins were separated along the second dimension by SDS-PAGE in a denaturing 12 to 20 % polyacrylamide gel containing 7 M urea (50). Proteins separated by SDS-PAGE were stained with Coomassie Brilliant Blue (CBB) or SYPRO Orange afterwards. Mass spectrometry analysis of CBB stained protein bands/spots from the SDS-PAGE gels was accomplished essentially as described by Bučinská et al. (18).

For gel filtration analysis, the HemJ.f eluate prepared from ΔPSI genetic background was injected onto an Agilent-1200 HPLC and separated on a Yarra 3000 column (Phenomenex) using 25 mM HEPES buffer, pH 7.5, containing 0.25 % DDM at a flow rate of 0.2 ml min.⁻¹ at 10 °C. Fractions corresponding to HemJ.f were pooled and concentrated on a 100 kDa cut-off microconcentrator (Millipore).

Identification and quantification of the HemJ.f-bound heme

For the analysis of heme, the HemJ.f protein was isolated by the affinity chromatography and subsequently further purified by gel filtration (see above). Heme was extracted from the isolated and concentrated protein by 90 % of acetone/2 % HCl, passed through a 0.22 µm filter and separated by HPLC on Nova-Pak C18 column (Waters) using a 25 - 100 % linear gradient with H₂O (A)/acetonitrile (B) both containing 0.1 % trifluoroacetic acid as a mobile phase at a flow rate of 1 ml min.⁻¹ at 40 °C. Protoheme was detected using a diode array detector (Agilent-1200). The protein concentration was determined from sample absorbance at 280 nm using the ProtParam tool (<http://web.expasy.org/protparam>) and a calculated extinction coefficient for the HemJ.f protein of 41000 M⁻¹cm⁻¹. Full reduction of heme in HemJ.f eluate from the ΔPSI strain was achieved by adding a few grains of sodium dithionite. UV-VIS spectra were measured at room temperature with a Shimadzu UV-3000 spectrophotometer.

PPO assay

PPO activity was monitored using a continuous fluorometric assay as previously described (52). Production of the fluorescent Proto, from non-fluorescent Protogen was detected using a fluorescence plate reader at 25 °C. Reaction mixtures consisted of 50 mM MOPS (pH 8.0), 2 mM glutathione, 20 µM Protogen, 200 nM HemJ.f and 1 mM menadione or benzoquinone. Protogen oxidation by Human HemY was used as a positive control.

Isolation of Hardero and HPLC-HRMS/MS analysis

The unknown tetrapyrrole (peak 13.4 min., see Fig. 6A) was prepurified from 600 ml of $\Delta hemJ/hemG$ cells grown without glucose for three days. Harvested cells were extracted by 50 ml of 70 % methanol, the solvent was evaporated on a rotary evaporator and the dried pigments dissolved in 2 ml of methanol. This solution was separated on Agilent-1200 using the same solvents as described for the analysis of cellular tetrapyrroles but employing a semipreparative C8 column (Luna 5 μ m, 250 x 10 mm, Phenomenex).. The peak corresponding to unknown tetrapyrrole was collected and dried in a vacuum concentrator. For HPLC-HRMS/MS analysis sample was analyzed on Thermo Scientific Dionex UltiMate 3000 UHPLC+ (Sunnyvale, CA, USA) equipped with a diode-array detector. Separation of compounds was performed on reversed phase Phenomenex Kinetex C18 column (150 x 4.6 mm, 2.6 μ m, Torrance, CA, USA) using H₂O (A)/acetonitrile (B) both containing 0.1 % HCOOH as a mobile phase with the flow rate of 0.5 ml min⁻¹. For the separation, the following gradient was used: A/B 85/15 (0 min.), 85/15 (in 1 min.), 0/100 (in 25 min.), 0/100 (in 30 min.) and 85/15 (in 35 min.). Analysis of mass spectra was performed on a Bruker Impact HD high resolution mass spectrometer (Billerica, Massachusetts, USA) with electrospray ionization. The following settings were used: dry temperature 200 °C; drying gas flow 12 l min⁻¹; nebulizer 3 bar; capillary voltage 3800 V; endplate offset 500. Spectra were collected in the range 20 - 2000 *m/z* with precursor ion selection set to 550 - 700 and automatic exclusion after five spectra. The analysis was

calibrated using sodium formate at the beginning of the analysis. Collision energy for fragmentation was set to 60 eV.

Protein modeling

For modeling we have used HemJ peptide from *R. sphaeroides* (WP_023003745) with 4 transmembrane helices. The modeling was performed using several automatic structure prediction servers – Robetta (53), RaptorX (54) and RaptorX-Contact (55). The server COFACTOR was used for prediction of heme binding site (56) and PPM server (57) was used to predict the position of the protein structure model within membrane. The protein models were aligned and visualized using PyMOL software [[The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.](#)]

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Conflict of interests:

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURES

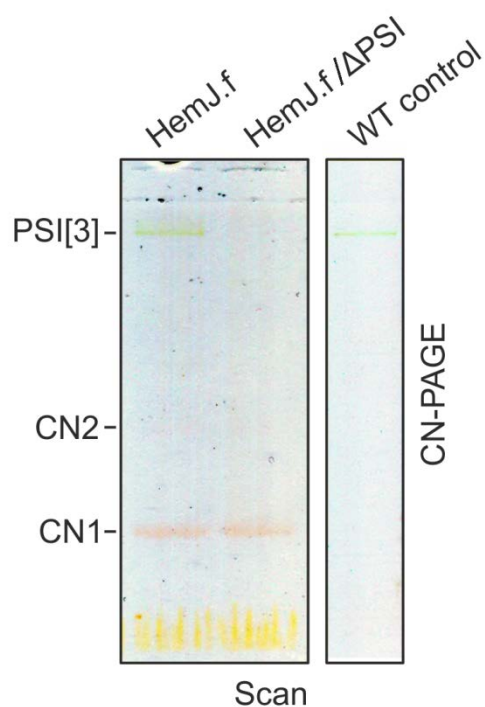


Figure 1.

Separation of the purified HemJ.f by clear native gel electrophoresis

Native isolations of HemJ.f from WT and Δ PSI backgrounds that resulted in reddish eluate were further separated by 4-14% clear native gel electrophoresis (CN-PAGE). Reddish bands CN1 and CN2 (poorly visible) were identified as HemJ.f. As already reported (18), a small amount of trimeric PSI (PSI[3]) is a typical contamination of FLAG eluates obtained from *Synechocystis*. The gel was scanned in transmittance mode (Scan) using an LAS 4000 Imager (Fuji).

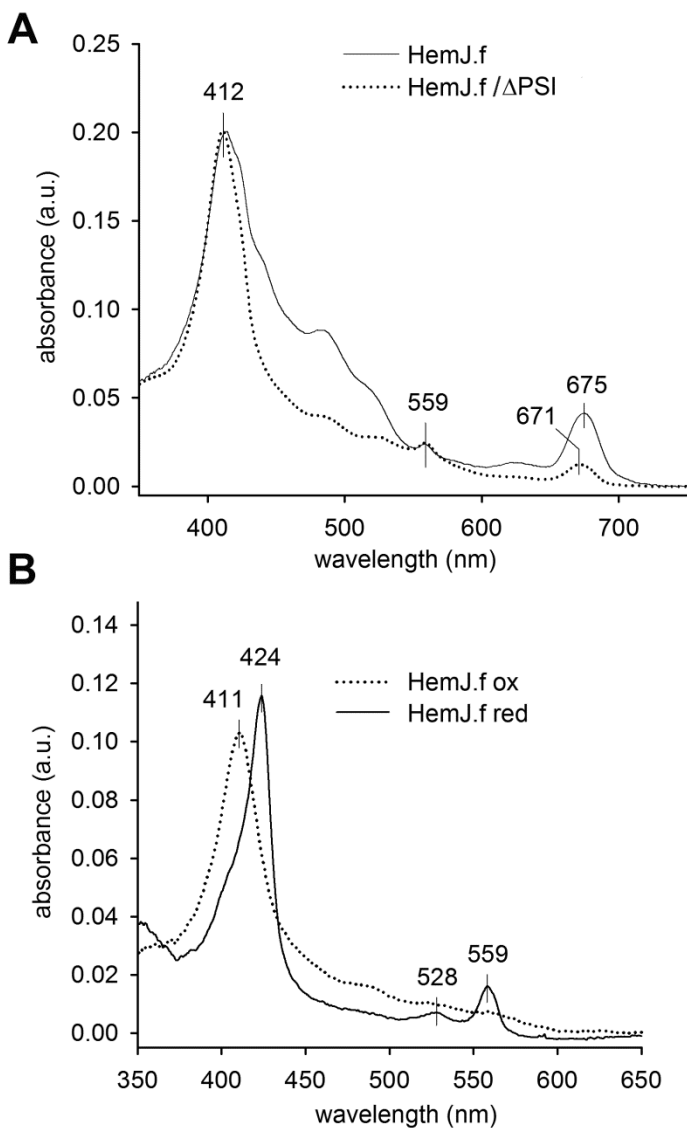


Figure 2.

Spectroscopy analysis of the purified HemJ.f

(A) Absorption spectra of HemJ.f eluate. Chlorophyll is represented by the peak at 675 nm, peaks at 412 and 559 nm are characteristic for heme. Amount of carotenoids visible at the region of 450 - 520 nm was variable in-between isolations. (B) Absorption spectra of oxidized and reduced eluate obtained from the Δ PSI genetic background.

a.u. – absorbance units

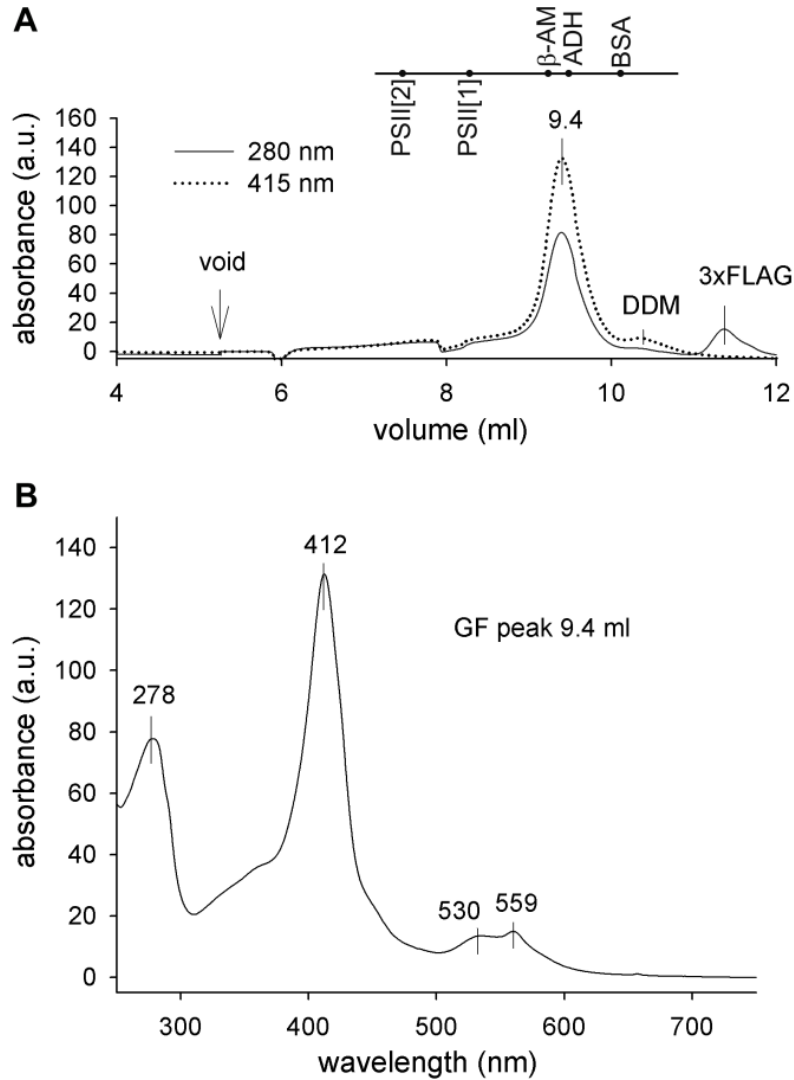


Figure 3.

Separation of the purified HemJ.f protein by gel filtration chromatography

(A) The HemJ.f pull-down obtained from the Δ PSI genetic background was loaded on a gel filtration column, and eluted proteins/complexes were detected by absorbance 280 and 415 nm.

Positions of standards are shown at the top of the graph: PSII[2] = PSII dimer (600 kDa); PSII[1] = PSII monomer (300 kDa); β -AM = β -amylase (200 kDa); ADH = alcohol dehydrogenase (150 kDa); BSA = bovine serum albumin (66 kDa); DDM marks the micelle of dodecyl- β -maltoside.

(B) Absorption spectrum of 9.4 ml gel filtration fraction was recorded by a HPLC diode array detector.

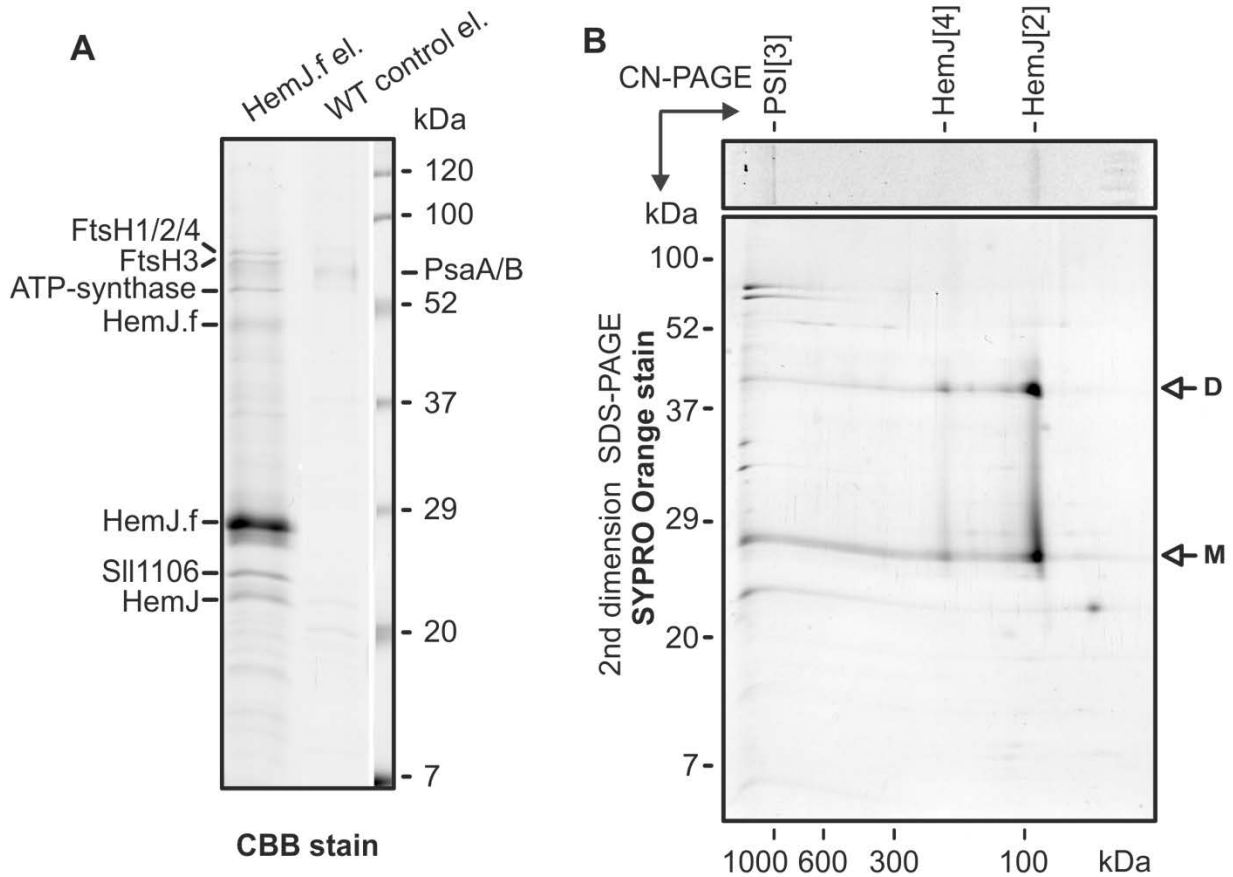


Figure 4.

One-dimensional SDS-PAGE and two-dimensional CN/SDS-PAGE separation of HemJ.f eluate

(A) Proteins isolated by affinity chromatography from HemJ.f strain and from WT control cells were separated by 12 to 20% SDS-PAGE, stained with Coomassie brilliant blue (CBB) and the individual proteins bands were identified by mass spectrometry (Table S1). (B) The gel strip from CN-PAGE (see Fig. 1) was further separated in a second dimension by 12 to 20% SDS-PAGE and stained with SYPRO Orange. HemJ.f bands (marked as CN1 and CN2 in Fig. 1) were tentatively assigned as dimeric (HemJ[2]) and tetrameric (HemJ[4]) HemJ.f oligomers, respectively.

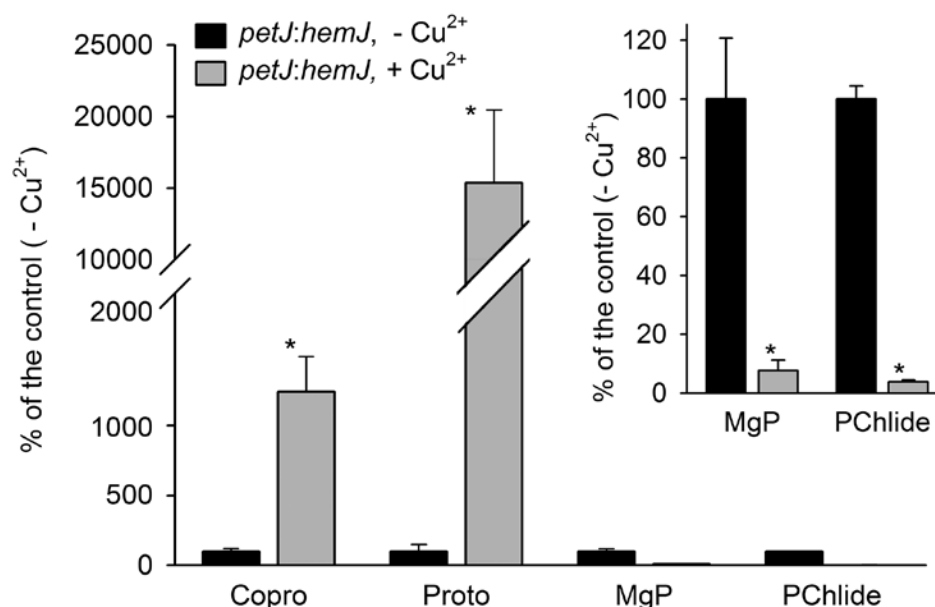


Figure 5.

Analysis of heme/chlorophyll precursors in the *petJ:hemJ* strain grown photoautotrophically in medium with or without copper

Heme/chlorophyll precursors were extracted with 70% methanol from the *petJ:hemJ* cells at $OD_{730} = 0.3 - 0.4$ and separated on a HPLC equipped with two fluorescence detectors (49). The amounts of later chlorophyll precursors Mg-protoporphyrin IX (MgP) and monovinyl protochlorophyllide (PChlide) were markedly reduced in cells cultivated with the repressed *hemJ* expression when compared with the same mutant cells grown without copper. On the contrary, Proto and Copro massively accumulated in the repressed cells. The inset shows a different scale for the less abundant precursors. *, significance difference tested using a paired *t* test ($P = 0.05$).

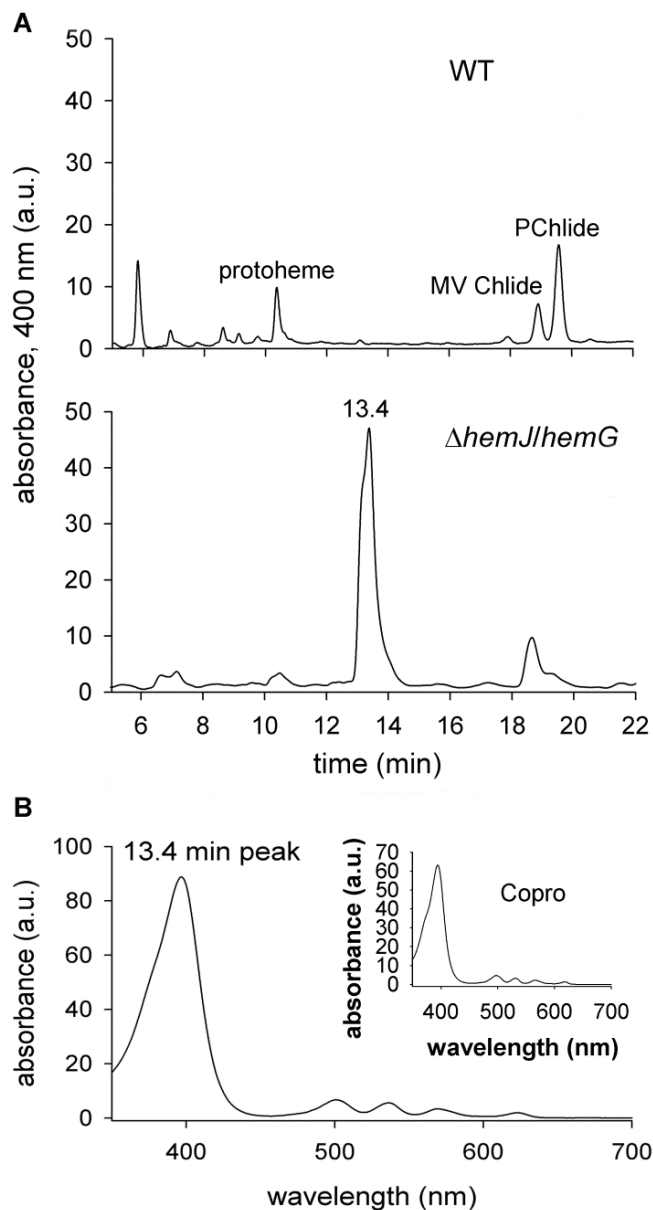


Figure 6.

Detection of an unusual tetrapyrrole in the $\Delta hemJ/hemG$ strain incubated without glucose

(A) Polar tetrapyrroles were extracted with 70% methanol from 50 ml of WT and $\Delta hemJ/hemG$ cells grown photoautotrophically for three days and harvested at $OD_{730} = 0.3 - 0.4$. The obtained extract was separated on a HPLC (see Experimental procedures) and eluted pigments were detected by a diode-array detector at 400 nm. MV Chlide – monovinyl chlorophyllide, Pchlide – monovinyl protochlorophyllide

(B) Absorption spectrum of the compound eluting at 13.4 min. The inset shows absorption spectrum of the Copro standard.

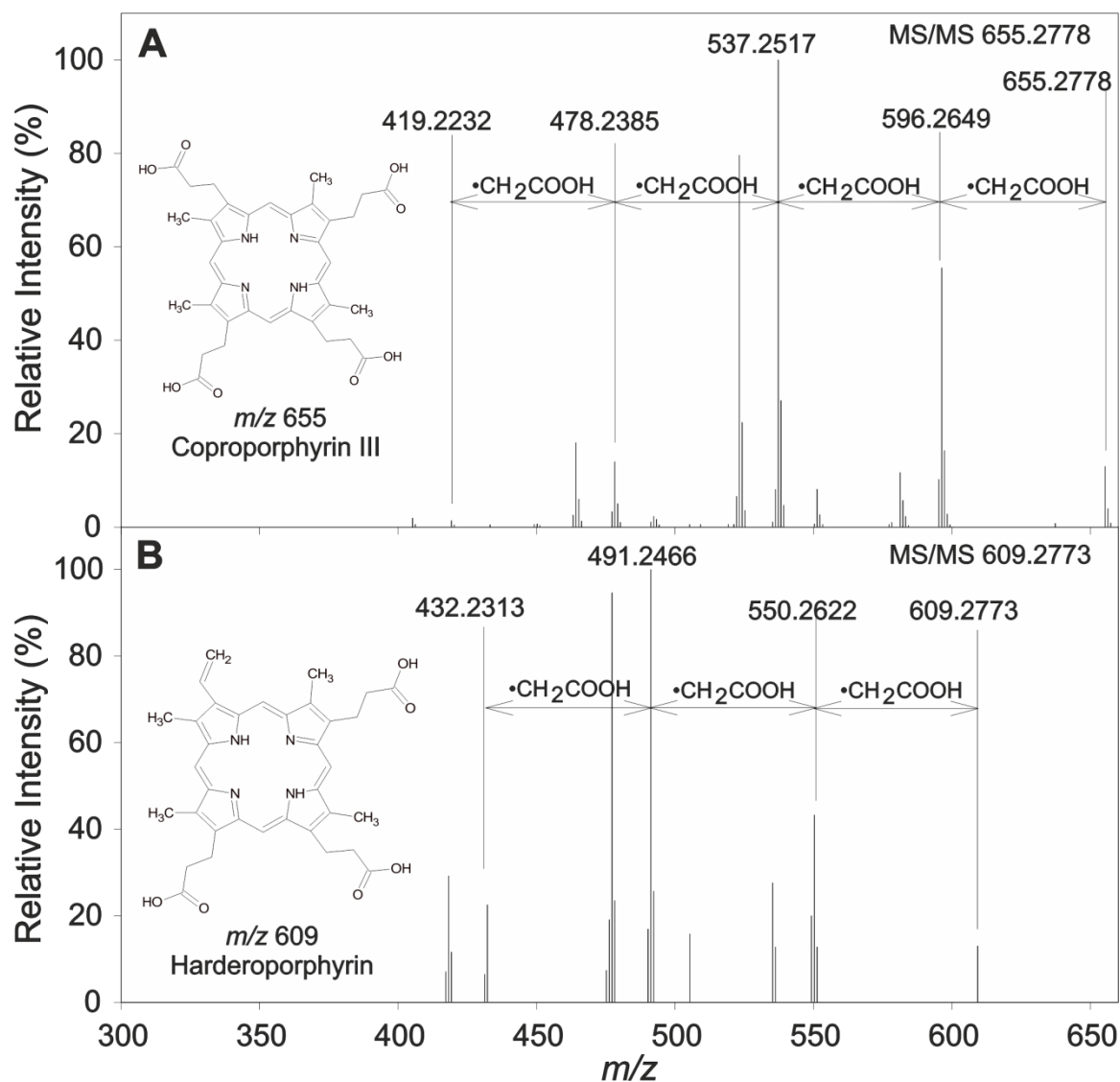


Figure 7.

HRMS/MS fragmentation of the Copro (A) and Hardero (B)

(A) Analytical standard of Coproporphyrin III (Copro) was subjected to HPLC-HRMS/MS analysis in order to provide background data for identification of the unknown tetrapyrrole found in $\Delta hemJ/hemG$ cells. It provided molecular ion at m/z 655 when subjected to HPLC-HRMS/MS and its subsequent fragmentation led to formation of ions corresponding to four consecutive losses of propionic radical. (B) Harderoporphyrin (Hardero) was isolated from $\Delta hemJ/hemG$ cells grown without glucose. The HPLC-HRMS/MS analysis provided molecular ion at m/z 609 with only three consecutive losses detected in its MS/MS spectrum. The fourth propionic group is substituted by a vinyl group as inferred from differences between Copro and Hardero m/z values.

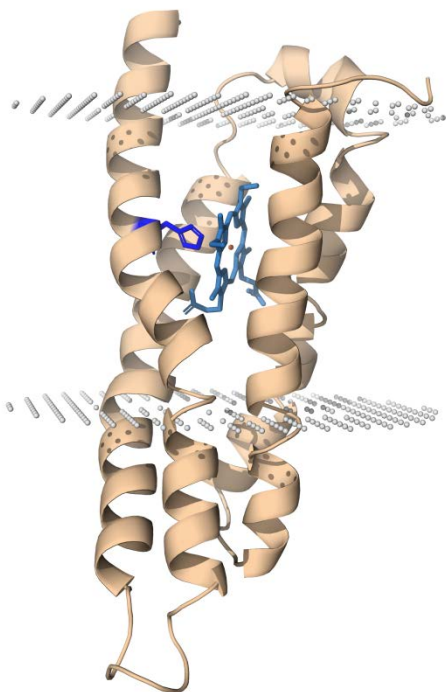


Figure 8.

Model structure of *R. sphaeroides* HemJ monomer

HemJ model predicted by automatic structure prediction server – RaptorX (54). The His16 proposed to bind heme is shown in blue. For prediction of heme binding site prediction the server COFACTOR was used (56). PPM server (57) was used to predict position of the protein structure model within membrane. Both N and C termini of the protein are on the periplasmic/luminal side of the membrane.