Running title:
Function of ferrochelatase C-terminal domains

Correspondence should be sent to:

Roman Sobotka

Institute of Microbiology,
Department of Autotrophic Microorganisms,
Opatovicky mlyn, 379 81
Trebon, Czech Republic

Tel: +420-384-340434 Email: sobotka@alga.cz
Functional assignments for the C-terminal domains of the ferrochelatase from *Synechocystis* PCC 6803: the CAB domain plays a regulatory role and region II is essential for catalysis

Roman Sobotka,¹,²,*  Martin Tichy,¹,²  Annegret Wilde,³ and C. Neil Hunter ⁴

¹Institute of Microbiology, Department of Autotrophic Microorganisms, Opatovicky mlyn, 379 81 Trebon, Czech Republic.

²Institute of Physical Biology, University of South Bohemia, 373 33 Nove Hrady, Czech Republic.

³Institute of Microbiology and Molecular Biology, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

⁴Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Sheffield S10 2TN, United Kingdom.

**Keywords:** ferrochelatase, tetrapyrrole biosynthesis, *Synechocystis* 6803, chlorophyll

This work was supported by Institutional Research Concept no. AV0Z50200510 (M.T.), by the project MSM6007665808 of the Ministry of Education of the Czech Republic (R.S.), by the Czech Science Foundation project P501/10/1000 (R.S) and by Deutsche Forschungsgemeinschaft Grant SFB429 (A.W). C.N.H. acknowledges financial support from the Biotechnology and Biological Sciences Research Council (UK).
Abstract

Ferrochelatase (FeCH) catalyses the insertion of Fe$^{2+}$ into protoporphyrin forming protoheme. In photosynthetic organisms FeCH and Mg-chelatase lie at a biosynthetic branchpoint where partitioning down the heme and chlorophyll pathways occurs. Unlike their mammalian, yeast and other bacterial counterparts cyanobacterial and algal FeCHs as well as FeCH2 isoform from plants possess a C-terminal CAB domain with a putative chlorophyll-binding motif. The CAB domain is connected to the FeCH catalytic core by a proline-rich linker sequence (region II). In order to dissect the regulatory, catalytic and structural roles of the region II and CAB domains we analyzed a FeCH ΔH347 mutant that retains region II but lacks the CAB domain and compared it with the ΔH324 FeCH mutant which lacks both these domains. We found that the CAB domain is not required for catalytic activity but is essential for dimerization of FeCH and its absence causes aberrant accumulation of chlorophyll-protein complexes under high light accompanied by high levels of the chlorophyll precursor chlorophyllide. Thus, the CAB domain appears to serve mainly a regulatory function, possibly in balancing chlorophyll biosynthesis with synthesis of cognate apoproteins. Region II is essential for the catalytic function of the plastid-type FeCH enzyme; although the low residual activity of the ΔH324 FeCH is more than sufficient to furnish the cellular demand for heme. We propose that the apparent surplus of FeCH activity in the wild type is critical for cell viability under high light due to a regulatory role of FeCH in distribution of chlorophyll into apoproteins.
Introduction

Cyanobacteria, algae and plants synthesize chlorophyll (Chl), heme and linear tetrapyrroles such as phycobilins via a common branched pathway. At the beginning of tetrapyrrole biosynthesis the initial precursor, 5-aminolaevulinic acid (ALA), is made from glutamate via glutamyl-tRNA, and is subsequently converted in several steps to protoporphyrin IX (PP\textsubscript{IX}), the last common precursor for both Chl and heme biosynthesis. Insertion of Fe\textsuperscript{2+} into this porphyrin macrocycle by ferrochelatase (FeCH) leads to heme, whereas insertion of Mg\textsuperscript{2+} by magnesium chelatase leads to Mg-protoporphyrin IX (MgP), the first biosynthetic intermediate on the ‘green’ Chl branch (reviewed in Tanaka and Tanaka 2007).

Because the levels of heme and Chl vary according to cell development, growth or light conditions, special regulatory mechanisms have evolved which control heme and Chl formation and co-ordinate their levels with the synthesis of the corresponding apoproteins (Müller et al., 1999). Stringent control of this pathway is particularly essential for oxygenic organisms such as plants and cyanobacteria that have to cope with the problem of photooxidation. Chl, as well as its intermediates, are readily excited by light and, unquenched, form reactive oxygen species under aerobic conditions. Therefore, for organisms carrying out oxygenic photosynthesis, it is essential to minimize cellular levels of unbound ‘free’ tetrapyrroles.

The mechanisms that control and regulate Chl/heme biosynthesis mostly remain to be elucidated, although it is well established that the total metabolic flow through the pathway is controlled at the point of ALA formation, a rate-limiting step for the whole pathway (reviewed in Tanaka and Tanaka 2007; Masuda and Fujita 2007). The biosynthetic step controlling ALA formation is most probably that catalyzed by glutamyl-tRNA reductase. This is the first enzyme in the pathway and it is modulated by a wide range of regulatory signals in line with the idea that this is the central controller of total tetrapyrrole flux (Kumar et al., 1996; Tanaka et al., 1996; McCormac et al., 2001). Another key regulatory site is at the branch point between Chl and heme biosynthesis, where distribution of PP\textsubscript{IX} has to be carefully balanced according to the actual demand for both essential pigments. These regulatory mechanisms that govern the distribution of PP\textsubscript{IX} remain to be elucidated; although the roles of both chelatases have become apparent (Papenbrock et al., 2000; Papenbrock et al., 2001; Sobotka et al., 2005).

FeCH is an enzyme of special interest regarding the regulation of tetrapyrrole biosynthesis. FeCH activity may regulate the flux down both the heme and Chl branches of the pathway via increased production of heme, which is expected to inhibit synthesis of ALA at the start of the pathway through feedback control (Goslings et al., 2004; Weinstein et al., 1993; Vothknecht et al., 1998). Differential consumption of the
common substrate PP_{IX} by FeCH and magnesium chelatase might also control partitioning at the Chl/heme branch point (reviewed in Cornah et al., 2003). Finally, a mechanism whereby the Chl branch exerts control over the heme pathway would provide an even more sophisticated mode of control at this biosynthetic branchpoint. We have recently demonstrated that FeCH activity influences Chl biosynthesis as decreased activity of this enzyme was followed by a significant increase in the rate of ALA formation, in the level of Chl precursors and in the accumulation of Chl-protein complexes (Sobotka et al., 2005; Sobotka et al., 2008a).

An intriguing feature of FeCH from cyanobacterial and algal sources is the hydrophobic C-terminal extension (CAB domain) with a high degree of similarity to the first and third helices of the plant light harvesting complex II (LHCII) including a Chl-binding motif (Fig. S1; Dolganov et al., 1995). Interestingly, the CAB domain is also a part of the FeCH isoform II responsible for production of the most of heme in chloroplasts of higher plants (Papenbrock et al. 2001). This highly conserved CAB domain is connected to the FeCH catalytic core by a more variable region II - a short, hydrophobic and proline-rich sequence (Fig. S1, Fig. 1A). This segment is preserved even in a few organisms harboring a plastid-type FeCH enzyme that lacks the CAB domain. This was shown for two cyanobacteria, *Synechococcus* JA-2-3Ab and JA-2-3B’a, (Kilian et al., 2008; Fig. S1) and for the green alga *Chromera velia* (Koreny L., Sobotka R., Obornik M., unpublished data; see Fig. S1). The only known plastid-type FeCH lacking both the region II and the CAB domain is from the cyanobacterium *Gloeobacter* which also lacks thylakoid membranes (Fig. S1).

Although the functions of both the CAB domain and the region II are unknown, we have recently demonstrated that deletion of both of these FeCH features in the ΔH324 strain of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) dramatically reduces the stability and activity of FeCH and leads to a large accumulation of its substrate, PP_{IX} (Sobotka et al., 2008a; see Fig. 1B). In addition, analysis of both full-length and truncated ΔH324 recombinant FeCHs demonstrated that the C-terminal extension comprising region II and the CAB domain is essential for dimerization of the enzyme (Sobotka et al., 2008a).

In order to dissect the functional and structural roles of the region II and CAB domains we have analyzed in the present work another FeCH mutant ΔH347 that retains region II but lacks the CAB domain (Fig. 1B). We have found comparable levels of the FeCH protein and of *in vitro* activity for the wild-type (WT) and truncated ΔH347-FeCH, in marked contrast with the very low residual activity of the ‘fully-truncated’ ΔH324-FeCH. However, deletion of the CAB domain prevented the ΔH347 strain from growing at higher light intensities and affected the cellular accumulation of tetrapyrroles. In particular, we have found that
although the ΔH347 mutant contains decreased level of PPIX it specifically accumulates chlorophyllide and fails to reduce cellular levels of Chl-protein complexes under high light. Analysis of the full-length and truncated FeCHs purified from *Synechocystis* under native conditions demonstrated that whereas the full-length enzyme forms a dimer, the ΔH347-FeCH is active as a monomer. The essential role of the CAB domain for the dimerization of FeCH *in vivo* was further confirmed by co-purification of the full-length FeCHs from the cell using the tagged C-terminal segment of FeCH as bait.

**Results**

**The C-terminal domains of FeCH are required for acclimation of *Synechocystis* to high light**

To address the proposed role of the FeCH CAB domain in the regulation of tetrapyrrole metabolism and also to elucidate the function of region II, we constructed the *Synechocystis* mutant ΔH347, which retains the 23-residue region II but lacks the last 40 residues that comprise the putative transmembrane CAB domain (Fig. 1). Another FeCH mutant, ΔH324, prepared for previous work (Sobotka et al., 2008a), was also included in this study. This mutant differs from the ΔH347 only by the additional absence of region II at the end of the FeCH catalytic domain (Fig. 1B) and thus a direct comparison of both strains should help to discriminate between effects of both region II and the CAB domain.

First, we compared the photoautotrophic growth of both mutants and of the control WT strain (WT<sub>zeo</sub>) (see Fig. 1B) under different light intensities. Under low light (5 µmol photons m<sup>-2</sup> s<sup>-1</sup>) all strains had comparable growth rates and also very similar levels of photosynthetic pigments (Table 1, Fig. S2). However, at normal light (40 µmol photons m<sup>-2</sup> s<sup>-1</sup>) the ΔH324 mutant grew significantly more slowly and with a further increase in light intensity to 150 µmol photons m<sup>-2</sup> s<sup>-1</sup> we observed a rapid loss of photosynthetic pigments. A further increase in light intensity abolished growth of this strain (Table 1, Fig. S2). The effect of increased light intensity on the ΔH347 mutant was much less pronounced; its growth was only slightly impaired at normal light and this strain was able to grow up to 200 µmol photons m<sup>-2</sup> s<sup>-1</sup> (hereafter high light; Table 1). Further increase in light intensity to 250 µmol photons m<sup>-2</sup> s<sup>-1</sup> also completely inhibited growth of the ΔH347 mutant, whereas the growth of the control WT<sub>zeo</sub> strain was not significantly affected (Table 1). Interestingly, the ΔH347 mutant did not exhibit any ‘bleaching’ at high light as we observed for the ΔH324; on the contrary, the ΔH347 mutant retains about 40% more Chl per cell than the WT<sub>zeo</sub> strain after ~ 40 hours at high light (Table 1, Fig. S2). This is noteworthy as a substantial decrease in Chl level is a typical response of *Synechocystis* to high light (Hihara et al., 1998; see control WT<sub>zeo</sub> strain in Table 1 and Fig. S2).
These results demonstrate that both the CAB and the region II domains are essential for photosynthetic growth of Synechocystis at high light intensities. However, the presence of region II of FeCH substantially improves acclimation to high light and furthermore the phenotypes of both FeCH mutants clearly differ under increased light intensities; whereas the ΔH324 strain loses most of its pigments probably due to destruction of thylakoid membranes the ΔH347 mutant retains a higher level of Chl, indicating that the viability of ΔH324 and ΔH347 mutants upon a shift to high light is abolished by different processes.

**The CAB domain is important for regulation of the tetrapyrrole pathway**

A remarkable feature of the ΔH324 strain was excretion of PP IX into the growth medium, at levels high enough to produce a brown colour (Sobotka et al., 2008a). As we did not observe this effect in ΔH347 we compared the accumulation of PP IX and Chl intermediates in both mutants grown at normal light levels to elucidate the effect of these two protein truncations on tetrapyrrole metabolism. Surprisingly, we found that levels of tetrapyrroles in these strains are very different; whereas the ΔH324 accumulates high levels of PP IX and also other intermediates in Chl branch (Fig. 2A; Sobotka et al., 2008a) ΔH347 contains less than 20% of the PP IX found in the control WT zeo strain. On the other hand, levels of magnesium protoporphyrins and protochlorophyllide in the ΔH347 strain are not significantly affected, with the exception of the ~ 2.5 fold increase in the content of the last Chl precursor, chlorophyllide (Chlide) (Fig. 2A).

The observed low PP IX content of the ΔH347 mutant could indicate reduced ALA formation at the beginning of the pathway; however measurements of ALA-synthesising capacity revealed only a slightly increased rate of ALA formation in the ΔH347 mutant in comparison to WT zeo (Fig 2B). This contrasts with a significantly elevated ALA formation in the ΔH324 mutant, which is probably responsible for the accumulation of PP IX in this strain (Fig. 2B; Sobotka et al., 2008a). So, detected changes in tetrapyrrole biosynthesis in the ΔH347 do not appear to be caused predominantly by elevated metabolic flow through the whole pathway as in the case of the ΔH324 mutant but rather by different events at the heme/Chl branch point between heme and Chl branches influencing PP IX pool and at final steps of the pathway resulting in accumulation of Chlide.

The low pool of PP IX in ΔH347 could be explained by a rapid ‘uncontrolled’ consumption of PP IX either by Mg chelatase or by truncated ΔH347-FeCH. The first possibility is not consistent with almost the normal level of magnesium protoporphyrin IX (MgP) in ΔH347. Moreover we did not observe higher cellular levels of the Mg chelatase subunits (Fig. 3A). The second possibility, a rapid consumption of PP IX by mutated ΔH347-FeCH, could be reflected in higher levels of cellular heme or phycobilins. To determine levels of heme B (protoheme) total noncovalently-bound heme was extracted by acetic acetone as described in
Materials and Methods, separated by HPLC and quantified spectroscopically. Indeed, the heme content of ΔH347 was significantly increased (Fig. 2C). Interestingly, the heme content of the ΔH324 mutant was not affected (Fig. 2C) suggesting that the ΔH324-FeCH is able to produce enough of heme required for normal cellular function, despite a barely detectable in vitro activity of this truncated enzyme (Sobotka et al., 2008a; see below). We did not observe significant changes in phycobilisome levels in any of the strains studied implying that the accumulation of the light-harvesting apparatus is under tight control and is not influenced by disturbances in tetrapyrrole biosynthesis (Fig. S2).

The enlarged pool of Chlide in the ΔH347 mutant is not easy to explain regarding the position of the FeCH in the pathway and normal or lower level of other Chl precursors. It should be also noted that the ΔH347 mutation did not significantly alter the accumulation or cell localization of enzymes in Chl pathway including the enzyme producing Chlide, protochlorophyllide oxidoreductase - (Fig. 3A). However, Chlide is the last precursor of the Chl biosynthesis pathway and thus its accumulation could be related to the failure of ΔH347 to adjust the levels of Chl-protein complexes according to light conditions. We therefore compared levels of Chl precursors in WTzeo and ΔH347 at high light when the growth rate of the ΔH347 is retarded (Table 1). Whereas the content of Chlide in the control strain decreased when compared to the situation at normal light, the content of Chlide in the ΔH347 remained almost unchanged (Fig. 2D), suggesting that the FeCH CAB domain is required for regulation of the final step(s) of Chl biosynthesis, which could include attachment of Chl to proteins and Chl turnover (see Discussion).

To summarize, it is evident that the two different truncations at the FeCH C-terminus have very different consequences for tetrapyrrole metabolism. Elimination of both region II and the CAB domain from FeCH results in a greatly elevated synthesis of PPIX and a consequent increase in the flux down the ‘green’ branch of tetrapyrrole biosynthesis. In contrast, the ΔH347 strain that retains region II contains only a small PPIX pool but exhibits increased accumulation of Chlide under different light regimes. These results highlight the importance of region II and the CAB domain of FeCH for both its enzymatic and regulatory functions.

Region II is critical for both the stability and enzyme activity of Synechocystis FeCH

We have shown previously that truncation of the whole C-terminal domain of Synechocystis FeCH strongly impairs the stability and activity of the resulting ΔH324 enzyme (Sobotka et al., 2008a). To elucidate the completely different effect of the ΔH347 mutation on tetrapyrrole metabolism, we compared the activity and stability of mutated and full-length FeCHs.
To assess the localization and cellular levels of the ΔH347-FeCH, a whole cell extract of the ΔH347 mutant was fractionated into cytosolic and membrane fractions, separated by SDS-PAGE, and subjected to immunodetection using anti-FeCH antibody raised against recombinant Synechocystis FeCH. The ΔH347-FeCH was detected only in the membrane fraction despite the lack of the putative transmembrane CAB domain. However, in contrast to the ΔH324 enzyme, both the FeCH levels and in vitro FeCH activities were comparable in membrane fractions from the ΔH347 and WT_{zeo} strains (Fig. 3 A,B). As we did not observe any in vitro activity for the ΔH324 enzyme, it is evident that the presence of region II dramatically improves both the stability and the activity of Synechocystis FeCH.

To further determine possible structural functions for both the region II and CAB C-terminal FeCH domains we analyzed enzymes purified under non-denaturing conditions from Synechocystis using the 3xFLAG-tag (FLAG hereafter). To show that the presence of the tag did not interfere with the enzyme function, we deleted the native hemH gene in the FLAG-hemH strain, resulting in a strain expressing just the tagged FeCH-version (Fig. 1B). The FLAG-hemH/ΔhemH strain did not display any apparent effects of FeCH deficiency such as retarded growth or accumulation of PP_{IX} and the in vitro activity of the FLAG-FeCH in the cell extract corresponded with the enzyme content (not shown).

Both full-length and truncated FeCHs were expressed in Synechocystis and purified from cell extracts under native conditions using anti-FLAG resin; the migration of purified proteins on SDS electrophoresis corresponded to theoretical masses of 46.2, 41.7 and 38.9 KDa for the FLAG-FeCH, FLAG-ΔH347 and FLAG-ΔH324, respectively (Fig. 4A). Activities of purified FeCHs were compared using an in vitro assay (Fig. 4B). Consistent with previous results on recombinant FeCHs (Sobotka et al., 2008a) deletion of both the region II and the CAB domains dramatically impaired activity of the ΔH324-FeCH (less than 5 % activity of the of the full-length enzyme; Fig. 4B). The specific activity of the FLAG-ΔH347 enzyme was also about 20 % of that of the WT enzyme. Intriguingly, the rate of incorporation of zinc into PP_{IX} by ΔH347 FeCH rapidly decreases in the first 50 seconds of the assay whereas the rate of incorporation of zinc by the full-length tagged enzyme is almost linear for at least 10 minutes (Fig. 4B). It should be noted that the activity of non-tagged ΔH347 FeCH in purified membranes exhibits the same decrease in rate of zinc- PP_{IX} formation (not shown). This implies that although the CAB domain is important for functioning of the FeCH, further truncation involving region II affects the FeCH accumulation and activity much more strongly.
The CAB domain is necessary for the dimerization of *Synechocystis* FeCH in vivo

Using recombinant *Synechocystis* FeCHs we have previously shown that deletion of the C terminus in ΔH324 FeCH abolishes dimerization of the recombinant enzyme and leads to loss of its activity (Sobotka et al., 2008a). This led us to an assumption that, in agreement with the situation in mitochondrial FeCH (Ohgari et al., 2005; Grzybowska et al., 2002), the dimer is the active form of the *Synechocystis* FeCH. Given that the purified ΔH347 FeCH is fairly active and the same enzyme is fully active in membranes (Fig. 3B), we speculated that region II might promote dimerization resulting in an active enzyme. However, on a native gel the purified FLAG-ΔH347 FeCH migrated strictly as a monomer in contrast to the dimeric full-length FLAG-FeCH (Fig. 5A). To facilitate further analysis of enzyme dimerization, all three purified tagged enzymes were separated using size exclusion chromatography and the FeCH activity of the column fractions was measured. According to the absorbance at 280 nm (Fig. 5B) the full-length FeCH migrates as a broad peak mainly as a dimer but with a significant fraction of monomers and also a small proportion of higher mass aggregates (compare with Fig. 5A), whereas both truncated enzymes migrate in a sharp peak corresponding to monomers with bound detergent molecules (Fig. 5B). Note that it is generally problematic to determine mass of membrane proteins on gel filtration as calibration of column with soluble proteins leads to inaccurate results (Zouni et al., 2005). However, the results in Fig. 5 are internally consistent and show that although the mass of the FLAG-ΔH347 protein is only 2.8 KDa larger than FLAG-ΔH324, its mobility is half a minute slower, which corresponds to ~25 KDa according to our column calibration (Fig. 5B). It could indicate that the loss of region II is accompanied by a significant difference in the conformation of the FeCH protein.

The FeCH activity profile for the collected fractions for the full-length enzyme corresponded well with the protein absorbance including an obvious shoulder at the position of a predicted monomer (Fig. 5C). This result indicates similar *in vitro* specific activities for both the dimeric and the monomeric forms of the enzyme. The maximum activity of the FLAG-ΔH347 FeCH was measured in the elution peak fraction resembling the monomeric form. We therefore expect that the FLAG-ΔH347 was active in the assay in a monomeric form. It should be noted that a substantial portion of the total FLAG-ΔH347 activity was lost during gel filtration suggesting a lower stability for this truncated enzyme when compared to the full-length FeCH which was still highly active when eluted from the column (Fig. 5C). As expected, no activity was detected when a similar amount of the FLAG-ΔH324 was analyzed by the same method (Fig. 5C).

Although previous results strongly support a model where the CAB domain is responsible for FeCH dimerization and the ΔH347 FeCH is therefore a partially active monomer *in vivo*, it cannot be excluded that dimerization of *Synechocystis* FeCH is driven by having a high concentration of pure protein. To obtain
further evidence that the full-length FeCH physically interacts with another FeCH molecule *in vivo*, we employed the *FLAG-hemH* strain expressing both the FLAG-FeCH and non-tagged WT FeCH. As these two enzyme forms have different mobility on SDS-PAGE they can be distinguished by immunodetection using a combination of anti-FeCH and anti-FLAG antibodies (Fig. 6A). After isolation of the FLAG-FeCH on anti-FLAG affinity gel the smaller native FeCH was specifically co-purified with FLAG-FeCH and it was the only co-purified protein visible on the Coomassie stained gel (Fig. 6A).

To resolve the question of whether the CAB domain alone is sufficient to promote formation of dimeric FeCH and to investigate whether another part of this enzyme is essential for FeCH-FeCH interaction *in vivo* we prepared a *Synechocystis* strains expressing the FeCH C-terminus consisting of region II and the CAB domain, as a small His<sub>6</sub>-tagged protein (His-C-tn; Fig. 1C). This artificial protein, lacking the extrinsic catalytic domain, was expressed both in the WT and the ΔH347 strains and used as *in vivo* bait for FeCH. If the CAB domain is the only segment required for the FeCH dimerization, the His-C-tn protein would form a stable complex only with the full-length FeCH but not with the truncated ΔH347 enzyme. The His-C-tn protein was purified on a nickel column under native conditions, subjected to gel electrophoresis and co-isolated FeCH was detected by anti-FeCH antibody (Fig. 6B). The full-length FeCH clearly co-purified with His-C-tn in contrast to the ΔH347 FeCH, confirming *in vivo* dimerization via the CAB domain (Fig. 6B).

Moreover, the His-C-tn protein formed a stable dimer with another His-C-tn molecule, which partly persisted even during separation by denaturing SDS-electrophoresis (Fig. 6B). Based on these data it can be concluded that the CAB domains have affinity for one another and that this domain is strictly required for dimerization of the *Synechocystis* FeCH *in vivo*.

**Discussion**

Cyanobacterial and algal FeCHs as well the plant FeCH2 isoform possess a unique conserved C-terminal CAB domain with a putative Chl-binding motif (see Fig. S1). The CAB domain and the FeCH catalytic core are connected by a ‘linker’ (region II), which is quite variable in its length and sequence among organisms (Fig. S1). Recently, we have demonstrated that the elimination of both region II and the CAB domain from FeCH in the *Synechocystis* ΔH324 mutant dramatically impaired activity and stability of the truncated FeCH (Sobotka et al., 2008a). Given the essential role of dimerization for the function of human and yeast FeCHs (Ohgari et al., 2005; Grzybowska et al., 2002), the low activity of the ΔH324 FeCH was originally attributed to the absence of the CAB domain yielding a monomeric enzyme (Sobotka *et. al.*, 2008a). However, newly discovered thermophilic *Synechococcus* strains possessing putative monomeric FeCHs lacking the CAB domain but still preserving the short region II (Kilian et al., 2008; Fig. S1) stimulated us to mimic these
naturally-occurring FeCH variants by preparing the ΔH347 strain lacking only the CAB domain. Importantly, the elimination of the CAB domain yielded an enzyme with comparable specific activity to the WT FeCH (Fig. 3A,B). Direct comparison of ΔH347 with the ΔH324 FeCH lacking both the region II and CAB domains reveals the crucial importance of the region II and enabled us, for the first time, to explore the role of the FeCH CAB domain in the regulation of tetrapyrrole biosynthesis.

Our earlier work on the ΔH324 strain (Sobotka et al., 2008a) posed an intriguing question as to why this mutant, possessing a strongly impaired FeCH, does not display any depletion in phycobilins, which are produced by oxidation of heme (Fig. S2). The present work helps to address this issue by demonstrating that the low activity of ΔH324 FeCH is more than sufficient to furnish the cellular demand for heme (Fig. 2C). However, it is still unclear why the WT cell maintains FeCH at levels much higher than those actually required by the demands placed on heme biosynthesis. It is noteworthy that under low light or microaerobic conditions the ΔH324 mutant resembles the WT phenotype; it exhibits normal pigmentation and growth rate and does not release PPIX into the growth medium (Table 1, Fig. S2, Sobotka, unpublished). These data suggest that under low-stress conditions, traces of FeCH activity are sufficient. Nonetheless, at increased light intensity the growth of the ΔH324 mutant is significantly retarded and a further increase in light intensity to 150 μmol of photons m⁻² s⁻¹ caused cell bleaching, probably due to destruction of thylakoid membranes (Table 1, Fig. S2). It appears that under such conditions the high content of FeCH found in the WT is essential for cell viability, to avoid accumulation of the high levels of phototoxic PPIX and other porphyrins found in the ΔH324 mutant.

Based on these data and on our previous results (Sobotka et al., 2005), we expect that the FeCH enzyme is involved in the regulation of tetrapyrrole biosynthesis and that the high content of the enzyme per cell, in relation to the biosynthetic demand for heme, has regulatory implications. Our previous work suggested a regulatory role for FeCH; the cellular FeCH activity increased 3 to 4-fold in Synechocystis mutants impaired in photosystem II assembly, and leads to a decrease in metabolic flow through the tetrapyrrole pathway (Sobotka et al., 2005). The cell could balance the distribution of PPIX between both chelatases and could also combat the detrimental effects of a stressful and fluctuating environment using a surplus of 'silent' FeCH to dissipate escalating levels of phototoxic PPIX. In the cyanobacterium Thermosynechococcus elongatus FeCH was found to physically interact with protoporphyrinogen oxidase, the enzyme which produces its substrate PPIX (Masoumi et al., 2008). It is possible that the delivery of PPIX to FeCH is controlled via formation of such complex - if needed, the FeCH quickly interacts with protoporphyrinogen oxidase and channels photosensitizing PPIX into the safer heme metabolite, with the bonus of inhibiting of the ALA synthesis via a heme feedback loop (see below). In our model, FeCH activity, which includes its access to PPIX, modulates
flow of this metabolite into the heme or Chl branches. The ΔH347 strain shows higher content of noncovalently-bound heme but has a low pool of PP IX although its ALA capacity is comparable to the WT (Fig. 2). This indicates that the ΔH347 mutant is deficient in the control of PP IX distribution and we speculate that deletion of the CAB domain facilitates access of truncated FeCH to PP IX.

According to current models of the regulation of tetrapyrrole biosynthesis, increased consumption of PP IX by FeCH should inhibit the GluTR enzyme via a heme feedback loop (for recent reviews see Tanaka and Tanaka, 2007; Masuda and Fujita, 2008). However, although an allosteric inhibition of GluTR by heme in plants is supported by convincing evidence, the model of ‘free’ heme directly interacting with the GluTR and modulating activity of this enzyme is probably over-simplified. In Chlamydomonas, inhibition of the GluTR activity by heme depends on the presence of an unidentified soluble protein (Strivastava et al. 2005) and heme paradoxically stimulates expression of the gene coding for GluTR (Vasileuskaya et al., 2005). In non-photosynthetic bacteria heme controls proteolytic degradation of GluTR and in the acidophilic bacterium Acidithiobacillus ferrooxidans heme also regulates activity of Glutamyl-tRNA synthase (Wang et al., 1999; Levican et al., 2007). The role of heme in the tetrapyrrole signaling network is thus quite complex and it is not so surprising that the increased protoheme level in the ΔH347 strain did not result in a decreased rate of ALA formation (Fig. 2B). It should be noted that the ΔH324 strain, where ALA synthesis is clearly stimulated, has a practically unchanged protoheme content per cell, so there might be a specific pool of heme used for signaling. However, as almost nothing is known about mechanisms balancing production of different heme forms (B, A, C) together with linear tetrapyrroles, a regulatory role of a putative heme pool remains speculative.

As we already discussed the ΔH324 dies at high light probably due to massive accumulation of PP IX and also other porphyrins. The growth of the ΔH347 mutant is also impaired by high light intensities although it does not accumulate any tetrapyrroles other than Chlide (Table 1) but, interestingly, at the same time this strain contains ~ 40% more Chl per cell than WT zeo. We expect that the failure of the ΔH347 to grow at high light is not caused primarily by a high concentration of phototoxic tetrapyrroles but due to an inability of this strain to control the synthesis and turnover of Chl-protein complexes. A similar ‘stay green’ phenotype was already described in the Synechocystis pmgA mutant that had lost the ability to regulate photosystem stoichiometry; particularly to decrease the level of photosystem I (PSI) relative to PSII upon a switch to high light (Hihara et al., 1998). Such an adjustment of the PSI/PSII ratio was shown to be essential for survival of Synechocystis when grown at 300 μmol of photons m⁻² s⁻¹ (Sonoike et al., 2001) and since PSI contains most of the Chl in the cell the process of high light acclimation results in remarkable decrease in total Chl content per cell (see WT zeo in Table 1). Interestingly, the downregulation of PSI was reported to be determined primarily by
limited availability of Chl for synthesis of PSI subunits (Muramatsu et al., 2009) and thus this process can be affected by an elevated level of Chlide - the last Chl precursor. On the other hand, the regulation of the PSI/PSII ratio cannot be accomplished simply by a total restriction of Chl formation but rather Chl redirection as, in contrast to PSI, the synthesis of PSII subunits is accelerated under high light stress due to a faster turnover of PSII (Nowaczyk et al., 2006). We expect that a sophisticated mechanism exists ensuring distribution of Chl to Chl-binding proteins under changing environmental conditions and that such apparatus has to recycle Chlide originating from degraded Chl-proteins via Chl turnover (Vavilin and Vermaas, 2007).

The presence of a Chl-binding motif in the FeCH CAB domain led us to the speculation that this domain is involved in the mechanism proposed above to balance Chl biosynthesis, Chl turnover and synthesis of particular Chl-proteins. Using the FLAG-tagged FeCH and its C-terminus as bait, we have provided clear evidence that the CAB domain mediates a specific interaction between FeCH molecules in vivo. In the FeCH dimer the interacting CAB domains should resemble the ‘cross helix‘ structure found in the light harvesting LHCII complex of higher plants; the Chl-binding motif ExxNGR is preserved in the CAB domain (Fig. S1, Liu et al., 2004). Our data thus support the possibility of Chl(ide) interacting with the CAB domain and may form an (alternative) route for transfer of Chl(ide) to photosystem apoproteins. In this context it is interesting that both cyanobacteria and plants contain high/early-light inducible proteins (ELIP/HLIP) which are structurally very similar to the FeCH CAB domain including a conserved Chl-binding motif (Dolganov et al., 1995). These proteins, which quickly accumulate at high levels upon a switch to high-light, might form part of a dynamic network of interacting CAB domains in the membrane that perhaps transiently form a complex with the FeCH CAB domain yielding a monomeric enzyme; virtually fully active but localized adjacent to different protein complexes, with different affinity to Chl or with different access to PPIX. Indeed, both ELIP and HLIP were found to regulate Chl biosynthesis (Xu et al., 2002; Tzvetkova-Chevolleau et al., 2007) and to physically interact with Chl-binding proteins (Promnares et al., 2006).

The CAB domain apparently has an important role also in the stability of Synechocystis FeCH; in contrast to the comparable membrane-associated catalytic activities of the WT and the ΔH347 FeCH, the specific activity of the purified FLAG-H347 FeCH was much lower (Fig. 4). This low specific activity is probably caused by lower stability of the enzyme as the purified FLAG-H347 enzyme lost its activity much faster than the full-length enzyme, which made it difficult for example to measure activity of FLAG-H347 FeCH separated by gel filtration. It is probable that one of the reasons for higher stability of the full-length FeCH is its dimerization.
The critical role of the region II for the activity of *Synechocystis* FeCH is noteworthy, regarding its rather low sequence similarity among different groups of organisms and its position at the end of the extrinsic catalytic domain of the enzyme. The only common features perceptible from amino-acid alignments are the rather hydrophobic nature of this sequence and a higher frequency of proline residues (Fig. S1). When compared to the structure of the spinach LHCII light harvesting complex (Liu et al., 2004), the FeCH CAB domain best corresponds to the third helix and the proline-rich region II can be aligned with the stromal loop connecting the second and third helices (Fig. S1). Indeed, this short sequence in LHCII contains five proline residues that bend this region to form a curled but relatively flat structure on the membrane surface (Fig. S3). So, the FeCH proline-rich domain could form a similar structure, although we cannot account for the importance of this domain for enzyme activity/stability. A clue could be an unusual kinetics of the ΔH347 FeCH in our assay (Fig. 4B). The rapid decline in the initial rate of chelation is specific only for the ΔH347 enzyme as the activity of recombinant ΔH324, although very weak, exhibits a linear rate of zinc-PPIX formation (Sobotka R., unpublished data). Also this effect is not caused by low stability of the H347 enzyme in the assay as the purified FLAG-H347 FeCH can be incubated in assay buffer without zinc for at least 15 min with no decrease in activity (not shown). The observed kinetics could be related to zinc uptake by FeCH, which is known to be quite complex as zinc, even at low concentrations, inhibits release of product from the enzyme (Davidson et al., 2009). To clarify this question a detail enzymological study is required.

We conclude that this study, based on a combined analysis of several *Synechocystis* strains and purified FeCHs, has demonstrated both regulatory and structural roles for the FeCH CAB domain and, unexpectedly, revealed a critical role of the region II for the catalytic function of the plastid-type FeCH enzyme. In addition, as the mitochondrial FeCH is known to be active only as a homodimer (Ohgari et al., 2005; Grzybowska et al., 2002), the *Synechocystis* FeCH can be described as a new type of dimeric FeCH since the deletion of the C-terminal CAB domain, responsible for dimerization, did not abolish catalytic activity of the resulting monomeric enzyme.
Materials and methods

Growth conditions

If not stated otherwise *Synechocystis* strains were grown photoautotrophically in liquid BG-11 medium (Rippka et al., 1979) supplemented by 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] at 30°C and 40 μmol of photons m\(^{-2}\) s\(^{-1}\) (normal light), on the rotary shaker. To induce expression of tagged FeCHs from the *petJ* promoter (see below), *FLAG-hemH*/*ΔhemH*, *FLAG-ΔH347* and *FLAG-ΔH324* strains were grown in BG-11 medium lacking copper in the trace metal mix.

Construction of *Synechocystis* mutants

Construction of the WT *zeo* strain and the ΔH324 mutant has been described in Sobotka et al., (2008a). Essentially the same approach was adopted for the preparation of ΔH347 mutant with stop codon in the *hemH* gene at amino acid position 347 (see Table S1 for all oligonucleotides used for strain preparation).

To obtain the *FLAG-hemH* strain expressing 3xFLAG-tagged FeCH (FLAG-FeCH), the coding sequence of the *hemH* gene (locus slr0839) encoding the FeCH was subcloned into the pDrive vector (Qiagen, Hilden, Germany). Subsequently, two complementary oligonucleotides encoding the 3xFLAG peptide (Sigma-Aldrich) were hybridized, thus creating *Nde*I-compatible overhangs, and then ligated into the *Nde*I site of the pDrive-*hemH* construct, thereby eliminating the second *Nde*I site. Using *Nde*I and *Bgl*II restriction enzymes (sites were introduced by the primers; Table S1), a DNA fragment encoding the FLAG-FeCH was excised and then ligated into pSK9 vector for chromosomal integration under the control of the copper dependent *petJ* promoter (Tous et al., 2001). The plasmid was transformed into *Synechocystis* WT and transformants were selected on BG-11 agar plates containing 7 μg ml\(^{-1}\) chloramphenicol.

In order to inactivate the entire FeCH gene and obtain thus a strain expressing the FLAG-FeCH as only FeCH enzyme in the cell (*FLAG-hemH*/*ΔhemH* strain) original *hemH* gene in the *FLAG-hemH* strain was replaced by erythromycin resistance cassette using PCR-mediated insertion (Lee et al., 2004; Sobotka et al., 2008a). To prepare the *FLAG-ΔH347* and *FLAG-ΔH324* strains expressing truncated FeCHs, modified *hemH* genes containing stop codons were amplified from the ΔH347 and ΔH324 mutants and cloned into the pSK9 plasmid as described above.

The *Synechocystis* His-C-tn strain expresses the 63-residue-long C-terminal fragment of *Synechocystis* FeCH as a His\(_{6}\)-tagged protein (His-C-tn) under the *psbAII* promoter. To prepare this strain, the *hemH* gene fragment was amplified by PCR using gene specific primers with artificial restriction sites for *Nde*I and *Bgl*II
and containing six histidine codons (CAC) in the forward primer (Table S1). After restriction, the PCR fragment was cloned into *NdeI* and *BamHI* sites of the pSBA plasmid containing the upstream and downstream regions of the *Synechocystis psbAII* gene (Lagarde et al., 2000). The plasmid was transformed into *Synechocystis* psbAII-KS strain as described previously (Lagarde et al., 2000).

**Fractionation of *Synechocystis* cells**

50 ml of cells (OD$_{750}$ ~ 0.5) were washed and re-suspended in the buffer A containing 20 mM Hepes pH 7.4, 10 mM MgCl$_2$, 5 mM CaCl$_2$ and 20% glycerol. The cell suspension was mixed with glass beads and broken in a MiniBeadBeater and the resulted homogenate centrifuged at 50,000 g for 30 min at 4°C. The supernatant containing soluble proteins was transferred to a new tube. Pelleted membranes were two times washed in buffer A, resuspended and solubilized in the same buffer containing 1% dodecyl-β-maltoside and unbroken cells and unsolubilized material discarded by centrifugation.

**Western blot and immunoblotting**

Unless otherwise stated, proteins were denatured by 2% SDS and 1% DTT for 30 min at room temperature and separated in a denaturing 12% SDS-polyacrylamide gel. Proteins separated in the gel were transferred onto a PVDF membrane. The membrane was incubated with specific primary antibodies and then with secondary antibody conjugated to horseradish peroxidase (Sigma, Germany). The anti-FeCH was raised in rabbits against recombinant *Synechocystis* GST-FeCH expressed in *E. coli*. The anti-FeCH C-terminus antibody (anti-C-tn) was raised in rabbits against residues 332-347 of the *Synechocystis* FeCH. The antibodies against *Synechocystis* Mg chelatase subunits, Gun4 and MgPMT were raised in rabbits using recombinant proteins prepared in *E. coli*.

**Affinity purification of 3xFLAG-tagged FeCHs from *Synechocystis***

2 L of FLAG-hemH/ΔhemH and FLAG-ΔH347 cells and 4 L of FLAG-ΔH324 cells harvested at OD$_{750}$ 0.7-0.8 were washed and re-suspended in buffer A (see above) containing protease inhibitors (complete protease inhibitor cocktail, Roche). The cell suspension was mixed with glass beads and broken in a Mini-Beadbeater-16 (BioSpec, USA) using 6 x 1 min cycles. Membranes were pelleted by centrifugation at 50,000 g for 20 min, 4°C, washed in buffer A and resuspended in 10 ml of the same buffer containing 1% dodecyl-β-maltoside. Membranes were solubilized by gentle mixing for 30 min at 10 °C and unbroken cells were discarded by centrifugation. Proteins were loaded onto a column containing ~100 µl of anti-FLAG M2 affinity gel (Sigma, Germany) and then washed with 15 ml of buffer A containing 0.04% dodecyl-β-
maltoside (buffer A-DDM). The FLAG-tagged FeCH was eluted into the buffer A-DDM by incubation of the M2 affinity gel with 3xFLAG peptide (100 μg/ml) for 30 min.

**Affinity purification of the His-tagged C-tn protein from Synechocystis**

For purification of the C-terminal fragment of *Synechocystis* FeCH (His-C-tn protein), membranes from 250 ml of cells (OD750 ~ 0.7-0.8) were washed and re-suspended in the buffer B (20 mM Hepes pH 7.7, 10 mM MgCl2, 5 mM CaCl2, 0.1 M NaCl and 10 % glycerol) containing protease inhibitor (complete protease inhibitor cocktail, Roche). Cells were broken and membrane proteins were prepared by the same way as already described for the purification of FLAG-FeCHs. Proteins were loaded onto a column containing ~75 μl of His-select resin (Sigma, Germany) charged with Ni2+ and pre-equilibrated with buffer B containing 0.04% dodecyl-β-maltoside (buffer B-DDM). To remove any loosely bound contaminants, the column was first washed with 8 ml of buffer B-DDM, and than successively with 1 ml of buffer B-DDM containing 10 mM, 20 mM and 30 mM imidazole. The His-C-tn protein was eluted with buffer B-DDM containing 150 mM imidazole.

**FeCH activity assay**

FeCH activity was monitored spectrofluorometrically at 35°C by directly recording the rate of zinc-PP IX formation using a Spectronic Unicam series 2 spectrofluorometer. The reaction mixture (1.5-ml final volume) contained 100 mM Tris-HCl (pH 8.0), 0.03 % Tween 80, 5 μM PPIX and 1 μM ZnSO4. The measurement of FeCH activity in the cell membrane fraction was initiated by adding of proteins in amount corresponding to 0.3 ml of cells at OD750 = 1. This value was calculated from the Chl content in the analyzed sample and Chl level per OD750 of the particular strain.

**Quantification of chlorophyll and chlorophyll precursors**

For Chl quantification, pigments were extracted from cell pellets (5 ml, OD750 ~ 0.4) with 100% methanol and Chl content was measured spectrophotometrically (Porra et al., 1989).

For quantitative determination of Chl precursors in the cells, 75 ml culture at OD750= 0.35-0.4 was filtered through 4 μm cellulose filter to remove all precipitated pigments in growth media and harvested. Pigments were extracted by 1 ml of methanol/0.2 % NH4OH using a MiniBeadBeater with 2 breaking cycles. Subsequently, the sample was centrifuged, and the supernatant containing extracted pigments was collected. The pellet was then extracted again by 0.3 ml of methanol/0.2 % NH4OH with one breaking cycle and combined supernatants mixed with 150 μl of 1M NaCl. This solution was extracted two times by 400 μl of
hexane to remove Chl and β-carotene, concentrated to 750 μl on a vacuum evaporator and extracted two times by 400 μl of petroleum ether (boiling range 45-60°C) to remove zeaxanthin. Remaining solution was evaporated to dry on a vacuum evaporator. Pigments were then resuspended in 140 μl of methanol/0.2 % NH₄OH, mixed with 60 μl of water and precipitated myxoxanthophylls discarded by centrifugation. Samples were immediately separated by HPLC (Agilent-1200, Agilent, USA) on a RP column (Nova-Pak C18, 4 μm particle size, 3.9 x 150 mm; Waters) using 30 % methanol in 0.5 M ammonium acetate and 100% methanol as solvent A and B, respectively. Porphyrins were eluted with a linear gradient of solvent B (65 % to 75% in 30 min) at a flow rate of 0.9 mL min⁻¹ at 40°C. HPLC fractions containing MgP (retention time ~6.5 min), Chlide (~8.5 min), PChlide (~11.5 min), MgPME (~13 min) and PP IX (~ 15 min) were collected and concentrations of the corresponding compounds determined fluorometrically using a Spectronic Unicam series 2 spectrofluorometer.

Quantification of hemes and determination of ALA-synthesizing capacity
For quantification of heme B (protoheme) a sample of cell debris, already extracted by methanol/0.2 % NH₄OH for quantification of Chl precursors, was extracted further by 1 ml of acetone-water-HCl (90:8:2). After centrifugation the solution was evaporated to dry on a vacuum evaporator, resuspended in 100 μl of acetone and immediately separated by HPLC at Nova-Pak C18 column (4 μm particle size, 3.9 x 150 mm; Waters) using linear gradient from 50 % to 80 % of solvent B (40 % acetone in methanol) in solvent A (30 % methanol in 0.5 M ammonium acetate) in 30 min at a flow rate of 1 mL min⁻¹ at 40°C. Heme B was detected by diode array detector (Agilent-1200, Agilent, USA) and quantified using authentic hemin standard (Sigma, Germany).

To determine ALA-synthesizing capacity a total of 100 ml of Synechocystis cells at an OD₇₅₀ of 0.4 were supplemented with 5 mM glucose and with 15 mM levulinic acid/KOH pH 7.5 to enhance ALA formation but to inhibit its condensation into porphobilinogen. After 4 h cells were harvested, resuspended in 0.3 ml of double-distilled H₂O, and mixed with 20 μl of 50% trichloroacetic acid. Precipitated proteins were discarded by centrifugation and the supernatant was adjusted to pH 6.7 by 90 μl of 0.5 M Na₃PO₄. The supernatant was then mixed with 12 μl of ethylacetoacetate, boiled for 15 min at 100°C, cooled on ice and cleared by centrifugation. A total of 450 μl of modified Ehrlich’s reagent was added to the supernatant and content of ALA was determined by absorption at 553 nm (Mauzerall and Granick, 1956) on Shimadzu 2000 spectrophotometer.

Native electrophoresis and size-exclusion chromatography
Nondenaturing PAGE was performed with the pre-cast 4-16% NativePAGE Bis-Tris Gel (Invitrogen) at 10 V/cm at 4°C using XCell SureLock cell (Invitrogen). Cathode buffer contains 0.25 mM Tricine, 7.5 mM Bis-Tris/HCl, pH 7.0, 0.05% sodium deoxycholate, 0.02 % dodecyl-β-maltoside; anode buffer contains 0.25 mM Bis-Tris/HCl, pH 7.0. ~0.25 μg of protein in the buffer A-DDM was loaded for each sample and, after the electrophoresis, the gel was stained with Coomassie brilliant blue. Gel filtration chromatography was carried out on the BioSep SEC-S3000 300 x 7.80 mm column (Phenomenex, USA) connected to a photodiode array detector Agilent-1200 (Agilent, USA). The column was equilibrated with buffer A containing 0.1% dodecyl-β-maltoside. The flow rate was 0.15 ml/min.

Supplemental Data

The following materials are available in the online version of this article:

Supplemental Table I. Oligonucleotides used for construction of *Synechocystis* mutant strains.

Supplemental Figure 1. Amino acid alignment among the C-terminal end of FeCH proteins from evolutionary distant Chl-producing organisms.

Supplemental Figure 2. Whole cell absorbance spectra of *Synechocystis* strains grown under different light regimes.

Supplemental Figure 3. Crystal structure of the spinach LHCII light harvesting complex with the highlighted stromal loop connecting the second and third helices.

Acknowledgements

The authors thank Eva Prachova for her technical assistance and Ulf Dühring for preparation of the pSK9-FLAG-hemH construct. The vector psk9 was a kind gift of Prof. S. Zinchenko (Moscow State University).

References


Vasilukaya Z, Oster U, Beck CF (2005) Mg-protoporphyrin IX and heme control HEMA, the gene encoding the first specific step of tetrapyrrole biosynthesis, in Chlamydomonas reinhardtii. Eukaryot Cell 4: 1620-1628


Table I. Growth rate and chlorophyll content of studied *Synechocystis* strains under different light regimes

<table>
<thead>
<tr>
<th>Light intensity, μmol photons m⁻² s⁻¹</th>
<th>5</th>
<th>40</th>
<th>200</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Doubling time (h) (d.t.)</td>
<td>Chlorophyll (Chl) a</td>
<td>d.t.</td>
<td>Chl</td>
</tr>
<tr>
<td>WTzego</td>
<td>58</td>
<td>5.5 ± 0.1</td>
<td>13.7</td>
<td>5.1 ± 0.2</td>
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<tr>
<td>ΔH347</td>
<td>61</td>
<td>5.5 ± 0.15</td>
<td>14.6</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>ΔH324</td>
<td>56</td>
<td>5.8 ± 0.2</td>
<td>20.1</td>
<td>4.9 ± 0.3</td>
</tr>
</tbody>
</table>

a μg . ml⁻¹ OD₇₅₀⁻¹
b no growth (strain did not grow under these conditions)

FIGURE LEGENDS

Figure 1. A) Schematic presentation of the *Synechocystis* FeCH (coded by hemH gene) with catalytic and C-terminal CAB domains connected by the region II. B) Strains with truncated FeCH used in this study; WTzego and ΔH324 were already described in (Sobotka et al., 2008a). The ΔhemH deletion was combined with the FLAG-hemH background (see below) to construct the FLAG-hemH/ΔhemH strain that expressed the tagged version as the only FeCH enzyme in the cell. C) Constructs used to express full-length and truncated versions of FeCH fused to a protein tag in *Synechocystis*. 3xFLAG-tagged FeCHs were expressed under *Synechocystis* petJ promoter; His-C-tn strain expresses the 63-residue-long C-terminal fragment of *Synechocystis* FeCH as a small His±tagged protein (His-C-tn protein) under the *Synechocystis* psbAII promoter.

Figure 2. Analysis of pigments in *Synechocystis* mutant strains. A) Quantification of the relative contents of PP₅X, MgP, MgPME, PChlide and Chlide in *Synechocystis* cells growing at normal light (40 μmol of photons m⁻² s⁻¹) and harvested at OD₇₅₀ = 0.35 - 0.4. Chl precursors were extracted with methanol/ 0.2 % NH₄OH and quantified by a combination of HPLC and spectrofluorometry. Values in the table represent mean ±SD from three independent measurements; asterisk indicates a significant difference tested using a paired t test (p = 0.05). B) ALA synthesizing capacity as determined in 100 ml of cell suspension with OD₇₅₀ = 0.4 treated for
4 h with levulinic acid to inhibit ALA condensation into porphobilinogen. Representative absorption spectra of developed assay as obtained for each strain together with 5 μM standard of ALA are also presented. C) Quantification of noncovalently bound heme B (protoheme) in cells growing at normal light. Heme was extracted by 90% acetone/2% HCl and separated and quantified using HPLC. Error bars and the t test of statistical significance are as in A. D) Quantification of the relative contents of chlorophyll precursors in *Synechocystis* cells growing for ~40 hours at high light (200 μmol of photons m⁻² s⁻¹) and harvested at OD₇₅₀ = 0.35 - 0.4. Obtained values from three measurements were compared to values for the control WTₙzteo strain growing at normal light; dashed line indicates 100% as in (A). Asterisk indicates statistically significant difference in precursor levels between WTₙzteo and ΔH347 at high light as tested using a paired t test (p = 0.05).

**Figure 3.** Accumulation of enzymes of tetrapyrrole biosynthesis and *in vitro* activity of FeCH in *Synechocystis* strains. A) Membrane and soluble protein fractions were prepared as described in Material and Methods, separated by SDS electrophoresis and blotted to a membrane. Enzymes were detected by specific antibodies. The amount of proteins loaded for 100% of each sample corresponded to 150 μl of cells at OD₇₅₀ = 1. ChlI, ChlD and ChlH are subunits of the Mg-chelatase (Jensen et al., 1996) and the Gun4 is another protein required for activity of the Mg-chelatase (Larkin et al., 2003; Sobotka et al., 2008b). MgPMT = Mg-protoporphyrin methyl transferase; POR = light dependent protochlorophyllide oxidoreductase. Below are blotted proteins stained with Ponceau red shown as a loading control; the peripheral subunit of ATPase is marked by an asterisk. B) *In vitro* FeCH activity in membranes as determined by a continuous spectrofluorometric assay. Activities were monitored as an increase in fluorescence of zinc-PP₉ using excitation and emission wavelengths of 420 and 590 nm, respectively.

**Figure 4.** Full-length and truncated FeCH enzymes purified from *Synechocystis*. A) FLAG-tagged full-length and truncated FeCHs were purified under native conditions on the anti-FLAG affinity gel, separated by SDS-electrophoresis and stained by Coomassie blue; ~ 0.5 μg of protein was loaded per each line. B) *In vitro* activities of purified FeCH enzymes as determined by continuous spectrofluorometric assay. Activities were monitored as an increase in fluorescence of zinc-PP₉ using excitation and emission wavelengths of 420 and 590 nm, respectively; 1x means that ~ 0.1 μg of enzyme was assayed. Relative activities of truncated enzymes in comparison to the full-length FeCH (100%) are also indicated.
Figure 5. Aggregation state of purified FeCHs. A) ~ 0.5 μg of the purified WT and truncated FeCH were separated by non-denaturing electrophoresis and stained by Coomassie blue; M and D letters indicate the positions of FeCH monomer and dimer, respectively. B) Gel filtration of the FLAG-FeCH and truncated FeCH enzymes on the BioSep SEC-S3000 column; ~ 1 μg of each protein was separated. Positions of standards are shown at the top of the graph. Standards: AM = β-amylase (200 KDa); ADH = alcohol dehydrogenase (150 KDa); BSA = bovine serum albumin (66 KDa); CA = carbonic anhydrase (29 KDa). C) FeCH activity in 0.5 min fractions as eluted from the gel filtration column during separation of purified FeCH enzymes (described in B). Whole volume of each fraction (75 μl) was assayed immediately after elution in in vitro FeCH assay. Obtained values for truncated enzymes were multiplied 10-fold to compare activity profiles.

Figure 6. A) Purification of the FLAG-FeCH in a complex with non-tagged FeCH. FLAG-FeCH was purified from the FLAG-hemH strain possessing both tagged and non-tagged form of this enzyme; purification was carried out under native condition on the anti-FLAG affinity gel as described in Materials and Methods. Each purification step was separated on SDS-electrophoresis, blotted and both FeCH forms detected by anti-FeCH antibody; eluted proteins separated on SDS-electrophoresis were also stained by Coomassie blue. The position of the FLAG-FeCH was resolved by anti-FLAG antibody. B) Purification of the FeCH C-terminal fragment and its co-purification with the full-length and the ΔH347-FeCH lacking the CAB domain. The 63-residue-long C-terminal fragment of Synechocystis FeCH was expressed as a His-tagged protein (His-C-tn) in the WT and in the ΔH347 backgrounds and purified from both strains using Ni²⁺ affinity chromatography. As the polyclonal anti-FeCH antibody recognized the C terminal parts of the protein only weakly, the His-C-tn was detected using an antibody raised against a synthetic peptide corresponding to region II of the Synechocystis FeCH (amino acids 333-348). The amount of membrane protein loaded for each sample corresponded to 150 μl of cells at OD₇₅₀ = 1, 1/50 of volume of each washing step (20 μl from 1 ml) and 1/25 of the total elution volume. The imidazole elution is described in Materials and Methods.