Complex formation between protoporphyrinogen IX oxidase and ferrochelatase during haem biosynthesis in *Thermosynechococcus elongatus*

Ava Masoumi,¹ Ilka Ursula Heinemann,¹ Manfred Rohde,² Michael Koch,³ Martina Jahn¹ and Dieter Jahn¹

Correspondence
Dieter Jahn
djahn@tu-bs.de

¹Institute of Microbiology, Technical University of Braunschweig, Spielmannstr. 7, D-38106 Braunschweig, Germany

²Division of Microbiology, Helmholtz Centre for Infection Research (HZI), Inhoffenstr. 7, D-38124 Braunschweig, Germany

³Laboratoire de Biologie et de Génomique Structurales, IGBMC, Parc d'Innovation, 1 rue Laurent Fries, BP 10142, F-67404 Illkirch Cedex, France

During haem and chlorophyll biosynthesis, flavin-dependent protoporphyrinogen IX oxidase catalyses the six-electron oxidation of protoporphyrinogen IX to form protoporphyrin IX. In the following step, iron is inserted into protoporphyrin IX by ferrochelatase. Based on the solved crystal structures of these enzymes, an *in silico* model for a complex between these two enzymes was proposed to protect the highly photoreactive intermediate protoporphyrin IX. The existence of this complex was verified by two independent techniques. First, co-immunoprecipitation experiments using antibodies directed against recombinantly produced and purified *Thermosynechococcus elongatus* protoporphyrinogen IX oxidase and ferrochelatase demonstrated their physical interaction. Secondly, protein complex formation was visualized by *in vivo* immunogold labelling and electron microscopy with *T. elongatus* cells. Finally, oxygen-dependent coproporphyrinogen III oxidase, which catalyses the formation of protoporphyrinogen IX, was not found to be part of this complex when analysed with the same methodology.

Received 20 March 2008
Revised 7 August 2008
Accepted 19 August 2008

INTRODUCTION

Tetrapyrroles participate in fundamental biological processes. They are essential components of the metabolism of practically all organisms on earth as they play a central role in electron-transfer-dependent energy-generating processes such as photosynthesis and respiration. They also function as prosthetic groups for a variety of enzymes. The most commonly found tetrapyrroles in nature are chlorophylls and bacteriochlorophylls. These magnesium-containing tetrapyrroles are essential for light harvesting and energy transduction during photosynthesis. Iron-chelating haems represent the prosthetic group of haemoglobin, where they are responsible for the transport of oxygen and carbon dioxide in the blood. They are also integral parts of the electron-transfer chains for the generation of respiratory energy (Heinemann *et al.*, 2008).

The structural core of tetrapyrroles implies a highly conserved biosynthetic pathway. The formation of protohaem comprises at least seven enzymic reactions starting

from the common precursor molecule δ -aminolaevulinic acid (ALA) (Frankenberg *et al.*, 2003). The three terminal steps of the haem biosynthetic pathway are executed by coproporphyrinogen III oxidase (CPO, EC 1.3.3.3), protoporphyrinogen IX oxidase (PPO, EC 1.3.3.4) and ferrochelatase (FC, EC 4.99.1.1) (Fig. 1A). CPO oxidatively decarboxylates the propionate side chains on rings A and B of coproporphyrinogen III (coprogen) to the corresponding vinyl groups (Dailey, 2002). The oxygen-dependent enzyme lacks obvious cofactors (Labbe, 1997; Medlock & Dailey, 1996). The crystal structures of the human and yeast enzymes have been solved (Phillips *et al.*, 2003, 2004). PPO catalyses the aromatization of protoporphyrinogen IX (protopogen), producing protoporphyrin IX (proto). The six-electron oxidation of proto is accomplished using a flavin cofactor. In eukaryotes PPO is associated with the outer surface of the inner mitochondrial membrane (Deybach *et al.*, 1985), while in plants the enzyme is additionally located in chloroplasts (Jacobs & Jacobs, 1987). When PPO is inhibited, protogen is exported into the cytoplasm and oxidized to proto, where it accumulates with detrimental consequences upon light exposure. The ultimate step in haem biosynthesis is catalysed by FC, with the insertion of

Abbreviations: coprogen, coproporphyrinogen III; CPO, coproporphyrinogen III oxidase; FC, ferrochelatase; PPO, protoporphyrinogen IX oxidase; proto, protoporphyrin IX; protogen, protoporphyrinogen IX.

Fig. 1. Late steps of haem biosynthesis and model for a complex of the enzymes involved. (A) The oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX catalysed by the enzyme coproporphyrinogen III oxidase (CPO). Protoporphyrinogen IX oxidase (PPO) catalyses the six-electron oxidation of non-fluorescent protoporphyrinogen IX to protoporphyrin IX. The chelation of ferrous iron into protoporphyrin IX via ferrochelatase (FC) forms protohaem. (B) A structural model of the PPO–FC complex based on the crystal structures of tobacco PPO and human FC. The two horizontal lines mark the membrane; inserted are PPO (yellow) and FC (red). From left to right the process of protoporphyrinogen IX release after production in PPO through the open cavity into FC and the further iron insertion and release of protohaem into the membrane are shown. According to our model the complex has to disassemble after protoporphyrinogen IX has been transferred into FC in order to release the product protohaem into the membrane. The model outlined served as a working hypothesis for our experimental investigation.

ferrous iron into the porphyrin macrocycle to form protohaem (Porra & Jones, 1963).

In mammals, the enzymes of haem biosynthesis are associated with the inner mitochondrial membrane (Harbin & Dailey, 1985); their localization in plants is less well characterized (Lister *et al.*, 2001). There is conflicting evidence regarding the inner-membrane localization of PPO and FC. Two isomers of PPO and FC have been reported to be targeted to either chloroplasts or mitochondria, where they are associated with membranes (Lermontova *et al.*, 1997; Suzuki *et al.*, 2002; Watanabe *et al.*, 2001).

Kinetic studies using solubilized membrane-bound and vesicle-reconstituted enzymes from mouse mitochondria suggested possible complex formation between PPO and FC in order to protect this sensitive biosynthetic intermediate proto (Ferreira *et al.*, 1988). Based on the crystal structures of tobacco PPO (Koch *et al.*, 2004) and human FC (Wu *et al.*, 2001), Koch and coworkers suggested a tight complex between these two enzymes. CPO was also

associated with the *in silico* model complex. The proposed model allows for metabolic channelling via an overlap of the active-site channel openings of tobacco PPO and human FC. Here, we experimentally investigated the existence of protein complexes between CPO, PPO and FC in the phototrophic cyanobacterium *Thermosynechococcus elongatus* using co-immunoprecipitation experiments and immunoelectron microscopy.

METHODS

Chemicals. Proto and coproporphyrin III were purchased from Sigma-Aldrich. Tween 80 was obtained from Carl Roth GmbH. The remaining chemicals were purchased from Sigma-Aldrich unless specified otherwise. Protino Ni-IDA resin was from Macherey Nagel. All chemicals were reagent grade and all solutions were made with molecular biology water (Sigma).

Employed bacterial strains and constructed plasmids. *Escherichia coli* strain DH10b [F^- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *endA1* *ara* Δ 139 Δ (*ara*, *leu*)7697 *galU* *galK* λ^- *rpsL* (Str^r) *nupG*] was used for cloning purposes. Strain

BL21(DE3)RIL [$F^-ompT hsdS(r_B^- m_B^-) dcm^+ Tet^r gal \Delta(DE3) endA Hte(argU ileY leuW Cam^r)$] was the host for gene overexpression and protein production. *Thermosynechococcus elongatus* BP1 cells were cultivated in a 5 l fermenter at 55 °C under continuous illumination from fluorescent white lamps ($\sim 80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The cells were grown in D-medium supplemented with micronutrients in a CO₂-enriched (10%) atmosphere to an OD₇₀₀ of 2.0 as previously described (Schulze *et al.*, 2006; Zouni *et al.*, 2001). After harvesting by centrifugation, 1.4 g wet cell mass (1 culture)⁻¹ was obtained.

A 1059 bp fragment encoding all 352 amino acid residues of *T. elongatus* CPO was produced by PCR using primers 5'-GCAAGCGC-ATATGGACACTA-3' and 5'-GGTTAAGCTTGACTGGCCAATT-GACC-3' with *T. elongatus* BP1 genomic DNA as template. The resulting fragment was digested with *NdeI* and *HindIII* and ligated into appropriately cut pET22b (Novagen) to generate pET22bcpo. The construction of the *T. elongatus* PPO-overproducing *E. coli* strain is previously described (Layer *et al.*, 2005). A DNA fragment encoding the 392 amino acid residues of *T. elongatus* FC was generated by PCR using primers 5'-CCGGAATTCGGGTGATTTGGGAATGGCGA-GCC-3' and 5'-CCGCTCGAGCGGACTGATGAGTTCAATCATC-AAC-3' with *T. elongatus* BP1 genomic DNA as template. The resulting 1179 bp fragment was digested with *EcoRI* and *XhoI* and ligated with the appropriately digested vector pET32a (Novagen) to yield pET32afc. Oligonucleotides were purchased from Biomers.net GmbH.

Recombinant production and purification of active *T. elongatus* CPO, PPO and FC. All three proteins were recombinantly produced in *E. coli* as His-tag fusion proteins and purified to apparent homogeneity using affinity chromatography.

Recombinant *T. elongatus* CPO was produced with *E. coli* BL21(DE3)RIL cells (Novagen) carrying the vector pET22bcpo, supplemented with 100 μg ampicillin ml⁻¹ and 34 μg chloramphenicol ml⁻¹. Protein production was induced at OD₅₉₅ 0.6 with the addition of 500 μM IPTG. After induction, cells were further grown at 37 °C for 4 h, harvested by centrifugation and washed in a buffer containing 20 mM Tris (pH 8.0), 350 mM NaCl, 10 mM imidazole, 0.2% Triton X-100. Subsequently, the cells were broken by sonication (Bandelin HD 2070; 0.5 s sound, 0.5 s pause, MS73 tip, with 50% amplitude) and cleared by centrifugation at 150 000 g at 4 °C for 45 min. The cell-free extract obtained was further filtered through a 0.2 μm sterile filter. Purification of recombinant *T. elongatus* CPO was performed using Ni-NTA Sepharose chromatography (GE Healthcare). Solubilized proteins were loaded onto a Ni-NTA column and bound proteins were eluted with 200 mM imidazole in chromatography buffer.

Recombinant *T. elongatus* PPO was produced with *E. coli* BL21(DE3)RIL cells carrying vector pET32appo. Protein production was induced at OD₅₉₅ 0.6 with the addition of 500 μM IPTG and 5 μg riboflavin l⁻¹. After induction, cells were grown at 25 °C overnight. The cells then were washed three times in lysis buffer [50 mM Tris (pH 8.0), 10 mM MgCl₂, 350 mM NaCl, 0.5% (w/v) Triton X-100, 0.5% (w/v) NP-40, 10 U benzonase ml⁻¹, 10 μg RNase A ml⁻¹, 0.05% (w/v) Tween 80, 8 mg lysozyme ml⁻¹]. Cells were broken by sonication (Bandelin HD 2070; 0.5 s sound, 0.5 s pause, MS73 tip, 50% amplitude) and cleared by centrifugation at 150 000 g at 4 °C for 40 min. Purification of recombinant *T. elongatus* PPO was performed using the MagneHis Protein Purification System (Promega) according to the manufacturer's instructions. After purification, the protein was dialysed against 20 mM Tris (pH 8.0), 50 mM NaCl and 0.1% (v/v) Triton X-100 and stored at 4 °C. Approximately 20 mg purified PPO protein per 1 l shake-flask culture was yielded. MALDI-TOF mass spectrometry confirmed purified PPO identity (Layer *et al.*, 2005).

Recombinant *T. elongatus* FC was produced with *E. coli* BL21(DE3)RIL cells carrying vector pET32afc. Protein production was induced at OD₅₉₅ 0.6 with the addition of 500 μM IPTG, 100 μg ampicillin ml⁻¹ and 34 μg chloramphenicol ml⁻¹. Cells were further grown at 37 °C for 2.5 h. Growth medium was exchanged with fresh LB medium including 100 μg ampicillin ml⁻¹, 34 μg chloramphenicol ml⁻¹ and 10 μg tetracycline ml⁻¹. Further cultivation was performed at 25 °C overnight. Cells were harvested, disrupted via sonication as described above and cell debris was removed by centrifugation at 12 000 g at 4 °C for 30 min. Cells were suspended in 50 mM Tris (pH 8.0), 150 mM KCl and 0.2% Triton X-100 and subjected to three rounds of sonication and centrifugation as described above. Solubilized proteins containing recombinant *T. elongatus* FC were loaded onto a cobalt-Sepharose column (Chelating Sepharose Fast Flow; GE Healthcare). Bound proteins were eluted with 140 mM imidazole in chromatography buffer and dialysed against assay buffer [50 mM MES-buffer pH 6.0, 1% (v/v) Triton X-100, 20 mM EDTA] for further analyses. The use of cobalt-Sepharose instead of Ni-NTA significantly enhanced FC solubility during chromatography and as a consequence improved the overall yield of the purified recombinant FC.

Catalytic activity for all three recombinant proteins was verified for CPO activity testing as follows. Coprophen was generated as described previously (Layer *et al.*, 2002). Proto was detected via its typical fluorescence at 633 nm (Breckau *et al.*, 2003; Heinemann *et al.*, 2007). The oxygen-dependent PPO assay was originally developed by Brenner and Bloomer for the mammalian enzyme and modified as previously described (Brenner & Bloomer, 1980; Heinemann *et al.*, 2007). Specific FC activity was monitored by recording the decrease of proto, which was detected fluorimetrically at 633 nm (data not shown).

Co-immunoprecipitation assays using whole-cell *T. elongatus* extracts. Approximately 1 mg purified recombinant CPO, PPO and FC was used for polyclonal antibody generation in rabbits by Eurogentec. In order to solubilize hydrophobic, membrane-bound or associated proteins, 5.5 g *T. elongatus* cells was suspended in 6 ml HEPES buffer (pH 8.0) containing 5 mM MgCl₂, 300 mM NaCl, 0.5% Triton X-100 and 1% n-dodecyl- β -D-maltoside. Cells were disrupted by sonication (Bandelin HD 2070, 0.5 s sound, 0.5 s pause, 70% amplitude) using a sonotrode (MS73 tip, Bandelin). The disrupted cells were treated in a Mikro-Dismembrator (Braun Biotech) for 30 min. Cell debris was removed by centrifugation at 4000 g at 4 °C for 15 min. The detergent-containing supernatant was again subjected to the dismembrator treatment for 40 min. Cell debris was removed by ultracentrifugation. After incubation at 4 °C for 60 min, cell debris was removed by centrifugation at 4 °C and 100 000 g for 45 min. About 300 μl Protein A-Sepharose CL 4B was pre-equilibrated with cell suspension buffer under gentle shaking at 4 °C overnight according to the manufacturer's instructions (Seize X Protein A Immunoprecipitation Kit; Pierce). Then 2.5 μl of either anti-CPO, anti-PPO or anti-FC antibodies at dilution of 1/100 000 was added and adsorbed to the Protein A-Sepharose at 4 °C for 12 h. About 500 μl of the clear *T. elongatus* cell extract (the equivalent of 500 mg wet cells) was incubated with the immobilized antibodies at 4 °C for 1–2 h. The immuno-adsorbant was recovered by centrifugation at 10 000 g for 1 min and washed four times by resuspension in a buffer containing 150 mM NaCl, 8 mM Na₃PO₄, 2 mM K₃PO₄ (pH 7.4), 10 mM KCl and subsequent centrifugation. Unfortunately, precipitated antibody heavy chains resulted in a diffuse protein pattern at a molecular mass of 50 000 Da in SDS-PAGE and subsequent Western blot analyses. Thus, they prevented the detection of signals for immunoprecipitated PPO with a calculated molecular mass of 50 199 Da and anti-PPO (data not shown). This problem was solved by using the Seize X Protein A Immunoprecipitation Kit (Pierce), which successfully removed most of the antibody heavy

chains. Protein samples eluted from Protein A-Sepharose were heated for 5 min at 95 °C and subjected to 8 % SDS-PAGE using standard techniques. The electrophoretically separated proteins were transferred onto PVDF membranes (Millipore) using a Trans-Blot apparatus (semi-dry transfer cell, Bio-Rad) according to the manufacturer's instructions. The membrane was incubated with anti-CPO, anti-PPO and anti-FC rabbit antibodies, respectively (1:5000 in PBS with 3 % skim milk powder from Fluka.), washed three times with PBS, and subsequently incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:20 000 in PBS with 3 % skim milk powder) from Pierce. The colorimetric detection of immunoreactive bands was performed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium from Promega according to the manufacturer's instructions.

Double-immunogold labelling. *T. elongatus* cells were fixed in a fixation solution of 0.5 % formaldehyde and 0.2 % glutaraldehyde in cacodylate buffer [100 mM M cacodylate (pH 6.9), 90 mM sucrose, 10 mM MgCl₂, 10 mM CaCl₂], for 1 h on ice. Fixed cells were washed with cacodylate buffer containing 10 mM glycine and embedded into 1.75 % aqueous agar and after solidification cut into small cubes. Samples were then dehydrated following the progressive lowering of temperature (PLT) method. For this purpose 10 % and 30 % ethanol treatments were carried out on ice for 15 min, followed by the 50 % step at -20 °C for 30 min. The subsequent ethanol steps (70, 90 and 100 %) were carried out at -35 °C, each step for 30 min, and the 100 % step was repeated twice. The Lowicryl resin K4M was infiltrated in several steps. First, 1 part K4M/1 part 100 % ethanol was applied overnight, followed by 2 parts K4M/1 part ethanol for 24 h and finally pure resin for 36 h with several changes. The resin was polymerized at -35 °C with UV-light treatment at 366 nm for 1 day and further polymerized at room temperature for another 2 days. Ultrathin sections were cut with a diamond knife and collected onto Formvar-coated 300 mesh nickel grids. Sections were incubated in a 1:25 dilution of Protein A-Sepharose-purified anti-PPO (stock solution 1.9 mg IgG protein ml⁻¹) at 4 °C for overnight. Sections were then washed with PBS and incubated in a 1:200 dilution of protein A/G gold (15 nm in diameter) for 30 min at room temperature. After washing with PBS containing 0.01 % Tween 20, sections were incubated with 100 µg protein A ml⁻¹ for 15 min at room temperature and washed again with PBS. Sections were then incubated with a 1:25 dilution of purified anti-FC (1.9 mg stock solution ml⁻¹) and incubated at room temperature for 3 h. After washing with PBS, sections were incubated with a 1:75 dilution of protein A/G gold (10 nm in diameter) at room temperature for 30 min. After washing with PBS containing 0.01 % Tween 20, sections were washed with TE, distilled water and air-dried. Sections were counterstained with 4 % aqueous uranyl acetate for 3 min and washed with water. After air-drying, samples were examined in a Zeiss EM910 transmission electron microscope with an acceleration voltage of 80 kV. Images were recorded onto negative film (Kodak SO-163). Prints were generated on Ilford Multigrade paper and then scanned and processed using Adobe Photoshop 6.0.

RESULTS

Proposed CPO-PPO-FC complex *in silico* and experimental approaches for its identification

In order to prevent the release of the photoreactive and toxic intermediate proto into cells, a putative protein complex between the last three enzymes in haem biosynthesis was proposed earlier (Koch *et al.*, 2004). Based on the crystal structures of the yeast CPO, tobacco

PPO and human FC, Koch and coworkers first suggested a tetrameric complex between the PPO and FC dimers (Fig. 1B). The part of the complex that is supposed to span the inner mitochondrial membrane has a length of 28 Å (2.8 nm), corresponding well to the assumed 30 Å for the lipidic part of a membrane. One appealing feature of the model is the overlap of the channel openings of PPO and FC that could facilitate substrate channelling between the active sites of the two proteins without solvent exposure. Koch and coworkers additionally proposed that the PPO substrate protogen could be taken up directly from CPO at the periplasmic side of the membrane, most likely with CPO as a third participant in the multienzyme complex.

Here, we sought biochemical evidence for the existence of the protein complex between CPO, PPO and FC in the cyanobacterium *T. elongatus*. We expected potential complexes to be associated with the cytoplasmic side of the thylakoid membrane as the thylakoids of cyanobacteria represent invaginations of the cytoplasmic membrane and the cytoplasmically synthesized enzymes do not carry obvious export signals. Co-immunoprecipitation analysis was combined with electron microscopy based localization of protein complexes in *T. elongatus* cells. These two methods provided independent evidence for formed complexes and their cellular localization. However, the exact positioning of these complexes, i.e. transmembrane or membrane-associated, requires additional biochemical approaches.

Recombinant CPO, PPO and FC production and antibody generation

The proteins of interest were produced and purified to apparent homogeneity as detailed in Methods. All purified recombinant proteins showed specific enzymic activity when tested with their natural substrates. Solutions containing 1 mg purified recombinant CPO, PPO and FC were supplied to Eurogentec for the generation of polyclonal rabbit antibodies. The specificity of antigen detection was evaluated by Western blot analysis. A dilution of 1/100 000 of anti-CPO, anti-PPO and anti-FC specifically detected 100 ng purified recombinant CPO, PPO and FC (data not shown). The results of the following immunoprecipitation experiment showed that the generated antibodies successfully precipitated recombinant CPO, PPO and FC from *E. coli* cell-free extracts and detected them in Western blots (data not shown).

The following strategy was employed for the detection of enzyme complexes in *T. elongatus* whole-cell extracts including solubilized membrane-bound proteins. The protein to be tested for potential binding partners was bound to its specific antibody. This antibody-antigen complex was immobilized on Protein A Sepharose, isolated via centrifugation and washed. In the case of a co-immunoprecipitated interaction partner, the second bound protein was visualized in Western blot experiments using a second antibody directed against it. Co-immunoprecipita-

tion experiments with anti-CPO, anti-PPO and anti-FC antibodies were performed with *T. elongatus* whole-cell extracts. The cells contained the natural amount of all three enzymes since none of the corresponding genes was overexpressed. Two of the complementary co-immunoprecipitation experiments resulted in the precipitation of the postulated PPO–FC complex. Significant amounts of complexed protein were detected with the anti-FC antibodies after immunoprecipitation with anti-PPO antibodies (Fig. 2B, lane 4) and with anti-PPO antibodies after the anti-FC-mediated precipitation (Fig. 2C, lane 6). The control experiments performed with whole-cell extracts with either pre-immune serum or Protein A-Sepharose ruled out non-specific cross-reactivities of the antibodies employed with PPO (Fig. 2C, lanes 2 and 3) and FC (Fig. 2B, lanes 2 and 3), respectively. The removal of the antibody heavy chains from the anti-PPO preparation was necessary to allow detection of the PPO due to almost identical molecular masses (Fig. 2C, lanes 5 and 6). The *in silico* model by Koch and coworkers additionally suggested that CPO could also be a partner of the enzyme complex. Thus, our co-immunoprecipitation results exclude CPO from the stable complex; however, they do not rule out transient interactions. Although anti-CPO antibodies precipitated CPO from *T. elongatus* whole-cell extracts (Fig. 2A, lane 4), no co-immunoprecipitated proteins were observed using anti-FC or anti-PPO antibodies (Fig. 2A, lanes 5 and 6). In summary, these results confirm the existence of a complex of PPO with FC in *T. elongatus* cells.

PPO and FC co-localize in *T. elongatus* cells

The purpose of double-immunogold labelling on ultrathin sections is to differently label the antigens of interest (CPO, PPO and FC) with specific antibodies for each antigen followed by protein A-conjugated immunogold markers of different sizes (10 vs 15 nm) on one ultrathin section. This allows the detection of co-localized antigens. Fig. 3(A) shows a longitudinal section of a *T. elongatus* cell, conventionally embedded in epoxy resin. It shows that the bacterial cytoplasm is mostly occupied by thylakoid membranes, which are most clearly visible only in such parts of the cytoplasm where the ultrathin section is perpendicular to the thylakoid membrane (arrows in Fig. 3A). First, we incubated the ultrathin sections of *T. elongatus* with anti-FC antibodies labelled with small 10 nm gold particles. Next, anti-PPO antibodies decorated with large 15 nm gold particles were applied. Differently sized gold particles less than 25 nm from each other were counted as protein–protein complexes since each of the two applied antibodies can maximally span a distance of approximately 12 nm due to the hinge in the Fab region. As evident from the statistical evaluation of 25 different longitudinal sections of *T. elongatus* and as shown in Fig. 3(B), more than $51.7 \pm 9.1\%$ of the detected anti-FC antibodies were significantly co-localizing with anti-PPO antibodies at room temperature (Fig. 3B, C). In total, for the 25 analysed longitudinal sections 330 out of 640 10 nm gold particle FC (FC-10 nm) molecules were found co-localized with PPO-15 nm molecules. On average, out of 25.6 ± 5.19 FC molecules 12.64 ± 2.32 were co-localized

Fig. 2. Detection of PPO–FC complexes in *T. elongatus* cell-free extract by co-immunoprecipitation analysis. Western blots were performed after separation of proteins by SDS-PAGE. Proteins were detected with anti-CPO (A), anti-FC (B) and anti-PPO (C) (1 : 5000), respectively, as primary antibodies, and with a secondary anti-rabbit antibody (1 : 20 000) coupled to alkaline phosphatase. In each panel, lane 1 shows the proteins of Page Ruler Prestained Protein Ladder. The relative molecular masses ($M_r \times 10^{-3}$) of the marker proteins are given. Lane 2 depicts an incubation of *T. elongatus* cell extract and Protein A-Sepharose. Lane 3 shows *T. elongatus* cell extract treated with pre-immune serum and Protein A-Sepharose (negative control). Lanes 4, 5 and 6 contain *T. elongatus* cell extract, Protein A-Sepharose and antibodies against the proteins given in the table above. The detecting antibodies for each panel are indicated below each panel. ABH, antibody heavy chain; CPO, coproporphyrinogen III oxidase; PPO, protoporphyrinogen IX oxidase; FC, ferrochelatase.

Fig. 3. Complex formation of *T. elongatus* PPO and FC analysed by double-immunogold labelling and electron microscopy. (A) Cross-section of an epoxy resin-embedded *T. elongatus* cell shows the distribution of thylakoid membranes throughout the cell (arrows point to perpendicular cut membranes). (B, C) Double-immunogold labelling of FC (10 nm) and PPO (15 nm); arrow-heads indicate 10 nm gold particles; complete arrows point to 15 nm gold particles. Complexes between FC and PPO are highlighted by circles. (D, E) Double-immunogold labelling of CPO (15 nm) and PPO (10 nm), and CPO (15 nm) with FC (10 nm), respectively. No obvious co-localization was observed.

with PPO on a single analysed section. These observations clearly indicated *in vivo* PPO–FC complex formation. It has to be taken into account that, due to the embedding procedure in low-temperature resin and counter-staining of the sections with uranyl acetate alone, the thylakoid membranes are only partially visible in the ultrathin sections compared to the osmium-fixed conventional embedding method (compare Fig. 3A, B). In cases where the thylakoid membranes were partly visible in conjunction with the gold particles, the PPO–FC complexes within *T. elongatus* were found localized to the cytoplasmic side of the thylakoid membrane. This is in good agreement with the cytoplasmic synthesis of the proteins as well as their missing export signals. The identical experiments were performed with the anti-CPO and either anti-PPO or anti-FC as the second antibody (Fig. 3D, E). In Fig. 3(D, E), less than 5 % of FC and PPO seem to co-localize with CPO. In total numbers for the 25 cut sections, only 26 out of 658 of the CPO-15 nm molecules were co-localized with PPO-10 nm. A similar situation was found for the CPO–FC analysis: in total numbers for the 25 cut sections only 25 out of 632 of the CPO-15 nm molecules were found co-localized with FC-10 nm molecules. For both CPO–PPO and CPO–FC the number of co-localizations was within the standard deviation, and therefore likely to be random. With regard to the size of the proteins, diameter of the section, cut orientation and antigen orientation with over 50 % pairs in double-immunogold labelling, the existence of a complex between PPO and FC seems very likely.

DISCUSSION

The results of both the co-immunoprecipitation and the double-immunogold labelling/electron microscopy analyses experimentally confirm the complex formation between PPO and FC in *T. elongatus*. Both enzymes have been shown to be located on the stromal side of the plant thylakoid membrane (Matringe *et al.*, 1992, 1994). However, this complex most likely is localized at the cytoplasmic side of the bacterial thylakoid membrane. The thylakoid membranes of cyanobacteria are invaginations of the cytoplasmic membrane. The proton gradient of photosynthesis is formed at this membrane as in non-phototrophic bacteria. Consequently, we expect an arrangement of the haem biosynthetic enzymes on the cytoplasmic side of the membrane similar to other non-phototrophic bacteria. This is in good agreement with the missing export signal for all three cyanobacterial enzymes. Nevertheless, the model proposed here based on the crystal structures of eukaryotic enzymes (Koch *et al.*, 2004) was only partly verified. The exact mode of interaction of the complex with the membrane, i.e. transmembrane versus membrane association, remains to be determined. However, amino acid sequence comparisons of *T. elongatus* FC with membrane-associated human FC, soluble *Bacillus subtilis* FC and other FCs revealed that the cyanobacterial FC possesses more sequence similarity to plant enzymes

than to bacterial or vertebrate counterparts. Since no structure for this type of FC is known, a prediction of the membrane localization behaviour remains difficult. The PPO-FC complexes allow the phototrophic cyanobacterium to protect the highly photoreactive intermediate protogen via channelling between the two enzymes. Earlier kinetic investigations of eukaryotic PPO and FC already suggested that substrate channelling occurs between these enzymes (Ferreira *et al.*, 1988). Similar conclusions were drawn from an investigation of various *B. subtilis* FC mutants (Olsson *et al.*, 2002). Channelling of potentially toxic or labile intermediates by enzymes seems to be a widespread solution in nature. Two other enzymes of haem biosynthesis, glutamyl-tRNA reductase and glutamate-1-semialdehyde 2,1-aminomutase, form a stable complex to protect the labile intermediate glutamate-1-semialdehyde (Lüer *et al.*, 2005; Nogaj & Beale, 2005).

Due to the unstable nature of pre-uroporphyrinogen III, complex formation between the producing porphobilinogen deaminase and accepting uroporphyrinogen III synthase was postulated, but has not yet been experimentally verified (Shoolingin-Jordan, 1995). Consequently, further biochemical and immunological experiments are required to solve all the protein-protein interactions required for high-fidelity haem biosynthesis.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (JA 470-7-3).

REFERENCES

- Breckau, D., Mahlitz, E., Sauerwald, A., Layer, G. & Jahn, D. (2003). Oxygen-dependent coproporphyrinogen III oxidase (HemF) from *Escherichia coli* is stimulated by manganese. *J Biol Chem* **278**, 46625–46631.
- Brenner, D. A. & Bloomer, J. R. (1980). The enzymatic defect in variegate prophyria. Studies with human cultured skin fibroblasts. *N Engl J Med* **302**, 765–769.
- Dailey, H. A. (2002). Terminal steps of haem biosynthesis. *Biochem Soc Trans* **30**, 590–595.
- Deybach, J. C., da Silva, V., Grandchamp, B. & Nordmann, Y. (1985). The mitochondrial location of protoporphyrinogen oxidase. *Eur J Biochem* **149**, 431–435.
- Ferreira, G. C., Andrew, T. L., Karr, S. W. & Dailey, H. A. (1988). Organization of the terminal two enzymes of the heme biosynthetic pathway. Orientation of protoporphyrinogen oxidase and evidence for a membrane complex. *J Biol Chem* **263**, 3835–3839.
- Frankenberg, N., Moser, J. & Jahn, D. (2003). Bacterial heme biosynthesis and its biotechnological application. *Appl Microbiol Biotechnol* **63**, 115–127.
- Harbin, B. M. & Dailey, H. A. (1985). Orientation of ferrochelatase in bovine liver mitochondria. *Biochemistry* **24**, 366–370.
- Heinemann, I. U., Diekmann, N., Masoumi, A., Koch, M., Messerschmidt, A., Jahn, M. & Jahn, D. (2007). Functional definition of the tobacco protoporphyrinogen IX oxidase substrate-binding site. *Biochem J* **402**, 575–580.
- Heinemann, I. U., Jahn, M. & Jahn, D. (2008). The biochemistry of heme biosynthesis. *Arch Biochem Biophys* **474**, 238–251.
- Jacobs, J. M. & Jacobs, N. J. (1987). Oxidation of protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis. Purification and partial characterization of the enzyme from barley organelles. *Biochem J* **244**, 219–224.
- Koch, M., Breithaupt, C., Kiefersauer, R., Freigang, J., Huber, R. & Messerschmidt, A. (2004). Crystal structure of protoporphyrinogen IX oxidase: a key enzyme in haem and chlorophyll biosynthesis. *EMBO J* **23**, 1720–1728.
- Labbe, P. (1997). Purification and properties of coproporphyrinogen III oxidase from yeast. *Methods Enzymol* **281**, 367–378.
- Layer, G., Verfurth, K., Mahlitz, E. & Jahn, D. (2002). Oxygen-independent coproporphyrinogen-III oxidase HemN from *Escherichia coli*. *J Biol Chem* **277**, 34136–34142.
- Layer, G., Grage, K., Teschner, T., Schünemann, V., Breckau, D., Masoumi, A., Jahn, M., Heathcote, P., Trautwein, A. X. & Jahn, D. (2005). Radical S-adenosylmethionine enzyme coproporphyrinogen III oxidase HemN: functional features of the [4Fe-4S] cluster and the two bound S-adenosyl-L-methionines. *J Biol Chem* **280**, 29038–29046.
- Lermontova, I., Kruse, E., Mock, H. P. & Grimm, B. (1997). Cloning and characterization of a plastidial and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. *Proc Natl Acad Sci U S A* **94**, 8895–8900.
- Lister, R., Chew, O., Rudhe, C., Lee, M. N. & Whelan, J. (2001). *Arabidopsis thaliana* ferrochelatase-I and -II are not imported into *Arabidopsis* mitochondria. *FEBS Lett* **506**, 291–295.
- Lüer, C., Schauer, S., Mobius, K., Schulze, J., Schubert, W. D., Heinz, D. W., Jahn, D. & Moser, J. (2005). Complex formation between glutamyl-tRNA reductase and glutamate-1-semialdehyde 2,1-aminomutase in *Escherichia coli* during the initial reactions of porphyrin biosynthesis. *J Biol Chem* **280**, 18568–18572.
- Matringe, M., Camadro, J. M., Block, M. A., Joyard, J., Scalla, R., Labbe, P. & Douce, R. (1992). Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether-like herbicides. *J Biol Chem* **267**, 4646–4651.
- Matringe, M., Camadro, J. M., Joyard, J. & Douce, R. (1994). Localization of ferrochelatase activity within mature pea chloroplasts. *J Biol Chem* **269**, 15010–15015.
- Medlock, A. E. & Dailey, H. A. (1996). Human coproporphyrinogen oxidase is not a metalloprotein. *J Biol Chem* **271**, 32507–32510.
- Nogaj, L. A. & Beale, S. I. (2005). Physical and kinetic interactions between glutamyl-tRNA reductase and glutamate-1-semialdehyde aminotransferase of *Chlamydomonas reinhardtii*. *J Biol Chem* **280**, 24301–24307.
- Olsson, U., Billberg, A., Sjövall, S., Al-Karadaghi, S. & Hansson, M. (2002). *In vivo* and *in vitro* studies of *Bacillus subtilis* ferrochelatase mutants suggest substrate channeling in the heme biosynthesis pathway. *J Bacteriol* **184**, 4018–4024.
- Phillips, J. D., Whitby, F. G., Kushner, J. P. & Hill, C. P. (2003). Structural basis for tetrapyrrole coordination by uroporphyrinogen decarboxylase. *EMBO J* **22**, 6225–6233.
- Phillips, J. D., Whitby, F. G., Warby, C. A., Labbe, P., Yang, C., Pflugrath, J. W., Ferrara, J. D., Robinson, H., Kushner, J. P. & Hill, C. P. (2004). Crystal structure of the oxygen-dependant coproporphyrinogen oxidase (Hem13p) of *Saccharomyces cerevisiae*. *J Biol Chem* **279**, 38960–38968.
- Porra, R. J. & Jones, O. T. (1963). Studies on ferrochelatase. 2. An investigation of the role of ferrochelatase in the biosynthesis of various haem prosthetic groups. *Biochem J* **87**, 186–192.

Schulze, J. O., Masoumi, A., Nickel, D., Jahn, M., Jahn, D., Schubert, W. D. & Heinz, D. W. (2006). Crystal structure of a non-discriminating glutamyl-tRNA synthetase. *J Mol Biol* **361**, 888–897.

Shoolingin-Jordan, P. M. (1995). Porphobilinogen deaminase and uroporphyrinogen III synthase: structure, molecular biology and mechanism. *J Bioenerg Biomembr* **27**, 181–195.

Suzuki, T., Masuda, T., Singh, D. P., Tan, F. C., Tsuchiya, T., Shimada, H., Ohta, H., Smith, A. G. & Takamiya, K. (2002). Two types of ferrochelatase in photosynthetic and nonphotosynthetic tissues of cucumber: their difference in phylogeny, gene expression and localization. *J Biol Chem* **277**, 4731–4737.

Watanabe, N., Che, F. S., Iwano, M., Takayama, S., Yoshida, S. & Isogai, A. (2001). Dual targeting of spinach protoporphyrinogen oxidase II to mitochondria and chloroplasts by alternative use of two in-frame initiation codons. *J Biol Chem* **276**, 20474–20481.

Wu, C. K., Dailey, H. A., Rose, J. P., Burden, A., Sellers, V. M. & Wang, B. C. (2001). The 2.0 Å structure of human ferrochelatase, the terminal enzyme of heme biosynthesis. *Nat Struct Biol* **8**, 156–160.

Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W. & Orth, P. (2001). Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution. *Nature* **409**, 739–743.

Edited by: J. W. B. Moir