Introduction of a new branchpoint in tetrapyrrole biosynthesis in *Escherichia coli* by co-expression of genes encoding the chlorophyll-specific enzymes magnesium chelatase and magnesium protoporphyrin methyltransferase

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Received 28 May 1999

Abstract The genes encoding the three Mg chelatase subunits, ChlH, ChlI and ChlD, from the cyanobacterium *Synechocystis* PCC6803 were all cloned in the same pET9a-based *Escherichia coli* expression plasmid, forming an artificial *chlH-I-D* operon under the control of the strong T7 promoter. When a soluble extract from IPTG-induced *E. coli* cells containing the pET9a-ChlHID plasmid was assayed for Mg chelatase activity in vitro, a high activity was obtained, suggesting that all three subunits are present in a soluble and active form. The *chlM* gene of *Synechocystis* PCC6803 was also cloned in a pET-based *E. coli* expression vector. Soluble extract from an *E. coli* strain expressing *chlM* converted Mg-protoporphyrin IX to Mg-protoporphyrin monomethyl ester, demonstrating that *chlM* encodes the Mg-protoporphyrin methyltransferase of *Synechocystis*. Co-expression of the *chlM* gene together with the *chlH-I-D* construct yielded soluble protein extracts which converted protoporphyrin IX to Mg-protoporphyrin IX monomethyl ester without detectable accumulation of the Mg-protoporphyrin IX intermediate. Thus, active Mg chelatase and Mg-protoporphyrin IX methyltransferase can be coupled in *E. coli* extracts. Purified ChlI, -D and -H subunits in combination with purified ChlM protein were subsequently used to demonstrate in vitro that a molar ratio of ChlM to ChlH of 1 to 1 results in conversion of protoporphyrin IX to Mg-protoporphyrin monomethyl ester without significant accumulation of Mg-protoporphyrin.

Key words: Chlorophyll biosynthesis; Mg chelatase; Methyltransferase; *Synechocystis*

1. Introduction

In (bacterio)chlorophyll biosynthesis, Mg chelatase catalyses the ATP-dependent insertion of Mg^{2+} into protoporphyrin IX leading to the formation of Mg-protoporphyrin IX. Subsequently, Mg-protoporphyrin IX methyltransferase converts Mg-protoporphyrin IX to Mg-protoporphyrin IX monomethylester using *S*-adenosyl-L-methionine (SAM) as a co-factor (Fig. 1A). The Mg chelatase enzyme consists of three different subunits, I (38–42 kDa), D (60–74 kDa) and H (140–150 kDa), in (bacterio)chlorophyll *a*-producing prokaryotes [1–3] and also in higher plants [4–7]. In *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Synechocystis*, the Mg-protoporphyrin IX methyltransferase (methyltransferase) is a single subunit enzyme of 25–27 kDa [8–10].

Recently, we have characterised the Mg chelatase enzymes from the cyanobacterium *Synechocystis* PCC6803 and the purple bacterium *R. sphaeroides* by purifying the constituent proteins from *Escherichia coli* and reconstituting highly active enzymes [11,12]. Based on this work, a provisional model of the Mg chelatase cycle was suggested in which the I and D subunits interact with MgATP and form a complex. The physical interaction between the I and D subunits from both species has been demonstrated by the work presented by Gibson et al. [12] and Jensen et al. [13]. It was also proposed that the H subunit with bound protoporphyrin IX reacts with this I-D-MgATP complex and a short-lived complex consisting of all three subunits and the three substrates is formed. Next, Mg^{2+} is inserted into protoporphyrin IX with concomitant hydrolysis of ATP resulting in another, probably short-lived, complex consisting of I, D, H, MgADP and Mg-protoporphyrin IX. The hydrolysis of ATP in parallel with Mg insertion was recently demonstrated by Jensen et al. [13]. This complex dissociates into I-D-MgADP and H-Mg-protoporphyrin which then can be re-charged with MgATP and protoporphyrin IX, respectively, and participate in a new reaction cycle following removal of the reaction products.

Protoporphyrin IX is the shared substrate of both Mg chelatase and ferrochelatase, which inserts Fe^{2+} into protoporphyrin. As an organism that synthesises protoporphyrin IX, *E. coli* should therefore be able to provide this substrate for Mg chelatase. In photosynthetic organisms, Mg chelatase is believed to play a key role in directing intermediates into the (bacterio)chlorophyll-specific part of the pathway. Thus, the consequence of assembling functional Mg chelatase and methyltransferase enzymes in *E. coli* should be the introduction of a new branchpoint in the tetrapyrrole biosynthesis pathway of *E. coli*. An *E. coli* strain that expresses all three Mg chelatase subunits will be a source for purification of a Mg chelatase complex and also for analysis of the requirements for a stable complex. Putative interactions between the Mg chelatase complex and the methyltransferase can also be studied.

2. Materials and methods

2.1. Construction of the pET9a-ChlHID, pET9a-ChlM and the pACYC-ChlM plasmids

For the plasmid expressing the three Mg chelatase subunits, the starting plasmid was pET9a-His_6-ChlH. This is a pET9a derivative

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PII: S0014-5793(99)00909-6

(Novagen) in which a *Xba*I-*Bam*HI fragment encoding the His_6-ChlH fusion from pET14b-ChlH [11] is cloned into the *Xba*I-*Bam*HI sites of pET9, resulting in His_6-tagged ChlH. Due to the way this construct was made, it has the following restriction sites downstream from the stop codon of the ChlH reading frame: *Hin*dIII, *Eco*RV, *Eco*RI, *Pst*I, *Bam*HI. Two gene-specific oligonucleotides containing *Pst*I and *Bam*HI sites, respectively, were designed to amplify *chlD* by PCR including the ribosomal binding site of the pET vector using pET3a-ChlD [2] as template. The resulting PCR fragment was cloned into the *Pst*I and *Bam*HI sites of pET9a-His_6-ChlH resulting in pET9a-His_6-ChlH-ChlD. Subsequently, *chlI* was amplified by PCR using gene-specific oligonucleotides containing *Pst*I sites and cloned into the *Pst*I site of the pET9a-His_6-ChlH-ChlD construct. The orientation of *chlI* with respect to the other reading frames was determined by restriction mapping. The final plasmid containing the artificial *chlH-I-D* operon was named pET9a-ChlHID (Fig. 1B). In this construct, the operon is under the control of the strong T7 promoter and each of the three individual reading frames has its own ribosome binding site.

The *Synechocystis chlM* gene was amplified by PCR using gene-specific oligonucleotides deduced from the known gene sequence [10] and *Synechocystis* genomic DNA as template and cloned into the *Nde*I and *Bam*HI sites of expression vector pET14b (Novagen). The insert was subsequently excised as a *Xba*I-*Bam*HI fragment and ligated into *Xba*I-*Bam*HI-digested pET9a, yielding the plasmid pET9a-His_6-ChlM. A fragment containing the T7 promoter followed by the *chlM* gene and T7 terminator was excised from pET9a-His_6-ChlM with *Bgl*II and *Eco*RV and ligated into the *Bam*HI and *Eco*RV sites of pACYC184 yielding pACYC-ChlM (Fig. 1B).

2.2. Expression of recombinant proteins and protein purification

Production of recombinant proteins was performed essentially as described by Jensen et al. [2]. *E. coli* BL21(DE3) cultures containing the respective plasmids were grown to an optcal density at 600 nm of 0.6–1 at 25°C and induced for 4–16 h by addition of 0.4 mM IPTG.

His_6-tagged ChlI, -D and -H proteins were purified as previously reported by Jensen et al. [11]. His_6-tagged ChlM was purified according to the manufacturer (Novagen) and the eluted protein was dialysed into buffer containing 50 mM tricine-NaOH, 300 mM glycerol, 1 mM DTT, pH 7.9 and stored at −20°C.

2.3. Enzyme assay

The Mg chelatase assay with purified protein subunits was performed in 100 μl buffer containing 50 mM MOPS-NaOH, pH 7.7, 300 mM glycerol, 16 mM $MgCl_2$, 5 mM ATP, 1 mM DTT, 4 μM protoporphyrin IX and protein amounts as indicated in the figure legends. Whenever total soluble *E. coli* extracts were used as protein source in assays, an ATP regenerating system consisting of creatine kinase (10 U/ml) and phosphocreatine (50 mM) was added.

The methyltransferase assay using purified ChlM was performed in 100 μl buffer containing 50 mM tricine-NaOH, pH 7.7, 300 mM glycerol, 1 mM $MgCl_2$, 1 mM SAM, 1 mM DTT, 4 μM Mg-protoporphyrin IX and protein amounts as indicated in the figure legends.

The coupled Mg chelatase and methyltransferase assay was performed in 100 μl buffer containing 50 mM tricine-NaOH, pH 7.7, 300 mM glycerol, 16 mM $MgCl_2$, 5 mM ATP, 1 mM SAM, 1 mM DTT, 4 μM protoporphyrin IX and protein amounts as indicated. As mentioned above, whenever total soluble *E. coli* extracts were used as protein source, the ATP regenerating system consisting of creatine kinase and phosphocreatine was added.

For all assays, the mixtures were incubated for 30 min at 34°C, stopped by addition of 900 μl acetone/water/32% ammonia (80:20:1, by volume) and centrifuged at $15\,000 \times g$ for 5 min. The fluorescence emission spectra of the extracted enzyme assays were recorded between 550 and 650 nm on a SPEX FluoroLog spectrofluorimeter (SPEX Industries, NJ, USA) using an excitation wavelength of 420 nm. Mg-protoporphyrin IX and protoporphyrin IX have characteristic emission peaks at 595 and 635 nm, respectively.

2.4. High performance liquid chromatography (HPLC) analysis of pigments

For HPLC analysis, the acetone phase was analysed for porphyrins by HPLC using a Beckman Ultrasphere ODS column (150×4.6 mm). The column was eluted with a 10 min linear gradient from 15% to 100% solvent B at 1 ml/min. Solvent A: 0.005% (v/v) triethylamine in water. Solvent B: acetonitrile. Elution of porphyrins was detected using a Waters in line fluorescence detector. Mg-protoporphyrin IX and its monomethyl ester was detected using an excitation wavelength of 420 ± 5 nm and an emission wavelength of 595 ± 5 nm. The fluorescence chromatograms at 595 nm were derived from the accumulated scans using the Millenium software (Waters). The retention times for the pigments analysed in this paper are 8.4–8.5 min for Mg-protoporphyrin IX, 8.8–8.9 min for protoporphyrin IX and 10.4–10.5 min for Mg-protoporphyrin monomethyl ester.

2.5. Porphyrin solutions

Protoporphyrin IX (Sigma) was prepared as outlined by Jensen et al. [11]. Mg-protoporphyrin IX (Porphyrin products, Logan) was resuspended in 0.1 M NH_4OH containing 1.5% (v/v) Tween-80. The concentrations of protoporphyrin IX and Mg-protoporphyrin IX were determined using ε_{mM} (554 nm) = 13.5 (2.7 M HCl) and ε_{mM} (419 nm) = 308 (EtOH), respectively. Mg-protoporphyrin monomethyl ester was prepared by extracting cells of the *R. sphaeroides* mutant N6 which has shown to accumulate Mg-protoporphyrin monomethyl ester [14]. Extraction was performed as described by Gorchein [15]. The concentration of Mg-protoporphyrin IX monomethyl ester was determined using ε_{mM} (419 nm) = 100 (ether).

2.6. Other methods

Recombinant DNA techniques, PCR and preparation of competent *E. coli* cells were all performed according to standard procedures [16]. SAM was prepared as a 100 mM stock and stored at −20°C.

3. Result and discussion

3.1. Construction of an artificial chlH-chlI-chlD operon expressing an active Mg chelatase

To reconstitute an active Mg chelatase in *E. coli*, the genes encoding the three Mg chelatase subunits (*chlI*, *chlD* and *chlH*) were used to construct an artificial operon under control of the T7 promoter (Fig. 1B). This expression plasmid, named pET9a-ChlHID, is based upon the pET *E. coli* expression system [17]. In order to test whether this plasmid produced active protein for all three subunits, preliminary experiments were performed in which cultures were grown to an A_{600} of 0.9 and induced with IPTG. Before induction with IPTG, one culture was induced for 2 h with 10 mM δ-aminolevulinic acid (ALA) to boost protoporphyrin biosynthesis. After 4 h induction with IPTG, soluble Mg-protoporphyrin IX did not accumulate in the *E. coli* cells. However, in soluble extracts from cells co-induced with 10 mM ALA, trace amounts of Mg-protoporphyrin IX were detected (results not shown). This suggests either that one or more of the substrates (protoporphyrin IX, Mg^{2+} or ATP) are limiting in *E. coli* or that the growth temperature of 25°C, which was chosen to achieve maximal expression of soluble proteins, was too low for optimal production of protoporphyrin IX. After sonication of the cell pellet from 100 ml of induced *E. coli* culture and centrifugation to obtain soluble proteins for assays, the remaining pellet of cell debris was extracted with NH_3/acetone and these extracts were analysed by fluorescence emission spectroscopy. Fig. 2A shows that Mg-protoporphyrin IX was present in both samples. However, the pellet from the culture co-induced with ALA (Fig. 2A, trace 2) contained at least six times more Mg-protoporphyrin IX than the pellet from the culture induced with IPTG alone (Fig. 2A, trace 1).

This indicated that expression of the *chlH-I-D* operon results in an active Mg chelatase complex and that Mg-protoporphyrin IX synthesised in vivo was found mainly in the membrane fraction. When equal amounts of soluble proteins from the two cultures where mixed with protoporphyrin IX, Mg^{2+} and an ATP regenerating system, virtually identical Mg chelatase activities were obtained in these in vitro assays (Fig.

2B, traces 1 and 2). This indicates that expression of soluble and active Mg chelatase subunits from genes within the pET9-HID plasmid is independent of the presence of increased levels of the protoporphyrin substrate.

3.2. Demonstration of in vitro Mg-protoporphyrin methyltransferase activity in strains containing either pET9a-ChlM or pACYC-ChlM

Having established that co-expression of *chlH-I-D* genes in *E. coli* yields the three active Mg chelatase subunits, the next step was to co-express *chlH-I-D* with *chlM* encoding Mg-protoporphyrin IX methyltransferase, the next enzyme in the pathway (Fig. 1A). Before this, the properties of pET9a-ChlM and pACYC-ChlM were examined in order to verify that these *chlM* constructs encoded active methyltransferase. In previous work, the *chlM* gene of *Synechocystis* PCC6803 was cloned and its function as the methyltransferase was established by complementation of a *R. capsulatus* mutant containing a defined mutation in the gene that encodes Mg-protoporphyrin IX methyltransferase [10]. In order to verify the enzymatic function in vitro, the *chlM* gene was PCR-amplified from *Synechocystis* genomic DNA using gene-specific primers and cloned into a pET9a expression plasmid. In vitro methyltransferase assays with extracts of soluble protein from IPTG-induced *E. coli* cultures and Mg-protoporphyrin IX and SAM as substrates revealed the formation of Mg-protoporphyrin IX monomethyl ester (Fig. 3) Since Mg-protoporphyrin IX (the substrate) and the monomethyl ester (the product) have the same spectroscopic properties, the assay mixtures were analysed by HPLC. It is clear that a product with the same retention time as authentic Mg-protoporphyrin monomethyl ester is formed specifically in the assay with protein from a *chlM*-expressing culture (Fig. 3, pET9a-ChlM) and no such product is formed when protein from a culture containing a control plasmid is used (Fig. 3, pET9a). The methyltransferase activity of ChlM protein produced from plasmid pACYC-ChlM (Fig. 1B) was also verified by an in vitro assay (Fig. 3).

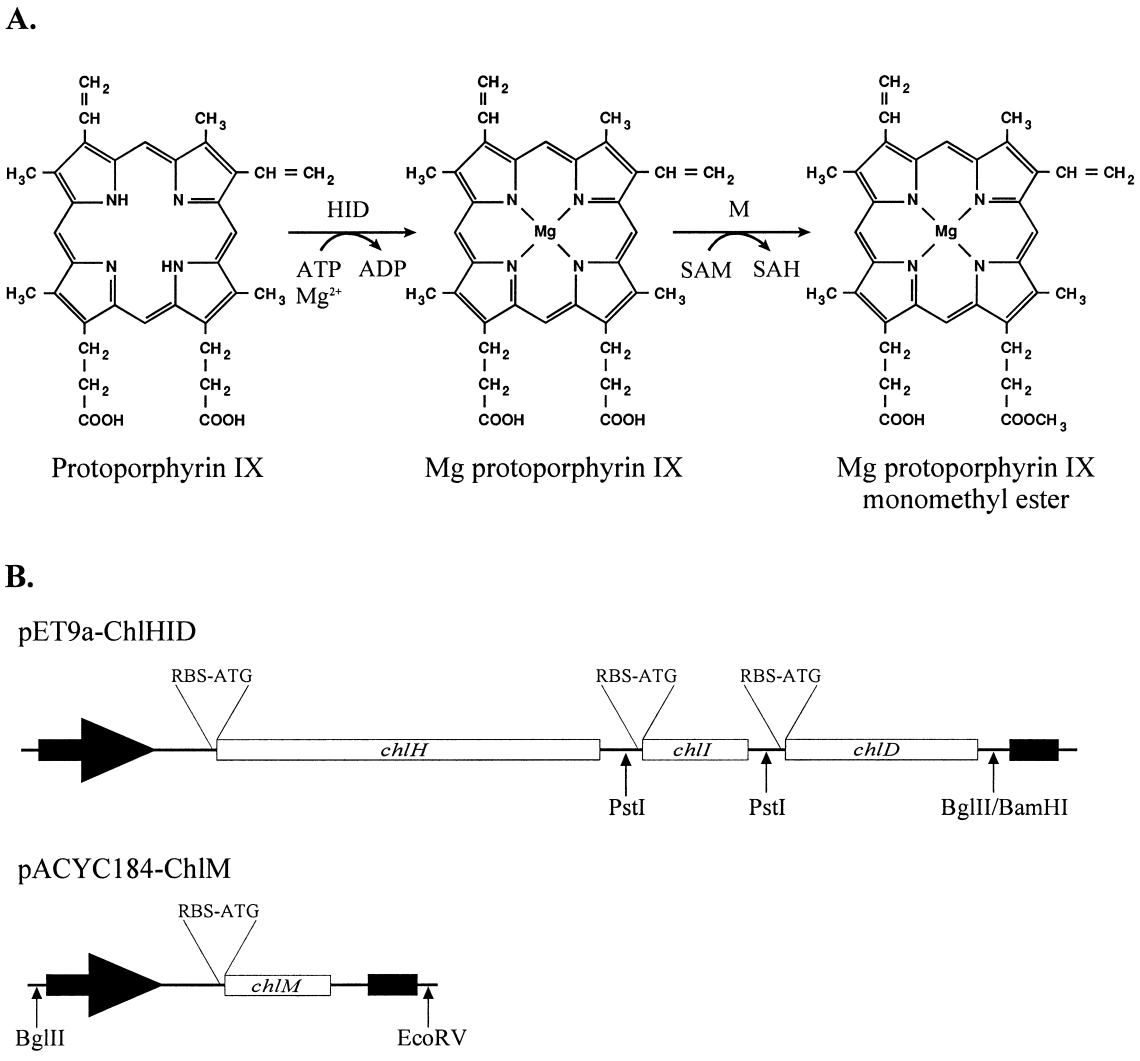

Fig. 1. (A) The Mg^{2+} insertion step of (bacterio)chlorophyll biosynthesis catalysed by Mg chelatase (ChlH, -I and -D) and the following step where the 6-propionic group is methylated in a reaction catalysed by SAM:Mg-protoporphyrin IX methyltransferase (ChlM). (B) Schematic representation of the pET9a plasmid containing the artificial *chlHID* operon which encodes the three Mg chelatase subunits and the structure of the pACYC-ChlM plasmid containing the *chlM* gene encoding the SAM:Mg-protoporphyrin IX methyltransferase. The black arrows and bars indicate the T7 promoter and terminator, respectively. RBS = ribosomal binding sites. Also shown are the relevant restriction sites used to make the constructs.

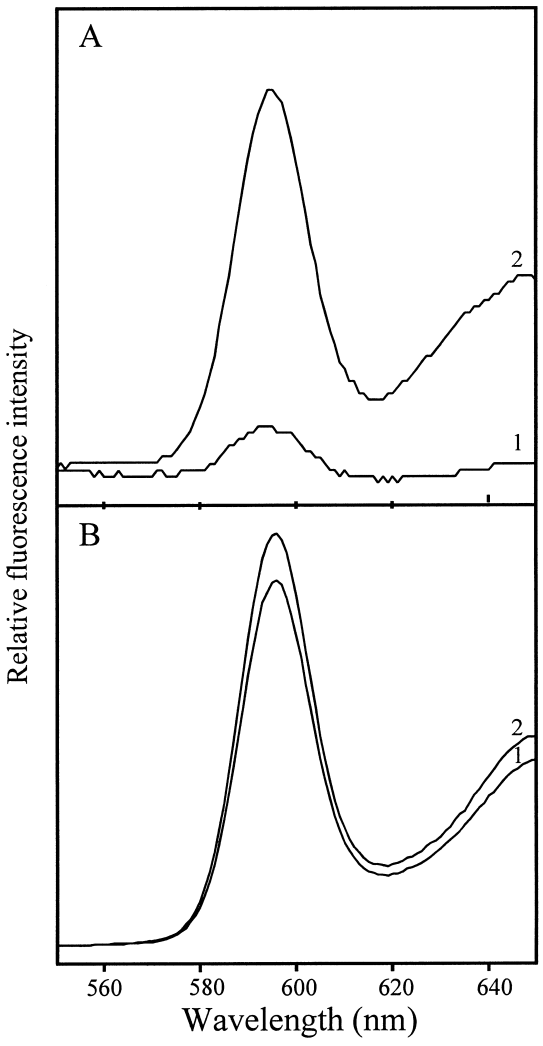

Fig. 2. The pET9a-ChlHID construct expresses all three Mg chelatase subunits in an active form as shown by fluorescence emission spectra of NH_3/acetone extracts of: (A) (1) Pellet of cell debris after sonication of the cell pellet from a 100 ml culture of *E. coli* containing pET9a-ChlHID and induced with IPTG. (2) The same as 1, but 10 mM ALA was added to the *E. coli*:pET9a-ChlHID culture, 2 h before induction with IPTG. (B) In vitro Mg chelatase assays with soluble proteins from cultures of *E. coli* (pET9a-ChlHID) treated with (1) IPTG and (2) IPTG+ALA. Approximately 300 μg total soluble protein was used in the standard assay. The excitation wavelength was 420 nm and emission was detected between 550 and 650 nm. Mg-protoporphyrin IX has a characteristic emission at 595 nm whereas protoporphyrin IX has maximum emission at 635 nm.

3.3. Co-expression of chlH, chlI, chlD and chlM in E. coli

pET-derived vectors contain the ColE1 origin of replication whereas pACYC184 contains the P15A origin of replication. Since these plasmids belong to different incompatibility groups, they can co-exist in the same *E. coli* cell. *E. coli* BL21 (DE3) cells containing pET9a-ChlHID were made competent and transformed with pACYC-ChlM. Cells containing both plasmids were selected on plates containing neomycin and chloramphenicol. Soluble protein extracts were prepared from IPTG-induced *E. coli* containing both plasmids and coupled Mg chelatase and methyltransferase assays were performed with added protoporphyrin IX as the sole porphyrin substrate SAM as the methyl donor, $MgCl_2$ and an ATP regenerating system. As seen in Fig. 4, protoporphyrin IX was converted in a time-dependent manner to Mg-protoporphyrin IX monomethyl ester. This clearly demonstrates that all four genes are expressed in *E. coli* and that the gene products are active. In the absence of added SAM, some accumulation of Mg-protoporphyrin IX occurs, since the cell extract cannot supply enough of this substrate for the amount of enzyme present. However, no such accumulation of the Mg-protoporphyrin IX intermediate is observed when SAM is added to the incubation. This could reflect the possibility that when the chelatase and the methyltransferase are coupled together, Mg chelatase is rate limiting, either because of some impairment of activity or because its turnover number is lower than that of the methyltransferase.

3.4. The activity of ChlH, -I, -D and -M reconstituted in vitro

In order to examine the interaction of the chelatase and methyltransferase in more detail, it is necessary to work with purified proteins, since it is not possible to define the amounts of enzyme in crude extracts. The purification of large quantities of His-tagged ChlI, -D and -H subunits from *Synechocystis* has allowed us to estimate the relative concentrations of these three protein components required for optimal Mg chelatase activity and to examine the biochemical and kinetic properties of the Mg chelatase [11]. Purified ChlH, -I, -D and -M proteins were therefore required in order to see if purified ChlM efficiently methylates newly synthesised Mg-protoporphyrin IX.

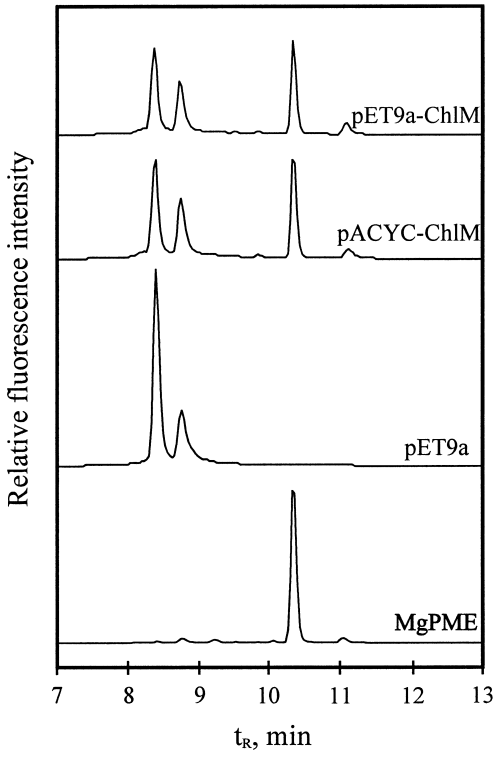

Fig. 3. In vitro assays demonstrating that ChlM is active when overproduced in *E. coli*. Soluble protein extracts (approximately 300 μg) from *E. coli* cells containing the pET9a-ChlM, pACYC-ChlM or pET9a plasmids were mixed with Mg-protoporphyrin IX and SAM and incubated for 30 min at 34°C. Subsequently, the assays were stopped and extracted with NH_3/acetone and analysed by HPLC. The HPLC traces of the three incubations and authentic Mg-protoporphyrin monomethyl ester (MgPME) as standard are shown.

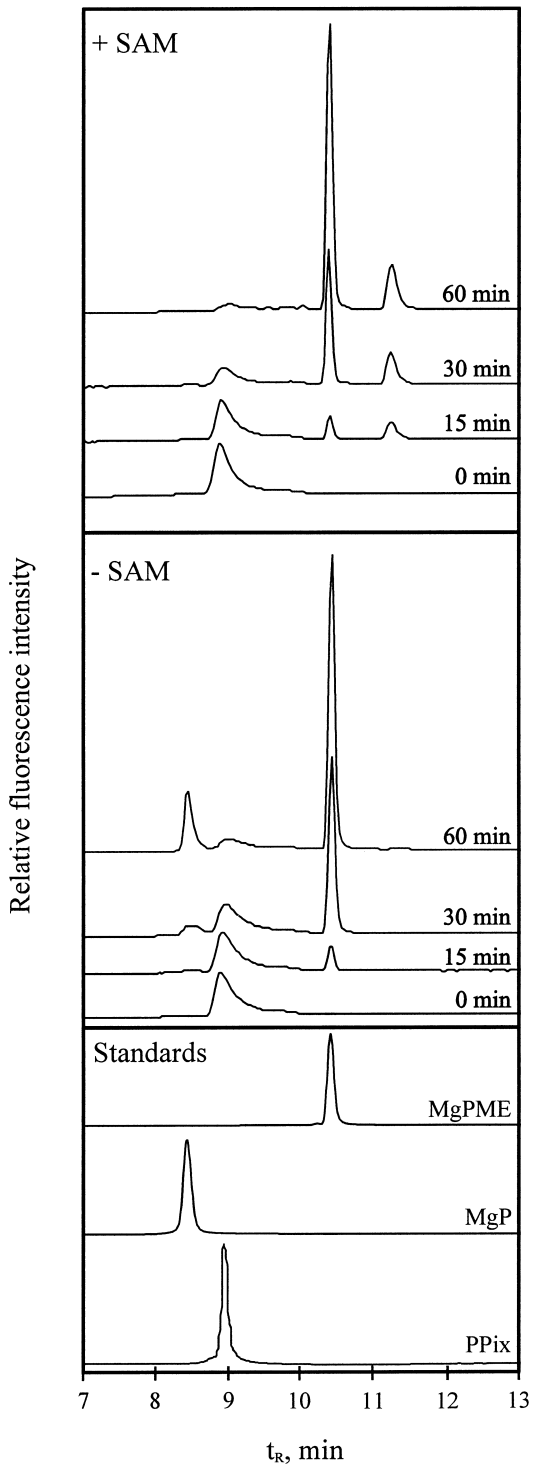

Fig. 4. *E. coli* cells containing both the pET9a-ChlHID and the pACYC-ChlM plasmids produce all four proteins in an active form. When soluble protein extracts (approximately 300 μg) from an IPTG-induced culture are mixed with protoporphyrin IX, ATP, $MgCl_2$ and SAM and incubated at 34°C, a time-dependent conversion of protoporphyrin IX to Mg-protoporphyrin monomethyl ester is clearly seen. Assays were performed with and without added SAM (+SAM and −SAM). Also shown are HPLC traces of authentic Mg-protoporphyrin monomethyl ester (MgPME), Mg-protoporphyrin IX (MgP) and protoporphyrin IX (PPix).

ChlM protein was purified on a Ni^{2+} agarose affinity column directly from the supernatant after sonication of the *E. coli* cell pellet and removal of cell debris. Coomassie-stained SDS-PAGE gels revealed that ChlM was more than 90% pure after this step (results not shown).

Coupled Mg chelatase and methyltransferase assays were performed with 0.28 μM ChlH in combination with 0.013 μM ChlD and 0.6 μM ChlI and concentrations of ChlM between 0.036 and 1.8 μM. The chosen concentration of ChlH has found to be limiting for Mg chelatase activity [11]. The

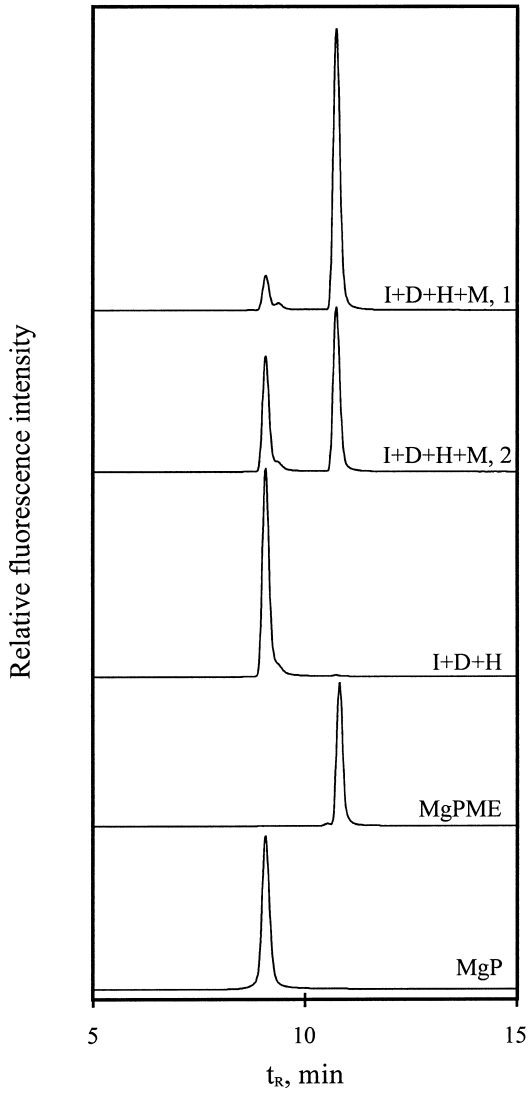

Fig. 5. Reconstituted ChlH, -I, -D and -M results in conversion of protoporphyrin IX to Mg-protoporphyrin monomethyl ester when ChlM and ChlH are present in a 1:1 ratio. Purified Mg chelatase subunits and methyltransferase protein were incubated with protoporphyrin IX, ATP, $MgCl_2$ and SAM and incubated at 34°C for 30 min. ChlM was tested with fixed amounts of the three Mg chelatase subunits. Coupled Mg chelatase and methyltransferase assays were performed with 0.28 μM ChlH in combination with 0.013 μM ChlD and 0.6 μM ChlI and concentrations of ChlM between 0.036 and 1.8 μM. Two of these traces are shown. Trace I+D+H+M, 1 corresponds to an assay in which the ChlH:ChlM ratio was 1:1. Trace I+D+H+M, 2 corresponds to an assay in which the ChlH:ChlM ratio was 3:1. HPLC traces of an assay with ChlI, -D and -H only (I+D+H) and of authentic Mg-protoporphyrin monomethyl ester (MgPME) and Mg-protoporphyrin IX (MgP) are also shown.

results in Fig. 5 demonstrate that when ChlM is present in a 1:1 stoichiometry with ChlH, almost all Mg-protoporphyrin IX is converted to the monomethyl ester whereas Mg-protoporphyrin accumulates when there is 3-fold less ChlM than ChlH.

Stimulation of methyltransferase activity by soluble BchH protein has recently been reported by Hinchigeri et al. [18]. In order to test this with purified ChlM and ChlH proteins from *Synechocystis*, an experiment was performed in which 0.36 μM ChlM was assayed with ChlH in the range from 0.093 to 1.12 μM, with added Mg-protoporphyrin IX as the porphyrin substrate. When the formation of the Mg-protoporphyrin monomethyl ester product was analysed by HPLC (results not shown), no effect of ChlH on methyltransferase activity was seen, suggesting that ChlH does not stimulate the methyltransferase activity of ChlM when purified proteins are used. In the study by Hinchigeri et al. [18], membranes of a *bchM* expression strain of *E. coli* were used in combination with soluble BchH and the presence of membranes might explain the observed stimulation either due to a specific requirement for a lipid environment or a non-specific effect. However, it may be that the converse occurs and that the presence of the methyltransferase effectively increases the turnover rate of Mg chelatase by removing the Mg-protoporphyrin product from the Mg chelatase.

3.5. Concluding remarks

We have demonstrated the synthesis of consecutive enzymes, Mg chelatase and Mg-protoporphyrin methyltransferase, from the chlorophyll biosynthetic pathway in *E. coli*. We have utilised the fact that *E. coli* naturally produces the substrate of the first enzyme, protoporphyrin IX. This work has necessitated the production of four active proteins, three for the chelatase and one for the methyltransferase. Each of these displays activity both in vivo and in vitro. These two biosynthetic reactions introduce a branchpoint in the tetrapyrrole metabolism in *E. coli*. This may be especially useful in the study of enzymes from organisms such as *Synechocystis*, since it facilitates genetic manipulation of enzymes and subsequent evaluation of altered or mutated enzymes.

Acknowledgements: This work was supported by Grants to Dr. Poul Erik Jensen from the Danish Agricultural and Veterinary Research Council (9600928) and to Prof. C.N. Hunter from the Biotechnology and Biological Sciences Research Council, UK.

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