Demonstration that the BchH protein of *Rhodobacter capsulatus* activates *S*-adenosyl-L-methionine:magnesium protoporphyrin IX methyltransferase

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Abstract **The *bchH* gene of *Rhodobacter capsulatus* has been cloned into an expression strain of *Escherichia coli*. Following induction of expression of the BchH protein, it was found that the *E. coli* strain also accumulated porphyrins with the fluorescence properties of protoporphyrin and zinc protoporphyrin. It was also found that the soluble BchH protein increased the activity of *S*-adenosyl-L-methionine:magnesium protoporphyrin IX methyltransferase, when mixed with membranes of an expression strain of *E. coli* into which the *bchM* gene (which encodes the methyltransferase) had been cloned, as well as membranes of a *bchH* mutant of *R. capsulatus*.**

Key words: BchH protein; *Rhodobacter capsulatus*; *S*-Adenosyl-L-methionine:magnesium protoporphyrin methyltransferase activation

1. Introduction

Most *bch* genes encoding enzymes of the magnesium branch of bacteriochlorophyll synthesis were initially assigned to particular enzymes on the basis of the observed phenotypes for *bch* mutants of *Rhodobacter capsulatus* [1–5] and *Rhodobacter sphaeroides* [6,7]. At the time of these assignments, the genes could not be directly related to enzymes because only one enzyme catalyzing a reaction in the magnesium branch had ever been assayed in these bacteria. Ironically, the assignment of this enzyme, *S*-adenosyl-L-methionine (SAM):magnesium protoporphyrin IX (Mg Proto) methyltransferase (MT) (EC 2.1.1.11), to a *bch* gene proved to be extremely difficult, and for years it was incorrectly assigned. A mutant lacking an active MT would be expected to accumulate Mg Proto; however, this phenotype was, initially at least, never observed. Three strains, with mutations, respectively, in the *bchD*, *-H*, and *-I* loci accumulated small amounts of Proto [5], which would be expected if the mutants lacked an active magnesium chelatase. However, since Gorchein [8] had observed that the MT and magnesium chelatase appeared to be very tightly coupled in whole cells of *R. sphaeroides*, it was possible that a mutant lacking an active MT might also be unable to catalyze magnesium chelation and, hence, accumulate Proto. As no MT activity could be demonstrated in *bchH* mutants (whereas it could be easily demonstrated in other *bch* mutants), the MT was assigned to the *bchH* gene [9,10]. Gorchein et al. [11] subsequently demonstrated that whereas both *bchD* and *bchI* mutants of *R. sphaeroides* contained an active MT but not an active magnesium chelatase, neither an active MT nor an active magnesium chelatase could be detected in a *bchH* mutant. These results were consistent with assignments of both the *bchD* and *bchI* genes to subunits of the magnesium chelatase and the *bchH* gene to the MT.

In the meantime, we had cloned the *bchH* gene into a strain of *Escherichia coli*, but were unable to demonstrate MT activity for the overexpressed BchH protein (unpublished observations). The situation was clarified when it was reported that the *bchM* gene actually encoded the MT [12,13], and that the products of all three genes, *bchD*, *-H*, and *-I*, were required for magnesium chelatase activity [14,15]. Because a *bchM* mutant was thought to have accumulated Mg Proto monomethyl ester (Mg ProtoME), this gene had been misclassified as one of the components of the oxidative cyclase enzyme system which catalyzes the conversion of Mg ProtoME to magnesium 2,4-divinylpheoporphyrin a_5 [16]. When it was shown that the mutant actually accumulated Mg Proto instead of Mg ProtoME, the *bchM* gene from both *R. capsulatus* [12] and *R. sphaeroides* [13] was cloned into strains of *E. coli* and the overexpressed BchM proteins shown to have MT activity. This left unanswered the question of why *bchH* mutants failed to demonstrate MT activity whereas the MT could easily be demonstrated in *bchD* and *bchI* mutants [11]. We demonstrate herein that the BchH protein acts as a stabilizer and an activator of the BchM protein, increasing its MT activity approximately 7-fold.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli BL21(DE3)/pET-3b (*E. coli*(pET-3b)) and *E. coli* BL21(DE3)/pAPUH (*E. coli(bchH)*) were grown at 37°C in LB medium [17] with 5 g l^{-1} of NaCl (in place of 10 g l^{-1}) and 200 μg ml^{-1} ampicillin. *E. coli* C600/pGP1-2/pT7-7::*bchM* (*E. coli(bchM)*) was grown at 30°C in LB medium [17] with 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin. *R. capsulatus* mutants ZY4 and ZY6 were grown aerobically at 30°C in RCV medium [18] with 10 μg ml^{-1} kanamycin.

2.2. Construction of the expression plasmid

The *R. capsulatus bchH* gene was amplified by PCR using 0.84 ng

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Abbreviations: ALA, δ-aminolevulinic acid; DE, dimethyl ester; DMF, *N*,*N*-dimethylformamide; IPTG, isopropyl-β-D-thiogalactopyranoside; ME, monomethyl ester; MT, methyltransferase; Proto, protoporphyrin IX; SAM, *S*-adenosyl-L-methionine

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(1.0 pM) of the linearized plasmid pFL137 (which contained the *R. capsulatus bchH* gene, base numbers 662–13826 of the photosynthetic gene cluster; Genebank accession number Z111653) as the template. Amplifications were performed using *Thermus aquaticus* Amplitaq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA) in 100 μl of the standard buffer [19] for 30 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1.5 min, and extension at 72°C for 2.5 min. Plasmid pAPUH (pET-3b::*bchH*) was constructed by ligating the PCR-amplified *bchH* gene into the *Nde*I and *Bam*HI restriction sites of the pET-3b vector [20]. The N-terminal primer (5′-CAAGGCCCCATATGCACGATGAGTCGATG-3′) contained an *Nde*I restriction site to allow the ligation of the amplified gene directly into the translation start site of the pET-3b expression vector. The C-terminal primer (5′-GTAGTCGTAGATCTCATTCTGCCGCAA-CG-3′) was designed to hybridize to a region immediately downstream of the gene, and to contain a *Bgl*II site to permit insertion into the *Bam*HI site of pET-3b. Plasmid pAPUH was cloned and maintained in *E. coli* strain BL21(DE3). *E. coli* containing pET-3b without the *bchH* gene was used as a control. All nucleic acid and enzymatic manipulations were performed according to standard published procedures [17] or manufacturers' protocols.

2.3. Induction of expression of BchH and BchM proteins

Cultures to be induced were grown as described above to an $A_{600} \simeq 0.6$. Cultures of *E. coli*(pET-3b) and *E. coli(bchH)* were induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by incubation for an additional 1.5 h at 37°C. Cultures of *E. coli(bchM)* grown at 30°C were heat induced for 30 min at 42°C, followed by incubation for an additional 1 h at 37°C. Induced proteins were analysed by SDS-PAGE in 5% (Fig. 1A) or 10% (Fig. 1B) polyacrylamide gels using the discontinuous buffer system of Laemmli [21]. For the analysis of Fig. 1A, intact *E. coli* cells were centrifuged from 1 ml of culture, resuspended in 400 μl of undiluted sample buffer [21], boiled for 4 min, and quenched on ice. For the analysis of Fig. 1B, the cells were centrifuged for 3 min at 13 000 rpm in a Heraeus Biofuge 15 table-top centrifuge, washed twice with 50 mM potassium phosphate buffer, pH 7.5, and resuspended in buffer A (50 mM potassium phosphate buffer, pH 7.5, 4 mM dithiotheitol, 10 mM EDTA, plus 2 mM phenylmethylsulfonyl fluoride). They were then sonicated at 4°C with four 20-s bursts (with 1 min intervals in between) at 80% maximum power with a FisherSonic Dismembrator model 300 sonicator fixed with a medium-size probe. The lysed cells were centrifuged for 20 min at 11 000 rpm in a Sorval SS34 rotor, and the pellet (the cell debris fraction) collected and resuspended in buffer A. The supernatant was recentrifuged for 90 min at 49 000 rpm in a Beckman Ti70 rotor, and the resulting supernatant and membrane fractions collected (the latter after washing once in buffer A, and resuspending in buffer A). Supernatants and resuspended cell debris and membrane fractions were analyzed by SDS-PAGE, using Kaleidoscope prestained proteins (Bio-Rad Laboratories Inc., Hercules, CA) as M_r standards. Unstained proteins were visualized with Coomassie Blue R250.

2.4. MT and protein assays

MT activity was assayed by the method of Hinchigeri et al. [22] using 7.6 nmol of Mg Proto (Porphyrin Products Inc., Logan, UT) and 100–140 nCi (2.1–2.9 nmol) of [*methyl*-^{14}C]SAM (48.2 mCi mmol^{-1}; DuPont NEN Research Products, Lachine, Que.) in final volume of 1 ml of buffer A containing 0.007% (v/v) Triton X-100. Incubation was for 2 h at 37°C. Radioactivity was determined with a Beckman series LS6000 liquid scintillation counter. Protein content was determined by the method of Bradford [23].

2.5. Analysis of porphyrins accumulated in E. coli(bchH)

Cultures (2 ml) of *E. coli*(pET-3b) or *E. coli(bchH)* were grown and induced by the addition of 0.4 mM IPTG as described above. In some incubations, 10 mM δ-aminolevulinic acid (ALA) alone or with 0.74 μM *N*-methyl-Proto (Porphyrin Products Inc., Logan, UT) were added at the same time as the inducer, followed by incubation for an additional 3 h at 37°C. The cultures were then centrifuged and washed once with 50 mM potassium phosphate buffer, pH 7.5, as described above, and the cell pellet extracted in a hand-held glass homogenizer with 1 ml of *N,N*-dimethylformamide (DMF). The DMF extract was then centrifuged as above to remove the cells, diluted to 4 ml with DMF, and the room temperature fluorescence spectra recorded in a 1 cm square, four-sided quartz cuvette by a Perkin-Elmer model MP-44B spectrofluorometer. Fluorescence standards employed were Proto, Mg Proto, Zn Proto (formed from Proto with a saturated solution of zinc acetate in methanol), and hemin. Kieselgel 60 silica gel TLC plates (EM Separations, Gibbstown, NJ) were used with two solvent systems: system A, 2,6-lutidine [24]; and system B, toluene/ethyl acetate/95% ethanol (4:1:1, v/v) [25]. Partial acid hydrolysis of Proto dimethyl ester (ProtoDE) by the method of Ellsworth [24] was used to prepare a solution of porphyrin standards (containing Proto, ProtoME, and ProtoDE), and Mg ProtoME was prepared enzymatically from Mg Proto and SAM.

3. Results

3.1. Recombinant expression of the BchH protein

Analysis by SDS-PAGE demonstrated that while the BchH protein was greatly overexpressed after addition of IPTG (Fig. 1A, lanes 4–5), it was nevertheless moderately expressed in *E. coli(bchH)* even before the onset of induction (Fig. 1A, lane 6). Its apparent M_r was 124 000 when compared to stand-

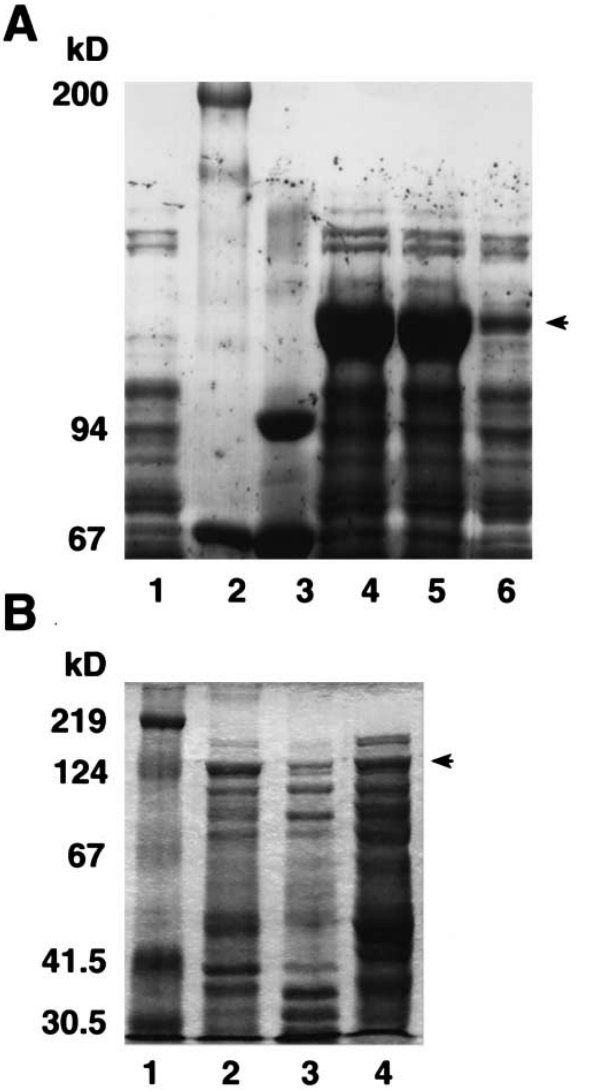

Fig. 1. SDS-PAGE analysis of the expression of the BchH protein in A: whole cells and B: broken cell fractions. A: Lanes: 1, induced control strain *E. coli*(pET-3b); 2 and 3, protein M_r standards; 4 and 5, induced *E. coli(bchH)*; 6, uninduced *E. coli(bchH)*. B: Lanes: 1, protein M_r standards; 2, cell debris; 3, membrane; and 4, soluble fractions of induced *E. coli(bchH)*.

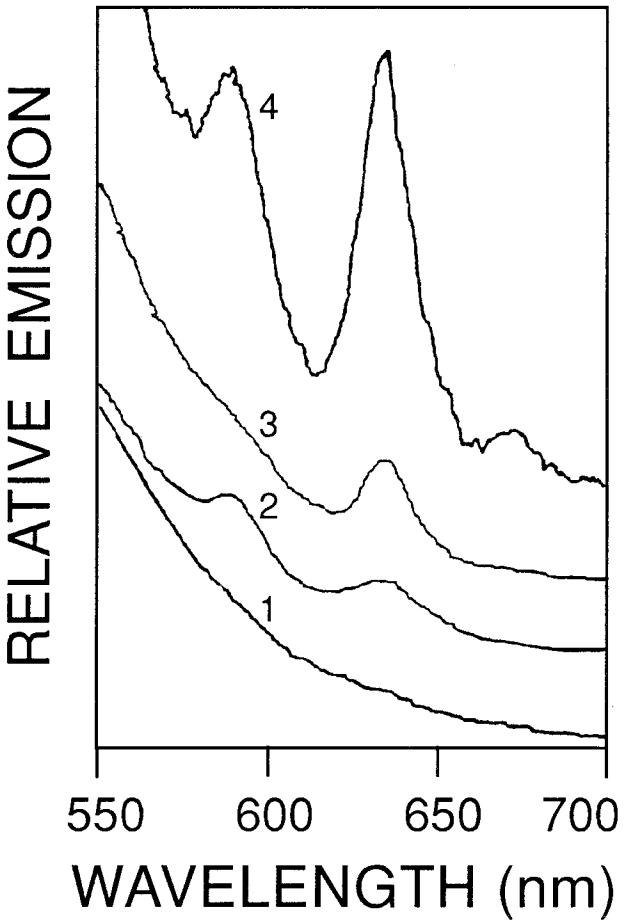

Fig. 2. Fluorescence emission spectra during excitation at 419 nm of DMF extracts of *E. coli*(pET-3b) induced in the absence (line 1) and presence (line 2) of 10 mM ALA, and *E. coli(bchH)* induced in the absence (line 3) and presence (line 4) of 10 mM ALA.

ard proteins (Fig. 1B, lanes 1–2); the nucleotide sequence of the *bchH* gene would encode a protein of ca. 129 000. Ultracentrifugation of the cell extract indicated that most of the BchH protein remained in the soluble fraction (Fig. 1B, lane 4), although some was still visible in the pellet following one wash, possibly in a denatured and/or aggregated state (Fig. 1B, lane 3).

3.2. Pigment production by *E. coli(bchH)*

It was observed that *E. coli(bchH)* produced pigment(s) with fluorescence similar to porphyrins or metalloporphyrins, while *E. coli*(pET-3b) produced little or no pigment(s), after both had been induced with IPTG. The cells of both strains were extracted with DMF; fluorescence emission spectra recorded during excitation at 419 nm revealed a band at 636 nm (F636) in the *E. coli(bchH)* extract (Fig. 2, line 3), but not in the *E. coli*(pET-3b) extract (Fig. 2, line 1). This emission band was observed with an authentic sample of Proto by excitation at 419 nm; hence, the accumulated porphyrin has been tentatively identified as Proto. Inclusion of ALA led to a much larger production of Proto (Fig. 2, lines 2 and 4). However, fluorescence spectra of DMF extracts of both strains after incubation with 10 mM ALA revealed an emission band at 590 nm (F590) as well as F636, although the bands were much more pronounced in extracts of the *E. coli(bchH)* strain. The F590 band was similar to the emission band of an authentic sample of Zn Proto, but not to that of Mg Proto (data not shown) or hemin which is not fluorescent. The F590 material may have been Zn Proto formed by the action of *E. coli* ferrochelatase on Zn^{2+} and excess Proto. Inclusion of 0.74 μM of the ferrochelatase inhibitor, *N*-methyl-Proto, abolished the formation of the F590 material, supporting this assumption (data not shown). The porphyrins were isolated from a large scale (500 ml) run of *E. coli(bchH)* induced in the presence of 10 mM ALA. Following induction, the porphyrins were extracted with DMF and run on silica gel TLC in two solvent systems as described in Section 2. In both systems, the extract exhibited fluorescent TLC bands corresponding to Proto and Zn Proto (or Mg Proto, as these metalloporphyrins are not separated in either system); however, no bands corresponding to Zn ProtoME (or Mg ProtoME) were observed (data not shown).

3.3. Activation of the MT by the BchH protein

Numerous unsuccessful attempts were made with the overexpressed BchH protein to demonstrate MT activity (data not shown). Subsequent to these attempts, Bollivar et al. [12] demonstrated that the MT was actually encoded in *R. capsulatus* by the *bchM* gene. The *E. coli(bchM)* overexpression strain was made available to us, and we confirmed that it contained an active MT in its membrane fraction (Table 1, run 8). We also had available a *bchH* mutant (ZY6) of *R. capsulatus* which had been constructed by Yang and Bauer [16] by inter-

Table 1
MT activities in membranes of *E. coli(bchM)* and the *R. capsulatus* mutant ZY6, with and without the addition of soluble fractions of mutant ZY6 and/or the overexpressed BchH protein

Run	Membrane fraction	Soluble fraction	MT specific activity[a]	Enhancement of MT activity
1	ZY6[b]	–	0	–
2	ZY6	–	38.1	1.0
3	ZY6	*E. coli*(pET-3b)	38.7	1.0
4	ZY6	ZY6	62.4	1.6
5	ZY6	*E. coli(bchH)*	144.2	3.8
6	ZY6	*E. coli(bchH)*+ZY6	154.1	4.0
7	ZY6	*E. coli(bchH)*[c]	54.1	1.4
8	*E. coli(bchM)*	–	17.2	1.0
9	*E. coli(bchM)*	ZY6	43.5	2.5
10	*E. coli(bchM)*	*E. coli(bchH)*	117.2	6.8

[a]In pmol h^{-1} mg membrane $protein^{-1}$.
[b]Control incubation (minus Mg Proto).
[c]MT activity in the membrane fraction following recentrifugation.

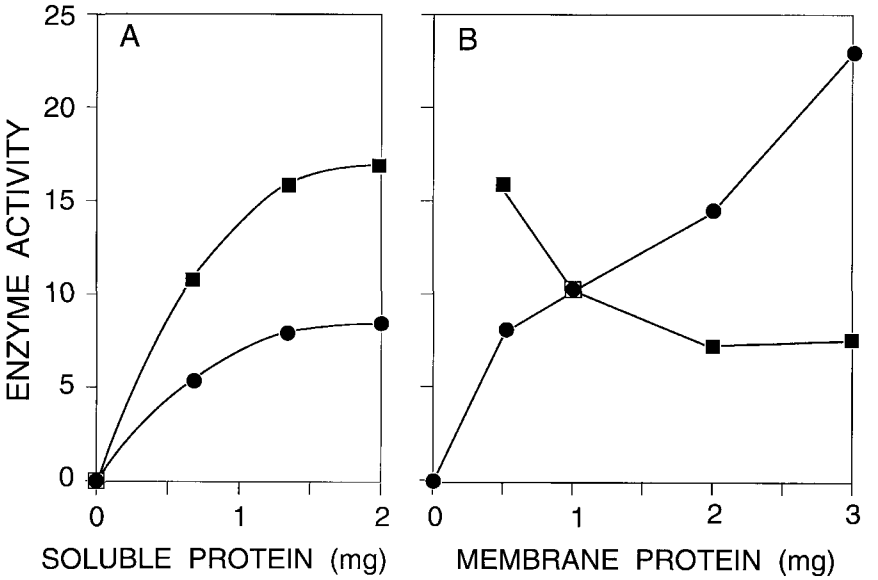

Fig. 3. Analysis of MT activity during recombination of membrane and soluble fractions of *R. capsulatus* mutant ZY6. A: Addition of increasing amounts of soluble fraction protein to a constant amount (0.5 mg ml^{-1}) of membrane protein. B: Addition of increasing amounts of membrane protein to a constant amount (1.3 mg ml^{-1}) of soluble fraction protein. (●) Total activity in pmol h^{-1} ml^{-1}. (■) Specific activity in pmol h^{-1} mg membrane $protein^{-1}$.

poson insertion into the *bchH* gene. The gene for the MT (*bchM*) had not been altered; nevertheless, when we at first looked for MT activity in the membrane fraction of ZY6, none was found. However, we made the interesting discovery that uncentrifuged crude cell extracts contained very high levels of MT activity (up to 50 pmol h^{-1} mg $protein^{-1}$), and it was only when the membrane and soluble fractions were *separated* that activity disappeared. We also found that MT activity could be reconstituted by recombining the membrane and soluble fractions (Fig. 3). Addition of increasing amounts of the soluble fraction to a constant amount of membrane protein (0.5 mg ml^{-1}) increased MT specific activity until it reached a maximum (Fig. 3A). However, while addition of increasing amounts of the membrane fraction to a constant amount of soluble protein (1.3 mg ml^{-1}) continuously increased the total activity of the MT, its specific activity decreased from the optimal value observed at a ratio of soluble/membrane protein of about 2.7 (Fig. 3B). It seemed likely, therefore, that ZY6 was producing a mutant BchH protein which had lost its ability to activate the MT in the membrane, but which could still activate it if the soluble and membrane phases were recombined. (The interposon was inserted into the coding sequence for only 25 amino acids (out of a total of 1194) of the C-terminus of BchH.) In order to test this hypothesis, we conducted the experiments shown in Table 1. The membrane fraction of a ZY6 culture, when prepared in the same day in which it was harvested, was found to have a fairly high level of MT activity (Table 1, run 2). This activity was extremely labile, however, and disappeared within a day of storage at either 4°C or −20°C. Whereas the soluble fraction from *E. coli*(pET-3b) gave no MT activation in the ZY6 membrane, the soluble fractions of *R. capsulatus* ZY6 and *E. coli(bchH)* activated it by factors of 1.6 and 3.8, respectively (Table 1, runs 3–5). The effects were not additive as addition of both of the latter two fractions yielded only a small further increase in MT activity (Table 1, run 6). None of the three soluble phases employed demonstrated MT activity by themselves (data not shown). In order to test whether the overexpressed BchH protein remained bound to the membrane-bound MT, the ZY6 membrane was recentrifuged following its mixture with the *E. coli(bchH)* soluble phase, and again tested for MT activity. The results (Table 1, run 7) revealed that the MT had lost most of its increased activity, but was still 1.4 times more active than it had been before contact with the BchH protein. We also added the soluble fractions of either ZY6 or *E. coli(bchH)* to the membrane fraction of *E. coli(bchM)*. It was found that these fractions increased the activity of the MT in the membrane by factors of 2.5 and 6.8,

Table 2
Radioactivity recovered in Proto and ProtoME bands following separation of magnesium-free porphyrins isolated from MT assays of membranes of *E. coli(bchM)*, with and without the addition of soluble fractions of *R. capsulatus* mutant ZY6 or the overexpressed BchH protein

Run	Membrane fraction	Soluble fraction	Enhancement of MT activity	dpm at R_f 0.03 (Proto)	dpm at R_f 0.50 (ProtoME)	Enhancement of dpm in ProtoME
1	ZY6[a]	–	–	83	31	–
2	*E. coli(bchM)*	–	1.0	113	350	1.0
3	*E. coli(bchM)*	ZY6	2.3	283	823	2.4
4	*E. coli(bchM)*	*E. coli(bchH)*	5.5	301	1253	3.6
5	*E. coli(bchM)*	*E. coli(bchH)*[b]	–	212	688	–

[a]Control incubation (minus Mg Proto).
[b]Mixed with an equal volume of a solution of porphyrin standards.

respectively (Table 1, runs 9–10). To confirm the absence of the MT in a *bchM* mutant, the *R. capsulatus bchM* mutant ZY4 was shown not to have MT activity in cell extracts or in the centrifuged membrane fraction, and the latter was not activated by the addition of the soluble fractions from ZY4 or ZY6 (data not shown).

3.4. Identification of the product of the MT assay as Mg ProtoME

In order to confirm that the product of the MT assay from *E. coli(bchM)* was indeed Mg ProtoME, the porphyrins were isolated after incubations similar to runs 8–10 of Table 1, but carried out in the presence of 2.1 μmol of [*Me*-^{14}C]SAM (48 μCi mmol^{-1}) instead of 2.1 nmol (48 mCi mmol^{-1}). The porphyrins (which had lost magnesium due to acetic acid treatment in the extraction procedure) were then separated by silica gel TLC in system A. A partially hydrolyzed sample of ProtoDE was used as a standard. It exhibited three bands corresponding to Proto, ProtoME, and ProtoDE at R_f-values of 0.03, 0.50, and 0.97, respectively. The enhancement of MT activity in the membrane fraction of *E. coli(bchM)* by the soluble fractions of *R. capsulatus* mutant ZY6 or *E. coli(bchH)* were by factors of 2.3 and 5.5, respectively (Table 2, runs 2–4). These factors were similar to the previously observed factors of 2.5 and 6.8, respectively (Table 1, runs 9–10). Following separation of the porphyrins by TLC, the bands at R_f 0.03 and 0.50 were scraped from the TLC plate and counted for radioactivity. The R_f-0.03 band (Proto) contained a relatively high but constant level of background counts (80–300 dpm) due, perhaps, to incomplete removal of [*Me*−^{14}C]SAM or its radioactive degradation products which would be relatively polar and remain near the origin. The R_f-0.50 band (ProtoME) contained a low background (Table 2, run 1) but exhibited increasing amounts of radioactivity in the three incubations with an active MT (Table 2, runs 2–4), which corresponded to the apparent increase in the amount of product as indicated from the enzyme assays. The enhancement of the dpm in the ProtoME band were calculated to be 2.4 and 3.6, for incubations containing the soluble fractions of *R. capsulatus* mutant ZY6 or *E. coli(bchH)*, respectively, compared to factors of 2.3 and 5.5 for the enhancement of MT activity calculated from the enzyme assays (Table 2, runs 3–4). When the sample of run 4 was mixed 1:1 with the standards solution (and the same volume was spotted), the dpm of the band which co-chromatographed with ProtoME was reduced by 55% (Table 2, run 5), confirming the identity of the radiolabeled compound in this band with ProtoME (derived from the true enzymic product, Mg ProtoME).

4. Discussion

Normally, porphyrins are not overproduced by *E. coli*. This is most likely due to a feedback inhibition by heme (when formed in excess of that required for insertion into hemoproteins) on the synthesis of ALA from glutamate, although such a regulation has so far only been conclusively demonstrated in higher plants, algae, cyanobacteria, and the photosynthetic green sulfur bacteria [26]. Such an accumulation of Proto was also observed [14,15] when the cloned BchH protein of *R. sphaeroides* was overexpressed in *E. coli*, and the Proto remained bound to the BchH protein following three chromatographic steps [15]. It is very likely, therefore, that the BchH protein contains a Proto binding site. The presence of the BchH protein in *E. coli* might, therefore, bind enough Proto to limit its availability to the action of ferrochelatase.

The primary function of the BchH protein in *Rhodobacter* is as one of the subunits of the magnesium chelatase enzyme complex, along with the BchD and BchI proteins [14,15]. Magnesium chelatase subunits homologous to the BchH, -I, and -D proteins have now been isolated from several plant and cyanobacterial species. The BchH protein has a 39% sequence homology with the *oli* gene product of *Antirrhinum majus* [27], 34% with the XanF protein of barley [28], and 40–41% with the ChlH proteins of *Synechocystis* PCC6803 [29] and *Arabidopsis thaliana* [30]. In addition, there is a 27% sequence homology with the *cobN* gene product of *Pseudomonas denitrificans* [27], a 140 000 M_r subunit of the cobaltochelatase enzyme complex. The latter is thought to provide the binding site for its tetrapyrrole substrate, hydrogenobrynic acid a,c-diamide [31]. As discussed above, the BchH protein also very likely provides a binding site for its tetrapyrrole substrate, Proto, in the magnesium chelatase enzyme complex. Neither the BchD nor BchI proteins were found to be homologous to the other two subunits (the products of the *cobS* and *cobT* genes) of the cobaltochelatase of *P. denitrificans* [5]. However, the BchI protein has a 49% sequence homology with residues 70–424 of the *ch42* nuclear gene product (the CS protein) of *Arabidopsis thaliana* [32–34], 49% with the XanH protein of barley [28], and 51% with the ChlI protein of *Synechocystis* PCC6803 [29]. Homologous chloroplast genes have also been identified in *Euglena gracilis* [35], *Cryptomonas phi* (EMBL accession number Z21976), and *Olisthodiscus luteus* (EMBL accession number S32166). The BchD protein has a 32% sequence homology with the ChlD protein of *Synechocystis* PCC6803 [29] and a possible homology with the XanG protein of barley [28]. In the case of *Synechocystis* PCC6803 [29], the ChlH, -I, and -D proteins were overexpressed in *E. coli* and shown to all be required to reconstitute magnesium chelatase activity. The other homologous proteins will, no doubt, eventually become known as the ChlH, -I, and -D proteins, respectively. It is known that both the magnesium chelatase of higher plants [36,37], *R. sphaeroides* [14,15], and *Synechocystis* PCC6803 [29], and the cobaltochelatase of *P. denitrificans* [31] require ATP hydrolysis for activity. Both the BchI and CS proteins have a putative ATP binding site [34], and this feature has now been found in all other BchI homologues [29]. Gorchein [38] has also recently demonstrated that magnesium chelation of Proto taken up by whole cells of *R. sphaeroides* required an energy source (presumably ATP). The product of the latter process was Mg ProtoME not Mg Proto, however, leading to a revival of the speculation [8] that the magnesium chelatase and MT were closely associated in vivo, perhaps as a complex. Hence, the BchH protein may also function to link the magnesium chelatase enzyme complex to the MT (the BchM protein) in the membrane, although the formation of such a complex remains to be demonstrated. In the process, however, it may also stabilize the MT and stimulate it to a higher level of activity.

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