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Effect of the PufQ protein on early steps in the pathway of bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus*

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Abstract The addition in *trans* of the *pufQ* gene to a strain of *Rhodobacter capsulatus* from which the entire *puf* operon had been deleted, increased its ability to synthesize coproporphyrinogen from both δ-aminolevulinic acid and porphobilinogen. Studies at the enzyme level indicated that the conversion of porphobilinogen to uroporphyrinogen III had about a 2-fold higher level of activity in the anaerobically-grown *pufQ*-containing strain. This increase in activity over the *puf*-deletion strain appeared to occur during transitions from aerobic to semiaerobic growth conditions. These results indicated that the PufQ protein may exert a stimulatory effect quite early in the pathway of bacteriochlorophyll biosynthesis.

Key words: PufQ protein; *Rhodobacter capsulatus*; Stimulation of bacteriochlorophyll biosynthesis; Porphobilinogen deaminase; Uroporphyrinogen III synthesis

1. Introduction

PufQ is the first gene of the *puf* operon, which also encodes genes for proteins of the reaction center and B870 light-harvesting complexes of *Rhodobacter capsulatus*. In *puf* operon-deletion mutants of *R. capsulatus* (such as strain ΔRC6), only a very small amount of bacteriochlorophyll (Bchl) is formed [1,2]. When the *pufQ* gene was added to these mutants in *trans*, the formation of Bchl (present in vivo as the B800-850 light-harvesting complex) was observed at near normal levels [1,2]. Hence, expression of the *pufQ* gene is apparently not absolutely required for Bchl synthesis, but seems to stimulate it to an optimum level. The PufQ protein appears to affect only that part of the tetrapyrrole biosynthetic pathway specific to Bchl, and does not affect the synthesis of hemes and cytochromes [1]. Furthermore, every step of the Bchl-specific pathway seems to be affected equally. Bauer and Marrs [1] carried out studies with seven strains of *R. capsulatus* which contained functional mutations in specific genes encoding enzymes of the magnesium branch of Bchl synthesis (the *bch* genes). These mutants formed no Bchl-protein complexes and accumulated elevated levels of Bchl intermediates. Deletions of the *puf* operon were then introduced into each strain. In all cases, the production of Bchl intermediates was greatly reduced, while the addition of the *pufQ* gene in *trans* restored the ability of all mutants to accumulate intermediates (which ranged from protoporphyrin IX to bacteriochlorophyllide).

The *puf* operon is almost completely repressed by oxygen [3,4], while *bch* genes are not repressed nearly as severely [5]. Sganga and Bauer [3] have recently shown that mRNA formed from two *bch* genes was induced normally by low oxygen in mutants of *R. capsulatus* unable to induce the *puf* operon. Therefore, if the PufQ protein is exerting its effect post-transcriptionally, it may act: (a) by stimulating an early step of the tetrapyrrole pathway to an increased level of activity required for Bchl synthesis; (b) by activating an enzyme at the branch point of the Bchl-specific pathway from the heme pathway; or (c) as a carrier protein of tetrapyrrole intermediate(s) in the Bchl-specific pathway. With respect to the latter hypothesis, we have recently presented evidence for specific (but substoichiometric) binding of the tetrapyrrole intermediate, protochlorophyllide, to the PufQ protein in reconstituted liposomes [6]. Kaplan and coworkers [7] have recently reported, however, that they have only found evidence for the involvement of the Q protein in the assembly of both the B875 and B800-850 light-harvesting complexes in the related bacterium *Rhodobacter sphaeroides*, rather than in the biosynthesis of Bchl itself.

Bchl synthesis is traditionally considered to occur in two stages. The first stage forms protoporphyrin IX from glycine and succinyl-CoA in a pathway common to the synthesis of both heme and Bchl. Biel [8] has recently postulated that the true committed step for the tetrapyrrole biosynthetic pathway may be porphobilinogen (PBG) synthase, rather than δ-aminolevulinic acid (ALA) synthase (since ALA appears to be involved in other reactions of general metabolism), and has presented evidence that PBG synthase is under oxygen regulation. The first step of the tetrapyrrole biosynthetic pathway committed to Bchl synthesis has traditionally been considered to be the branch point reaction catalyzed by magnesium chelatase. However, the true branch point for Bchl synthesis may occur earlier than magnesium chelatase. For example, Hunter and coworkers [9] have recently demonstrated that there are separate aerobic and anaerobic coproporphyrinogen (Coprogen) III oxidases in *R. sphaeroides*. Although no evidence has been presented for separate aerobic and anaerobic isozymes in earlier parts of the pathway, the question may now be asked as to exactly where the Bchl-specific pathway begins. It may be possible that the PufQ protein's presence is required to stimulate Bchl synthesis as a component of an induction system for one or more anaerobic (Bchl-specific) isozyme(s) in the early portion of the tetrapyrrole pathway. We present herein the results of whole-cell incubation studies which have demonstrated that the possession of the *pufQ* gene had a dramatic effect on the

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Abbreviations: ALA, δ-aminolevulinic acid; Bchl, bacteriochlorophyll; Coprogen, coproporphyrinogen; HMB, hydroxymethylbilane; PBG, porphobilinogen; Urogen, uroporphyrinogen.

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production of both Bchl and Coprogen from either ALA or PBG. In particular, the conversion of PBG to uroporphyrinogen (Urogen) III was found at a higher level of activity in cell extracts of the anaerobically-grown *pufQ*-containing strain ΔRC6(pΔ4) than in ΔRC6, indicating that the PufQ protein may affect the Bchl-specific pathway by stimulating increased production of an early enzyme in the tetrapyrrole pathway.

2. Materials and methods

2.1. Growth and incubation of strains ΔRC6 and ΔRC6(pΔ4) in the presence of bacteriochlorophyll precursors and inhibitors

R. capsulatus mutant strains ΔRC6 and ΔRC6(pΔ4) were grown anaerobically in 50-ml closed bottles in the dark at 30°C in RCV$^+$ medium [10] in the presence of kanamycin (10 $\mu g \cdot ml^{-1}$), or kanamycin 10 $\mu g \cdot ml^{-1}$) plus tetracycline (0.5 $\mu g \cdot ml^{-1}$), respectively. The bacteria were grown either with no additions or supplemented with one or more of the following as indicated in Table 1: (a) 1 mM ALA; (b) 0.25 mM PBG; (c) 0.07 mM *N*-methyl-protoporphyrin IX (Porphyrin Products Inc., Logan, UT); and (d) 12 mM nicotinamide. Following growth to the stationary phase, the in vivo spectra of the cultures between 600 and 900 nm were recorded with a Beckman model DU 640 spectrophotometer, which corrected for light scattering due to cell turbidity by automatic adjustment of the baseline. The relative contents of Bchl and protochlorophyllide were first normalized to constant turbidity by dividing the observed (corrected) A_{860} and A_{635} values, respectively, by (uncorrected) A_{680} values. These relative pigment contents were then normalized to constant (10^9) cell number by counting the number of cells contained in 10-fold dilutions of mid-log phase (A_{680} = 0.3–0.4) cultures of each strain with a La Fontaine 'Neubauer improved' hemocytometer using a Zeiss phase-contrast microscope at a magnification of 400-fold. Cultures of strains ΔRC6 and ΔRC6(pΔ4) were found to contain 3.44×10^8 and 5.58×10^8 cells per A_{680} unit, respectively. The formation of Coprogen was estimated after the cultures were centrifuged in a Sorval SS34 rotor at 17,500 × *g* for 20 min. Coprogen excreted into the culture medium was oxidized to coproporphyrin with 2.65 mM benzoquinone by the method of Jordan [11]. Following a 5-fold dilution with 1 M HCl, A_{405} values were measured and normalized to constant (10^9) cell number as described above, using A_{680} values of the cultures from which they had been excreted. Subsequently, the culture media were brought to pH 3.5 and extracted with ethyl acetate; porphyrins in the extract were examined by paper chromatography in 2,6-lutidine/water (5:3, v/v) in ammonia vapor as previously described [12]. Cultures of both strains were also grown in 25 ml of RCV$^+$ media in the absence of additions, and centrifuged as described above. The cell pellets were then resuspended in the same volume of 50 mM Tris-Cl (pH 8.0) containing either 1 mM ALA or 0.25 mM PBG plus 10 μM levulinic acid, incubated for 14 h at 30°C with gentle shaking (100 rpm), and subsequently analyzed for Coprogen synthesis as described above.

2.2. Preparation of cell fractions for PBG deaminase assays

R. capsulatus mutant strains ΔRC6 and ΔRC6(pΔ4) were grown anaerobically in 1-liter closed bottles in RCV$^+$ medium as described above. Cells were harvested when in the log phase (A_{680} = 0.4–0.6) and washed once in buffer A (0.05 M potassium phosphate, pH 7.5) as previously described [13]. The washed cells were resuspended in 12 ml of buffer A, disrupted by sonication, and separated into soluble and membrane fractions as previously described [13]. The membrane fractions were washed once, resuspended in buffer A to the same volume as before sedimentation, and used (along with the soluble fractions) for the PBG deaminase assays reported in Table 2. The mutants were also grown aerobically in 6-liter flasks in the dark at 30°C in RCV medium [14], supplemented with 0.1% (w/v) Bactotryptone, in the presence of the antibiotics described above. When still in the log phase, transition of the cells from aerobic to semiaerobic growth was accomplished by changing aeration and stirring rates from rapid to gentle. Analysis with a Beckman oxygen analyzer indicated that the dissolved oxygen content changed from 16–18% to 0–8% (calibrated with air saturation as 20%). Samples of 500 ml were collected at various times after the shift to semi-aerobic conditions, and cell extracts (prepared without separation into soluble and membrane fractions) were employed for the PBG deaminase assays of Table 3.

2.3. Preparation of the PufQ protein and reconstitution into liposomes

The assembly of expression vector pSF3, which carries a *malE-pufQ* gene fusion, has been previously described [15]. Recombinant fusion protein was expressed in a 15-liter culture of *E. coli* strain TB1 transformed with vector pSF3 and grown in 2 × TY medium at 37°C as previously described [15]. The fusion protein was purified by affinity chromatography and the PufQ protein liberated from the fusion protein by proteolysis and reconstituted into soybean phospholipid vesicles as previously described [6]. Liposomes containing 7 mg of phospholipids (±68 μg of hydrolyzed fusion protein) were combined with soluble fractions and employed for PBG deaminase assays reported in Table 2.

2.4. PBG deaminase and protein assays

Assays for PBG deaminase [16] were incubated for 30 min at 37°C, and contained 0.22 mM PBG, 0.17 to 0.65 mg of protein, and buffer A (to a final volume of 1.0 ml). Reaction was stopped by heating the sample for 2 min in boiling water followed by sedimentation of denatured proteins in a Heraeus Biofuge 15 table-top centrifuge at 12,000 rpm for 20 min. The product Urogen was then oxidized to uroporphyrin with 0.4 mM iodine in 0.5 M HCl by the method of Jordan and Shemin [16]. Following decolorization of the residual iodine with 5.3 mM $Na_2S_2O_5$ in 0.5 M HCl, the concentration of uroporphyrin was calculated from A_{405} values, using an extinction coefficient of 541 $mM^{-1} \cdot cm^{-1}$. Protein was determined by the method of Lowry et al. [17]. PBG deaminase specific activity was expressed as nmol Urogen $\cdot h^{-1} \cdot$ (mg protein)$^{-1}$, using values for *soluble* protein only (for the assays of Table 2), and total protein (for the assays of Table 3). Assay solutions following oxidation were brought to pH 1.8 and extracted with cyclohexanone; uroporphyrin in the extract was converted to its octamethyl ester and the isomer type (I or III) was estimated by the paper chromatographic system of Falk and Benson [18] as previously described [12].

3. Results and discussion

3.1. Growth and incubation of strains ΔRC6 and ΔRC6(pΔ4) in the presence of bacteriochlorophyll precursors and inhibitors

Tetrapyrrole production under various conditions of anaerobic growth was compared in the two strains of *R. capsulatus* (Table 1). Bchl formation was usually about 2 times higher in strain ΔRC6(pΔ4), which had the *pufQ* gene added in *trans* on the pΔ4 vector [19], than in the *puf*-deletion strain ΔRC6 (Table 1, run 1), although in some cultures of strain ΔRC6, Bchl synthesis was barely detectable (results not shown). The effects on Bchl synthesis by growth in the presence of the porphyrin precursors, ALA and PBG, were qualitatively similar, since Bchl synthesis was stimulated in both strains. Quantitatively, however, the presence of the *pufQ* gene consistently resulted in 2–3 times more Bchl being formed by strain ΔRC6(pΔ4) (Table 1, run 1). The ferrochelatase inhibitor, *N*-methyl-protoporphyrin IX, has been shown to stimulate porphyrin production by release of feedback inhibition by heme on ALA synthase [20], while nicotinamide has been shown to inhibit Bchl synthesis in *R. sphaeroides* [21] and cause the accumulation of a mixture of protochlorophyllide and its 4-desethyl-4-vinyl derivative. (The relative proportions of these components was not determined in the present work and the product is simply referred to below as 'protochlorophyllide'.) When nicotinamide was added to incubation mixtures containing either ALA or *N*-methyl-protoporphyrin IX, protochlorophyllide was formed by both strains; however, in this case the effect of *pufQ* was much more pronounced. The *pufQ*-containing strain formed approximately 5 (with ALA) to 20 (with *N*-methyl-protoporphyrin IX) times more than the *puf*-deletion strain (Table 1, run 2). It was also found that both ALA and PBG led to the accumulation of relatively large amounts of porphyrinogens, which were excreted into the growth medium. Following benzoquinone oxi-

Table 1
Analysis of Bchl, protochlorophyllide, and Coprogen synthesis in *R. capsulatus* strains ΔRC6(pΔ4) and ΔRC6

Run[a]	Additions	Pigment	Relative absorbance[b] (per 10^9 cells) in strain: ΔRC6(pΔ4)	ΔRC6	Ratio of relative absorbances of: ΔRC6(pΔ4)/ΔRC6
1	none	Bchl	0.280	0.147	1.9
	ALA	Bchl	0.376	0.195	1.9
	PBG	Bchl	0.501	0.186	2.7
2	none	Pchlide	ND[c]	ND[c]	–
	ALA + NA	Pchlide	0.076	0.014	5.4
	NMP + NA	Pchlide	0.104	0.005	≈20
3	none	Copro	0.17	0.12	1.4
	ALA	Copro	2.02	1.14	1.8
	PBG	Copro	2.76	1.63	1.7
4	ALA	Copro	2.88	0.47	6.1
	PBG + LA	Copro	3.62	0.83	4.4

[a] Runs 1 and 3 = cells were grown for 96 h with no additions or in the presence of either ALA or PBG; run 2 = cells were grown for 36 h with no additions or in the presence of nicotinamide (NA) plus either ALA or *N*-methyl-protoporphyrin IX (NMP); run 4 = fully grown cells were incubated for 14 h in the presence of either ALA alone or PBG plus levulinic acid (LA).
[b] The relative levels of Bchl or protochlorophyllide (Pchlide) were determined from A_{860} or A_{635} values of whole cells, respectively, while excreted Coprogen was determined from A_{405} values of centrifuged media after oxidation to coproporphyrin (Copro); all absorbance values were then normalized for constant cell number as described in section 2.
[c] ND = not detected.

dation and examination by paper chromatography, the resulting porphyrins were found to consist predominantly of coproporphyrin, with traces of uroporphyrin and porphyrins with 5–7 carboxy-groups also visible; however, the isomer types (I or III) were not determined. Again, the results were qualitatively, but not quantitatively, similar with the *pufQ*-containing strain forming 1.5–2 times more Coprogen than the *puf*-deletion strain (Table 1, run 3). In an attempt to eliminate the possibility of de novo porphyrin synthesis from succinyl-CoA and glycine in the presence of PBG, another experiment was conducted by incubation of fully grown cells in the presence of PBG plus levulinic acid. Levulinic acid inhibits PBG synthase in *R. capsulatus*, and also is a potent inhibitor of growth [22]. In this latter case, Coprogen production would be entirely from PBG. The results indicated that the presence of the *pufQ* gene resulted in 4 times more Coprogen production from PBG, as compared to 6 times more from ALA (Table 1, run 4). These results are consistent with an effect of the PufQ protein on the conversion of PBG to Coprogen III, a portion of the tetrapyrrole biosynthetic pathway which involves only three enzymes.

3.2. *Analysis of cell extracts of strains ΔRC6 and ΔRC6(pΔ4) for PBG deaminase activity*

The first two of the three enzymes required for conversion of PBG to Coprogen III are hydroxymethylbilane (HMB) synthase and Urogen III synthase. HMB synthase is an extremely slow enzyme (turnover number ≈0.5 s^{-1}), whereas Urogen III synthase is much faster (turnover number ≈200 s^{-1}) [23]. The third enzyme of the sequence, Urogen III decarboxylase, has recently been partially purified from *R. sphaeroides* by Jones and Jordan [24]. They determined some of its kinetic parameters, but a turnover number was not reported. Hence, although it is possible that it might be the rate-limiting step in the conversion of PBG to Coprogen III, we chose to examine the activity of the known slow enzyme, HMB synthase, as a likely candidate for metabolic regulation in this portion of the pathway. It is normally assayed by determining the rate of the conversion of PBG to Urogen III (referred to below as 'PBG deaminase') in which the activity of HMB synthase should be rate-limiting. We found the specific activity of PBG deaminase to be about 2 times higher in the ΔRC6(pΔ4) mutant than in the ΔRC6 mutant when both were grown anaerobically (Table 2, runs 1 and 2). Our assays were carried out at a PBG concentration about 10–17 times higher than the reported PBG deaminase K_m-values of 22 μM for *Rhodopseudomonas palustris* [25] and 13 μM for *R. sphaeroides* [16]; hence our activities should have been close to V_{max}-values.

This experiment could not distinguish whether the effect was due to an enhancement of the activity of one or both enzymes (present in constant concentrations in both strains), or to an

Table 2
The effect of the *pufQ* gene on specific activities of PBG deaminase in anaerobically-grown mutants of *R. capsulatus*

Run	Soluble fraction	Membrane fraction	PBG deaminase: Specific activity[a]	Relative activity
1	ΔRC6	–	25.8	1.00
	ΔRC6(pΔ4)	–	50.4	1.95
	–	ΔRC6	3.4[b]	–
	–	ΔRC6(pΔ4)	4.9[b]	–
2	ΔRC6	ΔRC6	24.7	1.00
	ΔRC6(pΔ4)	ΔRC6(pΔ4)	53.1	2.15
3	ΔRC6	ΔRC6	24.7	1.00
	ΔRC6	ΔRC6(pΔ4)	25.6	1.04
4	ΔRC6(pΔ4)	ΔRC6(pΔ4)	53.1	1.00
	ΔRC6(pΔ4)	ΔRC6	51.2	0.96
5	ΔRC6	–	24.6	1.03
	ΔRC6	liposomes	23.8	1.00
	ΔRC6	liposomes + PufQ protein	27.0	1.13
6	ΔRC6(pΔ4)	–	48.1	1.19
	ΔRC6(pΔ4)	liposomes	40.4	1.00
	ΔRC6(pΔ4)	liposomes + PufQ protein	43.8	1.08

[a] Results are the average of duplicate assays (in nmol Urogen · h^{-1} · (mg soluble fraction protein)$^{-1}$) with an average deviation of ±1.0.
[b] In nmol Urogen · h^{-1} · (mg membrane fraction protein)$^{-1}$.

increase in the concentration of one or both enzymes in the *pufQ*-containing strain, ΔRC6(pΔ4). We therefore attempted to demonstrate a *direct* effect of the PufQ protein on PBG deaminase activity, which might occur, for example, if the PufQ protein allosterically increased the V_{max} of HMB synthase. The presence of Urogen III synthase has been reported to facilitate release of HMB from the HMB synthase of *R. sphaeroides* [26]. If the two enzymes formed a complex in vivo, the transfer of the unstable intermediate, HMB, from one active site to the other might be facilitated [23]. Hence, another possible effect of the PufQ protein might be to promote complex formation between the two enzymes or otherwise aid in the transfer of HMB between them. If HMB is not used fast enough by Urogen III synthase, it would spontaneously cyclize and form Urogen I. We therefore studied the isomer composition of the Urogen produced in our PBG deaminase assays. Although not quantitative, the results (not shown) indicated that the Urogen produced by both mutants was predominantly the III-isomer, with only small amounts of the I-isomer visible. Hence, HMB was apparently being efficiently converted to Urogen III in both mutants.

HMB synthase and Urogen III synthase are both soluble. On the other hand, the hydrophobic PufQ protein has recently been demonstrated by Western blot analysis [13] to be contained in the membrane fraction of the ΔRC6(pΔ4) strain. In addition, we have found that washed membrane fractions separated from both strains contained ≤2.2% of the total PBG deaminase activity of supernatant fractions. We therefore mixed the supernatant fraction of the ΔRC6 strain (which lacks the *pufQ* gene) with the membrane fraction of the ΔRC6(pΔ4) strain, and vice versa. The results demonstrated only a marginal increase in PBG deaminase activity in the ΔRC6 soluble phase in contact with the ΔRC6(pΔ4) membrane (Table 2, run 3), and only a marginal decrease in PBG deaminase activity in the ΔRC6(pΔ4) soluble phase in contact with the ΔRC6 membrane (Table 2, run 4). Similar results were obtained when we used liposomes into which the overexpressed PufQ protein had been reconstituted (Table 2, runs 5 and 6). Empty liposomes led to a slight inactivation of PBG deaminase activity when mixed with the soluble fractions from both strains. However, inclusion of liposomes reconstituted with the PufQ protein again increased PBG deaminase activity in both cases, but only marginally (ca. 1.1 times). This result would have been expected in the case of PBG deaminase from the ΔRC6(pΔ4) strain if one or both of the enzymes had already been activated by being in contact with the PufQ protein. However, since the PBG deaminase from the ΔRC6 strain (which had not previously encountered the PufQ protein) was also not activated significantly, it is much more likely that the difference in the level of PBG deaminase activity in the two strains is due to an increase in the concentration of one or both of the enzymes in the ΔRC6(pΔ4) strain. However, in order to confirm this, their actual concentrations would need to be measured after purification to homogeneity of both enzymes from each strain. The effect of possession of the *pufQ* gene on Urogen III decarboxylase activity will also need to be investigated.

Table 3
Changes in PBG deaminase specific activities during adaptation of mutants of *R. capsulatus* from aerobic to semiaerobic growth conditions

Time after shift from aerobic (h)	PGB deaminase specific activity[a] in strain:		Ratio of PBG deaminase specific activities:
	ΔRC6(pΔ4)	ΔRC6	ΔRC6(pΔ4)/ΔRC6
0.5	16.4	17.1	0.96
1	15.3	14.6	1.05
2	16.0	13.8	1.16
3	18.7	17.1	1.09
4	22.3	15.3	1.46
5	18.9	14.4	1.31

[a] Results are the average of quadruplicate assays (in nmol Urogen · h^{-1} · (mg total protein)$^{-1}$) with an average deviation of ±1.8.

3.3. PBG deaminase activity during adaptations of strains ΔRC6 and ΔRC6(pΔ4) from aerobic to semiaerobic growth conditions

The pΔ4 vector was constructed with the *pufQ* gene immediately downstream of the oxygen-regulated *puf*-promotor [19], and should be induced by lowering the oxygen content of the medium. We found (Table 3) that after aerobic growth, PBG deaminase activity was similar in extracts from both strains. However, whereas the activity remained relatively constant in the ΔRC6 strain after lowering the oxygen content of the medium, the specific activity of PBG deaminase in the ΔRC6(pΔ4) strain had increased 1.3–1.4 times after 4–5 h, implying that the increase was due to some effect caused by increased transcription of the *pufQ* gene. Although both mutant strains can form the B800-850 light-harvesting complex, if the only effect of the PufQ protein is on the assembly of light-harvesting complexes (as proposed for *R. sphaeroides* by Kaplan and coworkers [7]), it is difficult to see how this early portion of the tetrapyrrole pathway could also be affected.

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