Rhodobacter capsulatus puf operon encodes a regulatory protein (PufQ) for bacteriochlorophyll biosynthesis

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ABSTRACT - Biosynthesis of the photochemical apparatus by purple nonsulfur photosynthetic bacteria is known to be inhibited by molecular oxygen and high light intensity. Polypeptides that bind bacteriochlorophyll (BChl) to form the light-harvesting I (LH-I) and reaction-center (RC) complexes are encoded by a single transcriptional unit termed the puf operon. In this investigation we demonstrate that the first structural gene in the puf operon (pufQ) of Rhodobacter capsulatus encodes a protein that is required for BChl biosynthesis and that there exists a linear relationship between the amount of pufQ expression and the level of BChl synthesis. Protein sequence similarity exists between PufQ and the region of RC polypeptides that are known to bind BChl and quinone. These observations suggest that pufQ may regulate BChl biosynthesis by a "carrier polypeptide" mechanism as originally proposed by Lascelles.

Conversion of light energy into useful forms of cellular energy requires the formation of membrane-bound pigment–protein complexes. Synthesis of the photochemical apparatus in purple nonsulfur photosynthetic bacteria is known to be regulated in response to oxygen tension and light intensity and is coordinated in such a way that neither free bacteriochlorophyll (BChl) nor free pigment-binding polypeptides accumulate during regulatory transitions. Several studies have established that synthesis of polypeptides that bind BChl to form the light-harvesting (LH) and reaction-center (RC) complexes is regulated by transcriptional control (1–5).

In contrast, evidence suggests that the flow of tetrapyroles through the BChl biosynthetic pathway may be controlled by a post-transcriptional mechanism of regulation (reviewed in ref. 6). This conclusion is based, in part, on the observations that when photosynthetically growing cells are subjected to environmental shifts, such as the introduction of oxygen (7), high light intensity (8), or inhibitors of protein synthesis (8, 9), there is an abrupt cessation of BChl biosynthesis that is too rapid to simply be attributed to inhibiting the synthesis of enzymes in the BChl biosynthetic pathway. In addition, classical forms of regulation such as feedback inhibition by BChl or its precursors do not occur (6, 10–12). Finally, intermediates in BChl biosynthesis are known to form insoluble tetrapyrole–protein–lipid complexes (6, 10–12) that are associated with a small (M, 9,000) hydrophobic protein (12).

These observations led Lascelles to propose that BChl biosynthesis could be controlled by regulating the synthesis or activity of a membrane-associated polypeptide that forms the tetrapyrole–protein complex required for BChl biosynthesis (10, 13). However, no direct evidence for or against this model has been presented.

In a previous study (14), we demonstrated that the oxygen and light-regulated puf operon of Rhodobacter capsulatus, which is known to encode BChl-binding polypeptides that form the LH-I and RC complexes (15), also includes a small gene (pufQ) of unknown function. In this investigation, we report that expression of the pufQ gene is required for BChl biosynthesis and that the level of BChl biosynthesis is directly dependent on the amount of pufQ expression. Finally, we note that the PufQ polypeptide exhibits significant protein sequence similarity to the membrane-spanning segments of RC polypeptides that bind both BChl and quinones. This similarity suggests that the role of PufQ in BChl biosynthesis could involve a tetapyrrole–protein interaction similar to that proposed for the carrier polypeptide by Lascelles (10, 13) and that pufQ may interact with the quinone pool.

MATERIALS AND METHODS

Growth Conditions. R. capsulatus was routinely grown either aerobically or photosynthetically in a complex medium composed of 0.3% peptone, 0.3% yeast extract, 2.0 mM CaCl2, and 2.0 mM MgSO4 (PYS medium) or in a minimum-salt medium (RCV medium; ref. 16). For spectral analysis, the cells were grown under low oxygen tension (14) in RCV medium supplemented with 0.6% glucose, 0.5% pyruvate, 50 mM dimethyl sulfoxide (RCV + medium; ref. 17). For anaerobic growth in the dark with different nitrogen sources, the cells were grown in screw-capped tubes filled with RCV + medium in which (NH4)2SO4 had been replaced by the indicated nitrogen source at a concentration of 8.0 mM.

Strain Construction. Mating conditions between Escherichia coli and R. capsulatus were as described by Marrs (18).

Mobilization of recombinant plasmids was accomplished by using the mobilizing plasmid pDPT51 (19). Construction of a defined chromosomal deletion of the puf operon [Δ(pufQ–pufC397/502)] was accomplished by GTA (gene-transfer agent)–mediated homologous recombination (20) of a DNA fragment (I segment) encoding spectinomycin resistance as described (14, 21).

Plasmid Construction. A plasmid was constructed that allowed us to both vary and assay the amount of puf operon transcription (pCB610). This plasmid was derived from pCB552, which is a construct containing the pufQ–B–A and -L genes followed by a translational fusion of pufM to the E. coli lacZ structural gene (14, 22). The puf operon promoter was replaced by the R. capsulatus nifHDK promoter by cloning a HindIII–NdeI restriction fragment obtained from pNF1 (23) into the Xho I–Nco I sites of pCB552 and by using T4 DNA polymerase to blunt-end the vector and insert DNA. Finally, an EcoRI restriction fragment (obtained from pUC4-KIXX; Pharmacia) encoding kanamycin resistance was inserted upstream from the nifHDK promoter in an opposite transcriptional orientation. The resulting construct (pCB610) contains the nifHDK promoter located 26 bp upstream of the puf operon.

Abbreviations: LH-I and LH-II, light harvesting complexes I and II; RC, reaction center; BChl, bacteriochlorophyll.

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upstream from the pufQ gene initiation codon followed by the pufB, -A, -L, and -M::lacZ genes.

Plasmid pDAY1 (D. A. Young, C.E.B., and B.L.M., unpublished data) was used for the trans addition of pufQ into strains containing a deletion of the puf operon. Briefly, pDAY1 is a pBR322 construct containing a derivative of the EcoRI–Q restriction fragment from the puf operon, which bears a kanamycin-resistance gene, the puf operon promoter, and the pufQ structural gene (14, 19), cloned into the EcoRI site of pBR322.

**BChL and β-Galactosidase Assays.** Spectral scans of tetrapyrroles were performed on crude membrane preparations obtained from cells grown in RCV + medium under low oxygen tension (14) and on pigments that were extracted from cell pellets with acetone/methanol (7:2) as described (14, 24). Fluorescence measurements of tetrapyrroles produced by bchD and bchH mutations (MB1008 and BR50, respectively), and β-galactosidase assays were performed as described by Biel and Marrs (25).

**RESULTS**

**BChL Biosynthesis Is Dependent on Expression of the pufQ Gene.** The puf operon has been shown to encode structural polypeptides for the LH-I (pufB and -A) and RC (pufL and -M) complexes (15) and an open reading frame (pufQ) encoding a protein of unknown function (14). Previously it had been established that point mutations that disrupt the formation of LH and RC complexes had little or no effect on BChl biosynthesis (26–28). However, it also had been observed that a deletion of the puf operon resulted in a reduction in the steady-state levels of BChl (29). These and other observations lead us to test whether proteins encoded by the puf operon are required for expression of the BChl biosynthetic pathway. We first constructed a strain containing a deletion of the pufQ through pufM genes (CB1064), which, as observed by Youvan et al. (29), exhibited a severe reduction in steady-state BChl levels (Fig. 1 Middle). However, we also observed that a normal amount of BChl was restored when we added back the first gene of the puf operon (pufQ) in trans with the plasmid pDAY1 (Fig. 1 Bottom). This result shows that BChl biosynthesis is dependent on expression of the pufQ gene but not upon synthesis of the LH-I and RC polypeptides encoded by the pufB through pufM genes. Furthermore, evidence is provided below that argues against this phenotype being a result of the inhibition of the synthesis or assembly of the LH-II complex.

**The Level of BChl Biosynthesis Correlates with the Amount of pufQ Expression.** To determine if the level of BChl is directly dependent on the amount of pufQ biosynthesis, we constructed a plasmid (pCB610) that allows us to both vary and measure the amount of puf operon expression. This plasmid contains the nifHDK promoter located just upstream from the pufQ, -B, -A, and -L genes followed by a translational fusion of pufM to the E. coli lacZ gene (see Fig. 2 A and Materials and Methods). We chose to drive expression of the puf genes with the nifHDK promoter because expression initiated from this promoter can be widely varied by growing the cells with different nitrogen sources (23). By introducing pCB610 into the strain of *R. capsulatus* that has a chromosomal deletion of the puf operon (CB1064), we could easily vary the amount of pufQ expression initiated from the nifHDK promoter by growing CB1064/pCB610 with different nitrogen sources and could quantitate the amount of puf operon (pufQ) expression by assaying the amount of β-galactosidase activity expressed from the pufM::lacZ fusion.† Fig. 2B shows the result of plotting puf operon expression.

†The assumption that the amount of PufQ synthesis is proportional to the amount of PufM::LacZ synthesis is supported by the observation that a plasmid that contains the nifHDK promoter upstream from a pufM::lacZ fusion (pNiF-Q-Z) exhibits similar levels of synthesis and regulation by nitrogen sources as does the pufM-lacZ fusion in pCB610 (23).
expression (β-galactosidase activity) vs. BChl levels in CB1064/pCB610 when grown under different nitrogen sources. The linear relationship shows that there is a direct correlation between the amount of puf operon expression and the amount of BChl that is synthesized. This relationship is not an artifact of growth conditions because substitution of pCB610 with a plasmid encoding the normal puf operon promoter, which is insensitive to nitrogen levels (pCB552; ref. 14), resulted in a constant level of puf operon expression and BChl levels when the plasmids were similarly grown with various nitrogen sources (data not shown). From these results we conclude that the level of BChl biosynthesis is dependent on the amount of pufQ expression.

**pufQ Functions at or Before the Mg–Fe Branchpoint in the Biosynthetic Pathway.** Heme and BChl biosynthesis are thought to share a common pathway up to protochlorophyll IX, at which point the pathways diverge, either by the addition of Fe to form heme or by the addition of Mg to form Mg-protoporphyrin, a precursor to BChl (see refs. 6 and 25 for the location of enzymes in the BChl biosynthetic pathway). It has been observed previously that mutants that disrupt enzymes in the BChl biosynthetic pathway build up easily detectable levels of intermediate tetrapyrroles that are characteristic of the location of the mutant enzyme in the biosynthetic pathway. For example, bchG mutants synthesize high amounts of bacteriochlorophyllide a, whereas bchC mutants synthesize high levels of 2-desacetyl-2-hydroxyethyl bacteriochlorophyllide a. This is in contrast to the phenotype of pufQ− strains, which synthesize reduced amounts of mature BChl without a detectable increase in intermediate tetrapyrroles. We could determine which stages in the pathway require PufQ by assaying whether the accumulation of tetrapyrrole intermediates by bch mutants requires pufQ expression. For example, the construction of a puf operon deletion in a bchA− strain (CB1101) resulted in a severe reduction in the biosynthesis of 2-devinyl-2-hydroxyethyl chlorophyllide a. This reduction is clearly due to the loss of PufQ biosynthesis because the trans addition of pufQ into CB1101 (pCB101/pDAY1) resulted in the restoration of intermediate tetrapyrrole production. Similar results were obtained with strains containing mutations in bchC, E, F, G, H, and D. Thus, synthesis of tetrapyrrole intermediates from Mg-protoporphyrin through bacteriochlorophyllide a is dependent upon the expression of the pufQ gene. Finally, it should be noted that cytochrome levels were not affected by the presence or absence of pufQ (data not shown), thus suggesting that the heme branch of the pathway is unaffected.

**DISCUSSION**

This investigation shows that expression of the pufQ gene is required for BChl biosynthesis. Several of our results suggest that pufQ encodes a polypeptide that controls the rate at which tetrapyrroles enter the biosynthetic pathway rather than simply an enzyme that catalyzes one of the steps in BChl biosynthesis. This conclusion is based on the observation that mutants that contain inactivated enzymes in the biosynthetic pathway characteristically accumulate increased levels of intermediate tetrapyrroles (6, 10–12, 25). This is in contrast to strains in which pufQ is deleted, which synthesize reduced amounts of BChl without a detectable increase in tetrapyrrole intermediates. Furthermore, synthesis of each of the known intermediates in the Mg branch of the BChl biosynthetic pathway is dependent on pufQ expression. This latter observation shows that PufQ does not simply affect turnover of BChl that has not properly assembled into the LH/RC components. Finally, the strongest evidence for our conclusion that pufQ encodes a protein that regulates the amount of BChl synthesis is based on the observation that there exists a linear relationship between the amount of pufQ expression and the level of BChl biosynthesis, indicating a rate-limiting role for PufQ. This finding shows that transcription of the puf operon fixes the amount of BChl biosynthesis relative to the synthesis of the LH-I and RC polypeptides that are also encoded by this operon.

Where in the biosynthetic pathway does PufQ impose its mode of regulation? Earlier investigations have established that heme and BChl biosynthesis share a common pathway up to protochlorophyll IX, at which point the pathways branch either through the addition of Fe to form heme or Mg to form Mg-protoporphyrin (Fig. 3; ref 6). Since strains deleted for the puf operon do not accumulate any of the intermediates in the BChl pathway but retain their normal complement of cytochromes, it seems most likely that regulation occurs at the stage where Mg is inserted into protochlorophyll IX.

How then does PufQ regulate the flow of tetrapyrroles into the Mg branch? Although direct evidence for the mechanism of PufQ is lacking, our data do support the "carrier polypeptide" model of regulation as originally proposed by Lascelles (10, 13). This model (Fig. 3) suggests that regulation of the Mg-tetrapyrrole branch is achieved, in part, by control of the synthesis and/or activity of a carrier polypeptide that acts as an obligatory membrane-bound cofactor for BChl biosynthesis. This model is based on observations that the Mg branch of the biosynthetic pathway forms insoluble tetrapyrrole–protein–lipid complexes (6, 10–12) that have been shown, in _R. sphaeroides_, to be associated with a low
molecular weight \( M, 9000 \) hydrophobic polypeptide (12). This is in contrast to earlier stages of the pathway that are common to heme and BChl biosynthesis, which involve soluble enzymes and substrates. We hypothesize that \( puQ \) encodes a carrier polypeptide similar to that proposed by Lascelles for several reasons. Strains with a \( puQ \) deletion exhibit the expected phenotype for a mutation in such a carrier polypeptide—i.e., a reduction in biosynthesis of all intermediate Mg-tetrapyrroles without an effect on the synthesis of heme. The polypeptide encoded by \( puQ \) is a small \((M, 8556)\) hydrophobic polypeptide (14) and thus exhibits the same physical properties as the putative carrier polypeptide previously isolated (12). Finally, as is shown in Fig. 4, \( puQ \) exhibits sequence similarity to the membrane-spanning region of RC polypeptides that have been shown by x-ray crystallography data (33-37) to interact noncovalently with the tetrapyrrole ring structure (20% amino acid identity to the \( R.\) \textit{capsulatus} RC-L and 30% to \( R.\) \textit{capsulatus} RC-M polypeptide sequences; ref. 15). Although these observations do not definitively show that \( puQ \) functions as the proposed tetrapyrrole carrier polypeptide, they do support such a model.

In addition to the putative tetrapyrrole-binding site, we note that the region of \( puQ \) that exhibits the highest sequence similarity with the RC polypeptides \((\text{PuF} \text{residues 48-74 show 42% identity with analogous residues in the RC M subunit})\) is an area known in RC polypeptides to form a binding pocket for quinone and competitive inhibitors of quinone (33, 34, 38, 39), thus suggesting that \( puQ \) may interact with the quinone pool. Such an interaction would be intriguing in view of the previous model that BChl biosynthesis is affected by the redox state of the electron-transport system. Indeed, a model proposed several decades ago (7), which is still credible, suggests that a BChl regulatory protein could be monitoring the electron-transport system whose redox potential could be affected by light intensity or by donation of electrons to oxygen through cytochrome oxidase (7). In support of this model are observations that introduc-

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**Fig. 3.** Carrier model of \( puF \) function. BChl and heme biosynthesis are thought to share a common soluble pathway up to the synthesis of protoporphyrin IX, at which point the pathways diverge either by the addition of Fe to form heme or Mg to form Mg-protoporphyrin IX. The dashed line denotes previously established feedback inhibition on \( 5\)-aminolevulinic acid synthetase activity by heme (30, 31). In the proposed model of BChl regulation, the hydrophobic \( puQ \) polypeptide interacts with protoporphyrin IX to form a membrane-associated complex that is required for the next step in BChl biosynthesis. Since different tetrapyrrole intermediates in the pathway are known to be membrane-associated, it is thought that subsequent steps involve similar interactions with the carrier polypeptide.

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**Fig. 4.** Amino acid similarity of \( puF \) to RC polypeptides: alignment of \( puF \) to the D and E membrane-spanning regions of the RC polypeptides from \( R.\) \textit{capsulatus} ([L156-232, M183-266] (positions of the L and M polypeptide subunits)), \( R.\) \textit{sphaeroides} ([L156-232, M185-268]), and \( R.\) \textit{viridis} ([L156-232, M183-266]). Bold italicized letters denote amino acid residues in \( puF \) that have identical matches to an amino acid residue in one or more of the RC polypeptides. Underlined residues denote conserved amino acid changes in \( puF \) relative to the RC residues. The numbers denote the amino acid position within the \( puF \) polypeptide. The similar RC segment was identified by using the Smith and Waterman (32) algorithm to probe for PuF similarity to proteins in a data bank composed of proteins exhibiting known interactions with tetrapyrroles (i.e., proteins encoded by the \( puf, puc, \) and \( puh \) operons from \( R.\) \textit{sphaeroides}, \( R.\) \textit{viridis}, and \( R.\) \textit{capsulatus}).
tion of oxygen or high light intensity into photosynthetically growing cultures causes a cessation of BChl biosynthesis that occurs much too rapidly to be simply attributed to inhibiting the synthesis of enzymes that catalyze one or more steps in the biosynthetic pathway (7). In addition, it has been demonstrated that insertion of Mg into the tetrapyrrole moiety is inhibited by the addition of quinone analogs and uncouplers of electron transport (40). The existence of a potential quinone-binding site in PufQ thus opens the possibility that not only is the level of PuFQ synthesis regulated by oxygen (14) but also its activity may be affected by the redox state of the quinone pool. These speculations suggest new experimental approaches to previous observations on the mechanism of light and oxygen regulation of BChl biosynthesis.

In summary, we have demonstrated that PuFQ is involved in regulating the flow of tetrapyrroles through the Mg branch of the BChl biosynthetic pathway. The existence of this protein within the puf operon appears to provide the cell with a way of coupling the amount of BChl biosynthesis to the synthesis of RC/LH-I polypeptides. Furthermore, the similarity between PuFQ and the RC polypeptides suggests that PuFQ functions as a tetrapyrrole-binding polypeptide whose activity may be regulated by the redox state of the quinone pool. Future mutational analysis on putative BChl- and quinone-binding sites should give a better understanding of the mechanism whereby PuFQ regulates BChl biosynthesis.

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