DNA Binding Characteristics of RegA*

A CONSTITUTIVELY ACTIVE ANAEROBIC ACTIVATOR OF PHOTOSYNTHESIS GENE EXPRESSION IN RHODOBACTER CAPSULATUS*

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In the purple non-sulfur bacterium *Rhodobacter capsulatus*, RegA and RegB comprise a two-component regulatory system that is required for maximal anaerobic transcription of key photosynthesis genes. RegB is a sensor kinase that uses ATP to phosphorylate its cognate response regulator, RegA. The mechanism under which RegA→P influences transcription of target genes has been unclear given that past attempts to demonstrate DNA binding activity by isolated RegA have failed. This led to a model invoking a role for RegA→P as an intermediate in a more complex multicomponent phosphoryl transfer cascade. In the present study, we describe the isolation of a mutant version of RegA (RegA*) which promotes high level expression of photo-synthesis genes independent of RegB. DNase I footprint analyses show that purified RegA* binds to the promoters of the *puf* and *puc* operons at locations that are consistent with RegA functioning as a transcriptional activator for these operons. We conclude that RegA functions, like most members of the response regulator family, as a DNA-binding protein that directly affects the expression of its target genes.

Oxygen tension is an important environmental factor in regulating synthesis of the *Rhodobacter capsulatus* photosystem (1). In the presence of oxygen, the cell suppresses synthesis of a photosystem and instead obtains cellular energy through respiration. In contrast, when oxygen tension is reduced to <1%, the cell synthesizes intracytoplasmic membranes that contain the photosystem used to convert light into chemical energy. The photosystem is a highly organized structure composed of complexes designated as light harvesting-I (LH-I), light harvesting-II (LH-II), and the reaction center. Each light harvesting complex is formed by the assembly of protein-pho-topigment subunits into intricate ring structures where bacteriochlorophyll molecules are oriented precisely to absorb and channel light energy to the reaction center.

Expression of the structural proteins for LH-I, LH-II, and the reaction center complexes, encoded by the *puf*, *puc*, and *puh* operons, is regulated coordinately with respect to oxygen (for review, see Refs. 2 and 3). At the onset of anaerobiosis, the transition to photosynthetic growth is accompanied by a very large induction of *puf*, *puh*, and *puc* transcription which is derived primarily from promoters specific to these operons. Anaerobic activation of these promoters is dependent on a two-component regulatory system comprised of the sensor kinase, RegB, and the response regulator, RegA (4, 5). Genetic evidence that RegA and RegB constitute a cognate two-component system includes the observation that knockout mutations in *regA* and/or *regB* abolish anaerobic induction of the *puf*, *puh*, and *puc* operons (4, 5). The *in vivo* evidence is supported by *in vitro* analyses that demonstrated that a truncated form of the RegB polypeptide autophosphorylates in the presence of ATP and transfers the phosphate moiety to RegA (6).

Questions that remain to be addressed are the mechanism whereby RegB senses alterations in the redox state of the cell and whether RegA activates transcription of photosynthesis genes directly. Early attempts by our laboratory failed to demonstrate DNA binding activity by RegA in *vitro*. However, because many response regulators exhibit poor binding affinities for DNA (7–10), these experiments were inconclusive. One strategy that has been very helpful in the characterization of response regulators has been the isolation and purification of constitutively active variants. Some constitutively active response regulators are known to exhibit much higher affinity for their DNA binding sites without the need for phosphorylation (11–14). For example, VirGN54D, a constitutive mutant of VirG, binds to its target site approximately 10-fold more tightly than wild type VirG (13).

Given the difficulties of demonstrating DNA binding activity with wild type RegA, we decided to search for a variant that would be more amenable to the characterization of *in vitro* DNA binding activity. This paper describes the successful isolation of a RegA mutant that activates transcription of photosynthesis genes in the absence of RegB. DNase I footprint experiments were used to provide the first direct evidence that RegA is indeed a DNA binding response regulator that interacts with clearly defined sites within the *puf* and *puc* promoters.

### MATERIALS AND METHODS

**Bacterial Strains, Media, and Growth Conditions—** *R. capsulatus* strains were routinely grown at 34 °C in PYS or RCV2/3PY as described previously (15). Spectinomycin and kanamycin were used at 10 µg/ml for the maintenance of plasmids and the construction of stable recombinants in *R. capsulatus*. A rifampicin concentration of 100 µg/ml was used for counterselection of transconjugants. RCV-lactose medium was the same as regular RCV medium (15) except that 0.2% lactose was used as the carbon source instead of maltose. *Escherichia coli* strains DH5α (Novagen), BL21(DE3) (16), and S17-1 (Δpir) (17) were grown at 37 °C in LB that contained ampicillin and kanamycin at 100 or 50 µg/ml, respectively.

**Disruption of the Chromosomal Copy of regB** —To create a *regB* mutant strain, two fragments were amplified from a genomic DNA preparation by polymerase chain reaction (PCR). One fragment encoded the NH$_2$ terminus of RegB, and the other encoded the carboxyl portion excluding the conserved histidine residue (5). The fragments were then ligated to either end of a kanamycin resistance cassette isolated from...
pBSL86 (18), and the resulting construct was cloned into pUC19 (19). Disruption of regB in strain SB1003/pCB5321 (20) was performed by gene transfer agent-mediated recombination of the plasmid-borne regB::Kn construct into the chromosome (21). Cells with the desired chromosomal insertion were selected by kanamycin resistance and then verified by PCR analysis. The regB-disrupted strain was designated SD01.

Isolation of Constitutively Active RegA Bypass Mutants (RegA*)—RegA mutants whose activities are independent of RegB were isolated by screening for mutants derived from SD01/pCB5321 which could grow aerobically on minimal lactose medium plates (22) (see “Results”). A culture of SD01/pCB5321 was grown until late log phase before the cells were harvested and magnetized with ethyl methanesulfonate for 30 min (23). The treated cells were then diluted serially and spread onto RCV-lactose plates. The plates were incubated aerobically at 34 °C until colonies appeared. Colonies with dark red color were restreaked several times onto RCV-lactose plates to isolate pure strains.

To determine which of the highly pigmented strains possessed regA mutations, DNA fragments carrying the regA gene were amplified from genomic DNA preparations and sequenced using the Applied Biosystems automatic sequencing system (Perkin-Elmer). PCR products derived from strains that contained RegA mutations were cloned into the suicide vector pZDJS (24), and the resulting constructs were then backcrossed into SD01/pCB5321 with the plasmid-mobilizing strain S17-1 (8pir) (17). The backcrossed strains contained a level of colony pigmentation similar to that observed with the mutant parent strain.

Spectral and Protein Analysis—Absorption spectra obtained from crude membrane preparations and the procedure used to measure β-galactosidase activity in R. capsulatus have been described previously (15).

Construction of a RegA Overexpression Vector—We combined features of the T7 RNA polymerase-based ϖ hepatoma expression system (Novagen) and the IMPACT™ I purification system (New England Biolabs) to overexpress and purify RegA*. A new vector pET9CBD was first constructed by isolating a 224-base pair fragment encoding a chitin binding domain from the vector pCYB1 (New England Biolabs) with a BamHI and NdeI site at the start codon and a HindIII site at the stop codon for RegA*. This construct contains the puf::lacZY reporter (see “Materials and Methods”) that reduces expression of the lacZY operon (180 base pairs) promoter regions were obtained by PCR and were 32P labeled as described previously (25, 26). Footprint assays were initiated by mixing binding reactions that contained 9.0 pmol of probe with various amounts of RegA* in buffer containing 40 mM Hepes (pH 7.8), 8 mM MgCl2, 7.5 mM KCl, 2 mM CaCl2, 1.5 mM dithiothreitol, 125 mg/ml bovine serum albumin, and 16% glycerol in a total volume of 20 μl. DNAse I digests of the binding reactions and the generation of DNA sequence ladders by the Maxam and Gilbert chemical cleavage method were carried out as described previously (27).

RESULTS

Isolation of Constitutively Active RegA Mutants—Normally, R. capsulatus is incapable of metabolizing lactose because it lacks lacZY homologs that encode the β-galactosidase and the lactose-specific permease. However, growth on minimal medium with lactose as a carbon source can support growth if the lacZY genes from E. coli are expressed heterologously (22). This property was exploited to select for constitutively active RegA mutants (RegA*) either as spontaneous mutants or from an ethyl methanesulfonate-treated culture of SD01/pCB5321. This strain contains a disrupted regB (see “Materials and Methods”) that reduces expression of the puf::lacZY translational fusion in pCB5321 (20) to a point that is too low to permit growth on lactose. Mutant strains with elevated photosynthesis gene expression can thus be isolated by selecting for growth on lactose. Such mutants would be expected to bypass a requirement for activation by RegB and consequently produce colonies under aerobic growth conditions which are more highly pigmented than the parental strain.

By this strategy, 14 dark red colonies were isolated from RCV-lactose plates that had been incubated aerobically. These strains appeared to be genetically stable when grown photoautotrophically (23). After 3 h of induction, cells from 5 liters of a culture of SD97* were harvested and the cells resuspended in 0.6 ml of ice-cold column buffer 20 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, 0.05 mM phenylmethylsulfonyl fluoride, and 50% glycerol and then stored at −80 °C.

DNAse I Footprint Analysis—DNA probes that contained the puf binding site upstream of the lacZY (180 base pairs) promoter were obtained by PCR and were 32P labeled as described previously (25, 26). Footprint assays were initiated by mixing binding reactions that contained 9.0 pmol of probe with various amounts of RegA* in buffer containing 40 mM Hepes (pH 7.8), 8 mM MgCl2, 7.5 mM KCl, 2 mM CaCl2, 1.5 mM dithiothreitol, 125 mg/ml bovine serum albumin, and 16% glycerol in a total volume of 20 μl. DNAse I digests of the binding reactions and the generation of DNA sequence ladders by the Maxam and Gilbert chemical cleavage method were carried out as described previously (27).

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By this strategy, 14 dark red colonies were isolated from RCV-lactose plates that had been incubated aerobically. These strains appeared to be genetically stable when grown photosynthetically (anaerobically) but were very unstable when grown aerobically as based on heterogeneity observed in colony pigmentation. Considering that regB has been inactivated in SD01, the mutants exhibiting aerobic synthesis of the photosystem might be (i) mutations in genes encoding alternate kinases resulting the activation of RegA or (ii) bypass mutations in regA (regA*) which allow RegA to influence puf promoter activity without a requirement for phosphorylation. Sequence analysis of the regA gene of all 14 mutants demonstrated that two spontaneously derived mutants contained the same G to T mutation at nucleotide 283 resulting in an alanine to serine substitution at position 95 of the RegA protein (Fig. 1). One of the regA*-mutated strains, SD97*, was chosen for further analysis (see below). Confirmation that the phenotype observed in SD97* was indeed caused by the mutation in RegA was shown by recombinating a copy of the regA gene.
isolated from SD97* back into the parent strain SD01/pCB532 (see "Materials and Methods"). This strain construction exhibited a phenotype identical to that observed with SD97* (data not shown).

**Phenotype of RegA**—Spectral analysis of photosynthetically grown anaerobic cultures demonstrated that SD97* cells had essentially the same amounts of photopigments as the wild type strain SB1003 (Fig. 2A). This is much higher than observed in SD01 (the parent of SD97*), which synthesizes low amounts of photopigments as a consequence of the chromosomal disruption of regB. Spectral analysis of cells grown under aerobic conditions demonstrated that, as expected, both wild type and SD01 cells synthesize low levels of photopigments (Fig. 2B). In contrast, aerobically grown SD97* cells synthesize significant elevated amounts of photopigments.

Considering that SD97* contains an inactivated regB gene, the results of the spectral analyses indicate that the mutation may result in a variant of RegA which promotes constitutive expression of photosynthesis genes. We subsequently tested the effect of the RegA* mutation on gene expression by measuring puf::lacZ activity in strains that harbored the puf::lacZ reporter plasmid pCB532V. As demonstrated in Fig. 3, puf activity in the wild type strain SB1003 was very low in aerobic cultures and increased significantly under anaerobic conditions. SD01 exhibited constitutively low puf activity as reported previously for a regB null strain (5). In contrast, strain SD97* exhibited high levels of activity when grown either anaerobically or aerobically. Compared with the wild type strain, SD97* exhibits a 3-fold increase in activity under anaerobic conditions and a dramatic 55-fold increase under aerobic conditions.

**Overexpression and Purification of RegA**—Previous in vitro studies with wild type RegA relied on protein purified from inclusion bodies that had to be solubilized and refolded (6). To overcome solubility problems, the regA* gene was translationally linked to an intein/chitin binding domain at its carboxyl terminus (see "Materials and Methods"). Upon overexpression, we observed that appreciable amounts of the RegA*-intein/CBD fusion protein were in the soluble fraction of crude cell lysates and that the protein could be purified easily using a chitin affinity column. Another advantage of this system is that the chitin-bound fusion protein can undergo self-splicing at the intein junction releasing native RegA* protein from the column. SDS-polyacrylamide gel electrophoresis revealed that RegA* purified with this expression system was the expected 21 kDa in size and that purity was greater than 95% (Fig. 4).

Previous studies in our laboratory indicated that misfolded RegA was incapable of being phosphorylated by RegB. Thus, we assayed whether the purified RegA* was capable of being phosphorylated by purified RegB* (RegB* is a soluble form of RegB which lacks the transmembrane domains) as a test for biological activity. This analysis demonstrated that a large percentage of RegA* (>30%) was phosphorylated, indicating that a significant proportion of the purified protein was indeed folded into its proper conformation (data not shown).

**DNA Binding Capability of RegA**—To examine whether RegA* exhibited DNA binding activity, we conducted DNase I protection assays using 32P-labeled probes of the puf and puc promoter regions. As indicated in Fig. 5, A and B, RegA* protected two regions of the puf promoter from DNase I digestion.

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tion including strong protection of nucleotides −22 to −51 and weaker protection of nucleotides −68 to −80 relative to the transcription start site. The −22 to −51 region overlaps a DNA sequence with dyad symmetry that is conserved for *puf* promoters from other species of photosynthetic bacteria (3, 28) and overlaps a conserved −24 sequence that has been proposed to be a recognition sequence for an alternative sigma factor for the *puf* promoter (2, 3, 28). Similarly, two areas of DNase I protection were evident for the *puc* promoter extending from −52 to −69 and −73 to −80 (Figs. 6 and 7). DNase I-hypersensitive sites were also evident within or flanking the RegA*-protected regions in both the *puf* and *puc* promoter regions (Fig. 7). Equivalent amounts of RegA* were required to obtain distinct regions of protection at the *puf* and *puc* promoters, suggesting that the protein had a similar binding affinity for both probes.

**DISCUSSION**

The exact role of RegA in promoting anaerobic induction of photosynthesis gene expression has been somewhat controversial. RegA and its homologs from other species exhibit high homology to other response regulators in the amino-terminal receiver domain and little homology to other response regulators in the short (50 amino acid) carboxyl effector domain (4, 29). In RegA the receiver domain is separated from the effector domain by a short hinge comprised of 2–4 prolines (Fig. 1), whereas in other response regulators the hinge is composed of a flexible 10-amino acid helix (29, 38). Careful analysis of the RegA effector domain reveals a sequence that resembles a helix-turn-helix DNA binding motif (30) (Fig. 1). However, previous attempts by our laboratory to demonstrate DNA binding with wild type RegA failed. This led to the speculation that RegA may be an intermediate in a multicomponent phosphotransfer cascade (2, 3, 5, 31). The results of this study provide the first direct evidence that RegA is indeed a DNA binding response regulator that directly affects the transcription of its target genes.

Although it is still a formal possibility that RegA* may be activated *in vivo* by an alternative kinase, the simplest explanation is that the mutant protein bypasses the necessity for phosphorylation to function as a transcriptional activator. How

![Fig. 5. DNase I footprint analysis of RegA* binding to the *puf* promoter. Panel A, top strand; panel B, bottom strand. G+A indicates a G+A Maxam and Gilbert chemical cleavage pattern. Lanes 2–6 are DNase I digestions of binding reactions that contained *puf* probe and increasing amounts (µg) of purified RegA*. Lane 7 has no RegA* added to the cleavage reaction.](image)

![Fig. 6. DNase I footprint analysis of RegA* binding to the *puc* promoter. Panel A is of the top strand, and panel B is the bottom strand. G+A and amounts of purified RegA* are as indicated in Fig. 5.](image)

![Fig. 7. Summary of the top and bottom strand DNase I protection patterns of RegA* binding to the *puc* and *puf* promoter regions. The large thick arrows denote the transcription start sites for the promoters; the thin arrows indicate the presence of putative CrtJ recognition palindrome. The brackets indicate regions of DNase I protection to top (above the sequence) or bottom (below the sequence) strands with asterisks representing DNase I-hypersensitive sites. The black boxes indicate putative sigma subunit recognition sequences.](image)
then could an alanine to serine substitution at position 95 result in a constitutively active RegA protein? Mutations in the same region of other DNA binding response regulators, such as BsuSpo0A (Q90K, 92Y), EcoNarL (V88A), and EcoompR (G94S, G94D, E96A), have been reported to influence the activities of the corresponding proteins dramatically (for review, see Ref. 29). According to crystallographic structural analysis of CheY and NarL (32, 33), these mutations all cluster in the α4 helix, which is located in close proximity to the hinge/linker region that functions to separate the receiver domain from the helix-turn-helix DNA binding domain. For RegA+, the substitution of serine for alanine creates an increase of the side group volume, as well as hydrophobicity, which could affect interactions of the α4 helix with the hinge region to lock the protein in its active conformation.

It is interesting that RegA binds much closer to the start site of transcription in the puf operon promoter (−22 to −80) than in the puc promoter (−52 to −80). The puc promoter has a sequence motif that is very similar to housekeeping genes, indicating that it most likely uses a “sigma-70” subunit for promoter recognition (see black boxes in Fig. 7). However, the puf operon does not exhibit a canonical sigma-70 sequence motif, leading to the speculation that it may use a secondary sigma factor for promoter recognition (2, 3, 20, 28). If so, then the close location of the RegA binding site to the puf transcription start site may be construed as additional evidence for the existence of a secondary sigma subunit that requires a different placement of RegA to promote activation of transcription. The RegA binding site on the puf promoter also overlaps a sequence that contains a dyad symmetry that is reasonably well conserved among puf promoters from different species (3, 20, 28). Mutational analysis has indicated that this palindromic sequence may bind an aerobic repressor (34, 35). Thus, regulation of puf expression may involve a competition between RegA and a repressor for overlapping binding sites. A similar situation exists for the puc operon whose expression is affected by an aerobic activation by RegA as well as aerobic repression by CrtJ (36). Footprint analysis indicates that the lack of sequence specificity of AbrB allows much greater flexibility in binding to a wide variety of promoters than would be observed with a sequence-specific DNA-binding protein (37, 38). This feature presumably allows the cell to control many different operons with a single regulator. Indeed, there is growing evidence that the RegA-RegB regulatory circuit in R. capsulatus affects several anaerobic physiological processes other than photosynthesis, including carbon and nitrogen assimilation (31, 39). Highly conserved homologs for RegA and RegB have also been found in several other species such as the related photosynthetic bacterium *Rhodobacter sphaeroides* and the non-photosynthetic purple bacterium *Rhizobium meliloti* (Fig. 1) (40, 41). Rather surprising is the observation that the putative helix-turn-helix motif in RegA is completely conserved among these different species (Fig. 1). This indicates that the mechanism of recognizing target promoters by RegA homologs is a highly conserved feature. Undoubtedly, continued studies of the RegA in these additional species will provide further insights into the extent and nature of the RegB-RegA regulon.

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