Autophosphorylation, Phosphotransfer, and DNA-binding Properties of the RegB/RegA Two-component Regulatory System in *Rhodobacter capsulatus*

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In the purple, photosynthetic bacterium, *Rhodobacter capsulatus*, the RegB/RegA two-component system is required for activation of several anaerobic processes, such as synthesis of the photosynthetic apparatus and assimilation of CO$_2$ and N$_2$. It is believed that RegB is an integral membrane histidine kinase that monitors the external environment. Under anaerobic growth conditions, it transduces a signal through phosphorylation of the response regulator, RegA, which then induces target gene expression. We used an *in vitro* assay to characterize the phosphorylation of wild-type RegA and a mutant variant (RegA*) that is responsible for abnormally high photosynthesis gene expression under both aerobic and anaerobic growth conditions. Phosphorylation assays indicate that phosphorylated RegA* (RegA*→P) is much more stable than RegA−P, indicating that it may be locked in a conformation that is resistant to dephosphorylation. DNase I footprint assays also indicate that unphosphorylated RegA* has a much higher affinity for specific DNA binding sites than the wild-type protein. Phosphorylation of RegA* increases DNA binding 2.5-fold, whereas phosphorylation of RegA increases DNA binding more than 16-fold. Collectively, these results support the hypothesis that RegA* is a constitutively active variant that does not require phosphorylation to assume a structural conformation required to bind DNA.

It has been known for many years that induction of photosynthesis gene expression in the anoxygenic phototroph, *Rhodobacter capsulatus*, is controlled by the RegB/RegA two-component regulatory system (1–4). This circuit functions as a signal transduction pathway that promotes synthesis of the photosynthetic apparatus in an anaerobic environment. RegB is believed to be an integral membrane histidine kinase that monitors the external environment and signals for a switch to photosynthetic growth when the oxygen concentration falls below a threshold level (∼1%) (1–3). This involves phosphorylation of the response regulator, RegA, which then stimulates the transcription of several key photosynthesis genes (2, 5). Recently, we reported the isolation of a mutant strain of *R. capsulatus* (SD97*) that exhibits constitutively high photosynthesis gene expression in the absence of a functional regB gene (11). The mutant strain was found to contain a RegA variant (RegA*) responsible for activation of operons (*puf, puc, and puh*) that encode structural proteins of the photosystem. DNase I footprint experiments with purified RegA* demonstrated that it is a DNA-binding protein that interacts with promoters for the *puf* and *puc* operons. This study also indicated that RegA* may recognize localized features in DNA structure rather than a specific nucleotide sequence to bind target promoters (11). In most cases, phosphorylation of a response regulator affects its ability to regulate gene expression. However, until experiments are carried out in parallel with wild-type RegA, it is not possible to determine whether RegA* activates gene expression because it is phosphorylated in vivo by an alternative kinase(s) or because it is a constitutively active DNA-binding protein.

To further probe the transcription activating function of RegA, we have characterized various properties of RegA* and wild-type RegA using an *in vitro* kinase assay. To facilitate this study, we constructed a vector that overproduces a soluble form of RegB (RegB*) that lacks all of its putative transmembrane domains. We demonstrate that purified RegB* will autophosphorylate *in vitro* when incubated with ATP and that it can donate a phosphoryl group to purified RegA or RegA*. By comparing the levels of RegA* and RegA phosphorylation and examining how phosphorylation affected its binding to the puc promoter, we have concluded that RegA* has an altered structural conformation that facilitates DNA binding, perhaps because it mimics the phosphorylated state of the wild-type protein. These results are consistent with the hypothesis that RegA* is a constitutively active transcriptional regulator that does not require phosphorylation to activate target genes.

**MATERIALS AND METHODS**

*Construction of RegB* and *RegA* Overexpression Vectors—We constructed a vector that could be used to overexpress a His$_6$-tagged, truncated form of RegB (RegB*), which lacks all of the putative transmembrane domains of the NH$_2$ terminus. A DNA fragment encoding the cytoplasmic portion of *regB* was amplified from the chromosome of *R. capsulatus* by polymerase chain reaction using the upstream primer 5′-CCATATGGGGATCCGGGGCTTTTGTGGC and the downstream primer 5′-CTCGAGAAGGATTGATTCATCGGCG. The upstream and downstream primers were designed to contain NdeI and XhoI restriction enzyme sites, respectively (*underlined* bases), to facilitate cloning. The polymerase chain reaction product was initially cloned into the plasmid, pCR2*11* (Invitrogen), and then subcloned into NdeI and XhoI restric-
tions of the expression vector pET28a (+) (Novagen). The resulting plasmid pET28a (+)/RegB was transformed into the Escherichia coli strain BL21(DE3) (12) to overproduce RegB as described below.

A clone that overexpresses wild-type RegA was also constructed using the same procedure as described previously for RegA (11). DNA sequencing of pET28a (+)/RegA and pET28a (+)/RegB confirmed the identity and were confirmed using the ABI automatic sequencing system (Perkin-Elmer).

Overexpression and Purification of RegB and RegA—A total of 5 litters of Terrific Broth was inoculated with the E. coli strain BL21(DE3)/pET28a (+)/RegB. The cultures were grown with vigorous shaking at 37 °C until they reached an A600 of 0.6. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1.0 mM. The cultures were grown for an additional 3 h before the cells were harvested by centrifugation at 7600 χ g at 4 °C for 10 min. The cell pellet was washed once and then resuspended in 50 ml of 1 M NaCl. Cells were lysed by three freeze-thaw cycles. The final lysate was centrifuged at 18,000 g for 30 min at 4 °C. The supernatant was filtered through a 0.45 μm Acrodisc (Gelman Sciences) and then loaded onto a 0.5 ml HiTrap chelating column (Amersham Pharmacia Biotech) that had been charged with NISO 4 and equilibrated with 1× start buffer. The column was washed with 5 ml of 1× start buffer before RegB protein was eluted with 1× start buffer supplemented with 400 mM NaCl. Elution fractions that contained significant amounts of RegB protein, as determined by the Bradford assay (Bio-Rad), were pooled and dialyzed first against 20 mM HEPES (pH 8.0), 400 mM KCl, 5 mM MgCl 2, 20% glycerol and then against 20 mM HEPES (pH 8.0), 400 mM KCl, 5 mM MgCl 2, 2 mM dithiothreitol, and 50% glycerol. The final RegB concentration was determined by the A280/A600 method described by Scopes (13), and the protein preparation was stored at −80 °C. The procedure used to overexpress and purify RegA and RegA* protein was the same as previously reported (11).

Protein Phosphorylation Assays—Protein phosphorylation assays were conducted by incubating purified RegB, ATP/γ-32P/ATP (a typical kinase reaction contained 1.0 mM ATP and 200–400 μCi of γ-32P/ATP (7000 Ci/mmol, ICN), and either wild-type RegA or RegA* in 1× footprint buffer (25 mM HEPES (pH 8.0), 150 mM KCl, 5 mM MgCl 2, 3 mM CaCl 2, 10 mM dithiothreitol, and 50% glycerol). The reaction was incubated at room temperature, and at various times, samples were removed, mixed with an equal volume of 2× SDS-loading buffer, (100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol), and placed on ice. The samples were later size fractionated by SDS PAGE using a 12% gel. The 32P-labeled protein bands were visualized by autoradiography and excised from the gel, and the amount of Cerenkov radioactivity they contained was measured by scintillation counting.

To compare the percentage of RegA/RegA* protein preparations that could be phosphorylated, 640 pmol of RegB in a 40-μl reaction was incubated for 2 h at room temperature in the presence of 50 mM ATP/γ-32P/ATP in 1× footprint buffer. The reaction was then diluted with 120 μl of 1× footprint buffer. Samples containing 5 μl of the diluted kinase reaction were mixed with an equal volume of 1× kinase buffer that contained various amounts of either RegA or RegA*. These phosphotransfer reactions were incubated at room temperature for 20 s before being terminated with the addition of 10 μl of 2× SDS-loading buffer. The reactions were then fractionated by SDS-PAGE, and the levels of RegA/RegA* phosphorylation were quantitated as described above.

To compare the relative stabilities of RegA–P and RegA*–P, approximately 175 pmol of RegB was mixed with ATP/γ-32P/ATP in 1× kinase buffer in a total volume of 175 μl. The mixture was incubated at room temperature for approximately 2 h to allow RegB to autophosphorylate. Then 50-μl samples were removed from the reaction and mixed with an equal volume of 1× footprint buffer that contained no protein, RegA, or RegA* (the final concentrations of RegB, RegA, or RegA* in these mixtures was 5 μM). At various times, 10-μl samples were removed from each of the three reactions and mixed with an equal volume of 2× SDS loading buffer and then placed on ice. These samples were later fractionated by SDS-PAGE, and the level of RegB, RegA, or RegA* was quantitated as described above at various times after the reactions were initiated by the addition of ATP.

DNase I Footprint Experiments—A 180-base pair DNA fragment that encompassed the puc promoter region was amplified by polymerase chain reaction and purified as described previously (14). The polymerase chain reaction product was stored in 25 mM HEPES (pH 8.0), 50 mM NaCOOH, and 0.1 mM EDTA.

To prepare RegA–P/RegA*–P for use in footprint reactions, RegB was incubated at room temperature for approximately 2 h in the presence of 1.0 mM ATP, RegA/RegA* protein was then added to the kinase reaction and incubated for 5 min before serial dilutions were made with 1× footprint buffer to obtain various RegA–P/RegA*–P concentrations.

RESULTS

Characterization of RegB Autophosphorylation—In previous studies, difficulties were encountered in the purification of RegB because overproduced protein formed insoluble inclusion bodies (6). To avoid this problem, we constructed a new vector, which expresses only the cytoplasmic portion of RegB (RegB') fused to a “His tag” at its NH2 terminus. SDS-PAGE analysis of total samples made from cultures that expressed His-RegB (hereafter called RegB') revealed that a significant proportion (>50%) of the polypeptide was soluble. The protein was subsequently purified to homogeneity by nickel column chromatography (see “Materials and Methods”).

Autophosphorylation of RegB' was assayed by incubating the purified protein with γ-32P/ATP at room temperature. At various times, samples were removed from the reaction, mixed with a denaturing dye solution, and then chilled on ice. After fractionation by SDS-PAGE, the level of RegB' phosphorylation was measured and the data plotted as a function of time (Fig. 1). Typically, we observed that the proportion of phosphorylated RegB' increased for approximately 2 h before the reaction appeared to equilibrate. We consistently observed that autophosphorylation reactions plateaued when 30–40% of the protein had become phosphorylated, indicating that a significant fraction of the RegB' preparation was biologically active. A 10-fold increase in ATP concentration did not significantly affect the kinetics of autophosphorylation or the final level at equilibration (data not shown). Therefore, the rate-limiting step in RegB' autophosphorylation does not appear to be binding of ATP. Rather, the rate may be limited by the ability of RegB' to catalyze the transfer of phosphate from bound ATP to its histidine phosphoacceptor site or possibly by the formation of RegB' dimers required for autophosphorylation to commence.

RegA Is Phosphorylated to a Greater Extent than Wild-type RegA—We examined the ability of RegB'–P to function as a substrate for the transfer of phosphate to RegA or RegA* by conducting kinase assays that contained mixtures of these proteins. RegA or RegA* was quantitated as described above at various times after the reactions were initiated by the addition of ATP.

The levels of RegA/RegA* phosphorylation in the samples were measured following SDS-PAGE (Fig. 2). In repeated experiments, we observed that phosphorylation of wild-type RegA increased for approximately 2 h after the addition of ATP. After this time, RegA phosphorylation usually plateaued at a level of

1 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.
5–10%. In contrast to wild-type RegA, reactions with RegA* did not plateau, even when incubated as long as 4 h (Fig. 2). Instead, RegA* continued to accumulate so that 20–30% of the protein had become phosphorylated by the end of the time course.

Despite differences in the extent of phosphorylation, we consistently observed that initial rates of phosphorylation were very similar for both RegA and RegA* suggesting that autophosphorylation of RegB might be a rate-limiting step in the process. To test this possibility, we attempted to measure the rate of phosphotransfer from RegB–P to RegA or RegA*. This experiment involved incubating RegB with [γ-32P]ATP for 2 h to allow the formation of RegB–P and then adding RegA or RegA* to the reaction. At various times samples were removed, and the extent of phosphotransfer was measured as before. In this analysis, we observed that phosphotransfer from RegB–P to RegA or RegA* was complete before the first sample was removed (10 s time point) from the reaction (data not shown). Although the rate of phosphotransfer was too fast to measure using this method, the experiment confirmed that the rate-limiting step for RegA/RegA* phosphorylation occurs during RegB autophosphorylation.

The Phosphoryl Group in RegA*–P Is Significantly More Stable than That of RegA–P—We interpreted the observation that a greater proportion of RegA* became phosphorylated than wild-type RegA as indicating one of two possibilities. The active fraction of the wild-type protein preparation might have been much lower than for RegA*. Alternatively, the phosphoaspartate bond might be more stable in RegA*–P than RegA–P. If so, it could have the effect of increasing the equilibrium level of RegA*–P over that of the wild-type protein.

We attempted to measure the percent active fraction of RegA and RegA* protein preparations by assaying DNA binding activity. However, these experiments did not yield consistent results, probably because unphosphorylated RegA has an extremely low affinity for its DNA substrate. To overcome this problem, we measured the catalyzing ability of RegA and RegA* to accept phosphate from a constant amount of preformed RegB–P. Several reports have concluded that the response regulator, rather than the histidine kinase, is responsible for catalyzing phosphotransfer (15–17). Consequently, a measure of phosphotransfer activity provided a method of comparing the relative proportions of active protein in RegA and RegA* preparations. The experiment determined the amount of RegA or RegA* needed for maximal phosphotransfer by mixing a constant amount of RegB–P with various amounts of either RegA or RegA*. Phosphotransfer reactions were terminated 2 min after mixing the proteins, and levels of RegA–P or RegA*–P were again measured after fractionation by SDS-PAGE. These data indicate that the percent active fractions for RegA and RegA* preparations are reasonably similar and, thus, cannot account for large differences in the extent of RegA or RegA* phosphorylation observed for the time course experiments described earlier.

Next we examined the relative stability of the phosphate group on RegA–P and RegA*–P by initiating a single round of phosphotransfer and then measuring the decay to RegA-OH or
RegA*-OH. The assays were initiated by mixing $^{32}$P-labeled RegB with a large excess of unlabeled ATP and either RegA or RegA* to allow rapid transfer of labeled phosphate to the response regulator while masking subsequent autophosphorylation or phosphorylations. At various times, samples were removed and quenched, and the level of RegA–P or RegA*–P that remained was measured. (Previous experiments had determined that when denatured and kept on ice, RegA–P/RegA*–P was stable for several hours, indicating that little additional loss of phosphate occurred in the interval between quenching reaction samples and separation by SDS-PAGE (data not shown).) Fig. 4 shows results obtained from one experiment that demonstrates an obvious difference in RegA–P and RegA*–P stability. Whereas the amount of RegA*–P was unchanged over a period of 4 h, the percentage of RegA–P that remained after the same time period was 6-fold lower than the original level.

**RegB Functions as a Phosphatase for RegA–P**—Instability of the phosphate in wild-type RegA–P could be a consequence of inherent phosphatase activity that is present in the wild-type but not the mutant RegA. Alternatively, RegB*-OH that is present in the kinase reactions may be able to remove phosphate from RegA–P but not from RegA*–P. To test these two possibilities we set up rapid RegB*-P to RegA phosphate transfer reactions as described above with the exception that various amounts of RegB*-OH were added to the reactions at the initial round of phosphorylation. If stability of the phosphate was dependent on an inherent phosphatase activity of RegA, then the stability would not be affected by the level of RegB*-OH. However, if RegB*-OH was also functioning as a phosphatase, then the half-life of phosphate on wild-type RegA would be dependent on the level of RegB*-OH. The results of this experiment indicate that the half-life of phosphorylated RegA–P is 120 min at a 4:1 ratio of RegA to RegB, 60 min at a 1:1 ratio, and 13 min at a 1:4 ratio. This indicates that increased stability of phosphate with RegA* is a consequence of reduced dephosphorylation by RegB.

**DNA Binding Affinities of RegA and RegA*—**Recently, we reported that purified RegA* bound to promoter regions of the puf and puc operons (11). Because phosphorylation has been reported to increase the DNA binding affinity of various response regulators, we decided to test whether phosphorylation affected this activity for wild-type RegA and RegA*.

For this analysis we performed DNase I protection assays on the puc promoter region using various amounts of RegA, RegA–P, RegA*, or RegA*–P. To obtain roughly equivalent levels of RegA or RegA* phosphorylation, samples from equilibrated RegB* autophosphorylation reactions were mixed with either RegB* or RegA*. The mixtures were incubated for a few minutes to facilitate phosphotransfer before serial dilutions were prepared and added to the footprint assays. Typically we observed that 15–25% of the RegA* protein were phosphorylated in kinase reactions that were conducted in this way. To test the effect of unphosphorylated protein, serial dilutions of RegA or RegA* were added directly to footprint assays.

As indicated by Fig. 5, RegA and RegA* protected identical regions of the puc promoter from DNase I digestion. This site is the same as we previously reported for RegA* binding to the puc promoter (11). The only apparent differences in these experiments was the amount of each protein required to obtain a similar extent of DNase I protection (note in Fig. 5 that different ranges of protein concentration were used in testing the various forms of RegA and RegA*).

In the case of unphosphorylated wild-type RegA, a protein concentration of 16 $\mu$M was required to obtain half-maximal protection. Even at the highest RegA concentrations, binding to the puc promoter was very weak and is observed by DNase I hypersensitivity at positions −56 and −57 rather than nuclease protection. Phosphorylation of RegA to a level of 20% substantially improved its affinity for DNA as demonstrated by significant nuclease protection observable between 0.5 and 2 $\mu$M of phosphorylated RegA. Analysis of half-maximal protection by PhosphorImager analysis indicates that phosphorylation increased RegA binding by approximately 16-fold.

In the case of RegA*, the concentration of unphosphorylated protein required for half-maximal DNase I protection was slightly less (2.5-fold) than that observed with phosphorylated RegA. Phosphorylation of RegA* provided a further 6-fold increase in binding resulting in the highest binding affinity as measured by half-maximal protection (0.8 $\mu$M). A comparison of the apparent binding affinities of these proteins thus indicates that there is a 15–20-fold difference in DNA binding affinity between unphosphorylated RegA and RegA*–P with the overall order of affinity being RegA ≪ RegA* < RegA–P < RegA*–P.
In addition to differences in binding efficiency, there is an apparent qualitative difference among the type of DNA-protein complexes produced by unphosphorylated wild-type RegA and other forms of the protein. This difference is indicated by a change in the DNase I hypersensitivity of protection patterns observed for RegA–P, RegA*, and RegA*–P. These patterns do not exhibit hypersensitivity at position −56 as seen in the RegA footprint, suggesting that the structure of a wild-type RegA–DNA complex may be slightly altered when the protein is not phosphorylated.

Collectively, the DNase I footprint data indicate that phosphorylation significantly increases DNA binding activity for wild-type RegA. The similarity in the apparent binding affinities of RegA–P and RegA* suggests that the mutant protein probably does not require phosphorylation to activate photosynthesis gene expression in vivo.

**DISCUSSION**

The RegB polypeptide lacks the first 175 amino acid residues of the wild-type protein. Despite this, it was evident in our experiments that the truncated protein retains the ability to undergo autophosphorylation and phosphotransfer. Therefore, the putative transmembrane domains located in the NH2 terminus of RegB are not essential for it to function as a kinase. Insertion of RegB into the cytoplasmic membrane may orient the protein to enhance dimerization, which is required for autophosphorylation and/or may be important for the regulation of kinase activity (15). It is apparent that the protein does not require an anaerobic environment to be activated because we did not take steps to ensure that RegB* was kept anaerobic during purification or in kinase assays. This suggests that RegB activity may be inhibited by some mechanism in vivo to prevent phosphorylation of RegA and induction of target genes under anaerobic conditions. Alternatively, the kinase and phosphatase activities of RegB may be reciprocally regulated as observed in other systems (15). It is possible that regulating RegB activity may involve an interaction of the NH2 terminus of RegB with some other protein. If so, the absence of the amino-terminal domain in RegB might render a constitutive kinase activity in vivo. We plan to test this hypothesis by expressing the truncated protein in R. capsulatus.

Strain SD97* was isolated by selecting for elevated photosynthesis gene expression in a regB null mutant grown under aerobic conditions (11). Consequently, RegA* must either be activated by a heterologous kinase or have the ability to bind efficiently to target promoters while unphosphorylated. Our DNase I footprint assays demonstrate that unphosphorylated RegA* was able to bind the puc promoter as well as RegA–P or RegA*–P. This suggests the view that RegA* is a constitutively active transcription factor that is directly responsible for abnormally high photosynthesis gene expression. Presumably, this is because RegA* adopts a conformation that resembles the activated structure of RegA–P.

Most response regulators are composed of a receiver domain at its NH2 terminus and an effector domain at its COOH terminus. They are believed to exist in dynamic equilibrium between at least two different structural conformations that constitute active and inactive states. It has been proposed that phosphorylation increases the proportion of protein in the active conformation by disrupting intramolecular interactions between the receiver and effector domains (15, 18–21, 23). For some response regulators this permits the protein to dimerize. For others, phosphorylation exposes the DNA-binding domain (18). In either case, the ultimate effect is to increase the DNA binding activity. The 10-fold increase in binding affinity that we observed for the wild-type protein probably underestimates the effect of phosphorylation in vivo where it is likely that the proportion of RegA–P during anaerobic growth is greater than the 15–25% obtained in kinase reactions.

The effector domain of RegA is relatively short but contains an amino acid sequence motif that is characteristic of a helix-turn-helix type DNA-binding domain. This region is separated from the receiver domain by a short “linker” comprised of four consecutive proline residues. Based on the structural model for CheY and NarL, the point mutation in RegA* (a serine for alanine substitution at position 95) is located in the α4 region of the protein (24, 25). In fact, constitutively active variants of other response regulators, including Spo0A, NarL, and OmpR, also contain mutations in this region (26). Because the α helix is proposed to lie in close proximity to the linker region of RegA, we suspect that the SD97* mutation may result in a realign-
ment of domains that is mediated by the stretch of prolines. This could produce a conformational change that resembles the effect of RegA phosphorylation and result in constitutive activation of genes within the RegA regulon.

Differences in the relative stabilities for RegA–P and RegA*–P indicate that the two proteins have different structures. Inherent rates of phosphatase activity for different response regulators have been found to vary widely, suggesting that they may be influenced by protein conformation (27, 28, 30). Often these rates are enhanced in vivo by phosphatase activity exhibited by the histidine kinase. The observation that dephosphorylation of RegA–P is enhanced by increasing amounts of RegB–OH indicates that RegB must indeed have an inherent phosphatase activity for RegA–P. Presumably the mutant protein assumes an altered conformation that inhibits the ability of RegB to bind and/or catalyze the dephosphorylation of RegA*–P.

Nothing is known about the mechanism used by RegA to activate transcription at target promoters. Various DNA-binding response regulators have been shown to activate transcription by recruiting RNA polymerase to the promoter or by catalyzing the formation of an open complex between the polymerase and promoter. However, in most cases that have been studied, phosphorylation of the response regulator does not directly affect these activities. Instead, phosphorylation simply influences the ability of the response regulator to bind target promoters (18, 19, 22, 29, 32, 34–37). In the case of RegA, there is an apparent correlation between the location of its binding sites at the puf and puc promoters and those of additional transcriptional regulators. For instance, puc promoter activity is known to be under the control of the aerobic repressor, CrtJ, which binds a palindrome that overlaps the RegA binding site for this promoter (31). In addition, the RegA binding site for the puf promoter is superimposed over a region of dyad symmetry proposed to be the binding site for a transcriptional repressor that functions under aerobic conditions (33). Thus, it appears that aerobic/anaerobic control of the puf and puc operons results from antagonistic interactions between RegA and various transcriptional repressors through competition for overlapping binding sites. If so, phosphorylation of RegA would have a direct affect on its ability to contend with or perhaps displace DNA-bound repressors. Consequently, the regulation of photosynthesis gene expression may then depend on the relative affinities of multiple regulators that may each be controlled in a redox-dependent manner. It will be interesting to examine whether this is a common feature for additional genes that fall within the RegB/RegA regulon.

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