Isolation and in Vitro Phosphorylation of Sensory Transduction Components Controlling Anaerobic Induction of Light Harvesting and Reaction Center Gene Expression in *Rhodobacter capsulatus*†

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ABSTRACT: Anaerobic induction of light harvesting and reaction center gene expression involves two *trans*-acting factors termed RegA and RegB. Sequence and mutational analysis has indicated that RegA and RegB constitute cognate components of a prokaryotic sensory transduction cascade with RegB comprising a membrane-spanning sensor kinase and RegA a cytosolic response regulator. In this study we have purified RegA, as well as a truncated portion of RegB (RegB′) and undertaken an *in vitro* analysis of autophosphorylation and phosphotransfer activities. Incubation of RegB′ with [γ-^{32}P]ATP and $MgCl_2$ resulted in phosphorylation of RegB′ (RegB′∼P) over a 20-min incubation period. Incubation of RegB′∼P with RegA resulted in rapid transfer of the phosphate from RegB′ to RegA. In analogy to other characterized prokaryotic sensory transduction components, mutational and chemical stability studies also indicate that RegB′ is autophosphorylated at a conserved histidine and that RegA accepts the phosphate from RegB at a conserved aspartate.

The purple photosynthetic bacterium *Rhodobacter capsulatus* synthesizes a photosystem that is composed of three membrane-spanning pigment−protein complexes: light harvesting-I, light harvesting-II, and the reaction center complex. One characteristic feature of anoxygenic purple photosynthetic bacteria is that the photopigment complexes are synthesized only under anaerobic growth conditions. Aerobic repression of photosystem synthesis presumably reflects a more efficient utilization of molecular oxygen (respiration) as an energy source rather than synthesis of a photosystem that utilizes light for cellular energy.

Genetic and molecular analyses have indicated that all of the known essential structural genes required for synthesis of the pigment and polypeptide components of the photosystem are linked to a region of the chromosome termed the photosynthesis gene cluster [Marrs, 1981; reviewed by Bauer et al. (1993) and Bauer (1995)]. Sequence (Alberti et al., 1995; Armstrong et al., 1989) and mutational analysis (Yen & Marrs, 1976; Taylor et al., 1983; Zsebo & Hearst, 1984; Guiliano et al., 1988; Yang & Bauer, 1990; Bollivar et al., 1994a,b) have indicated that the photosynthesis gene cluster is arranged such that the central portion codes for polypeptides that catalyze synthesis of bacteriochlorophyll and carotenoid photopigments. Pigment biosynthesis genes are in turn flanked by the *puh* and *puf* operons that code for the reaction center and light harvesting-I structural polypeptides. Separated from the photosynthesis gene cluster is the *puc* operon which codes for the light harvesting-II structural polypeptides. Studies from several laboratories have indicated that transcription of photosynthesis genes are induced under anaerobic growth conditions [reviewed in Bauer et al. (1993) and Bauer (1995)]. The level of anaerobic induction varies from 2- to 3-fold for bacteriochlorophyll and carotenoid biosynthesis genes to more the 30-fold for the light harvesting and reaction center genes coded by the *puf*, *puh*, and *puc* operons.

Anaerobic induction of light harvesting and reaction center gene expression has recently been shown by our laboratory to involve two *trans*-acting factors RegA and RegB (Sganga & Bauer, 1992; Mosley et al., 1994). Sequence analyses indicates that *regA* and *regB* code for sensory transduction polypeptides of the response regulator/sensor kinase class (Parkinson & Kofoid, 1992; Parkinson, 1993). Specifically, RegA is a 20 534 dalton polypeptide that exhibits sequence similarity to response regulators (Sganga & Bauer 1992) whereas RegB is a 47 394 dalton polypeptide that exhibits similarity to membrane-spanning histidine sensor kinases (Mosley et al., 1994). Mutational analyses also demonstrate that disruption of either RegA or RegB effectively disrupts the cells ability to induce the *puf*, *puc*, and *puh* operons upon a shift from aerobic to anaerobic growth conditions.

In this study we have undertaken an *in vitro* analysis of the RegA and RegB sensory transduction cascade. Specifically, we have purified RegA and a cytosolic truncated version of RegB (RegB′) from *Escherichia coli* cells that overexpressed these polypeptides using a T7 RNA polymerase expression system. After purification and refolding, we have been able to demonstrate that RegB′ has the capability to autophosphorylate as well as to transfer a phosphate to its cognate partner RegA. Chemical evidence for the nature of the phosphorylated residue is also presented.

MATERIALS AND METHODS

Plasmid Construction and Bacterial Strains. RegA and RegB′ were both heterologously overexpressed in *E. coli*

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using T7 RNA polymerase based expression systems. A plasmid harboring *regA* downstream of the T7 promoter region was constructed by cloning a 857 bp *Bam*HI−*Sal*I restriction fragment from pMWS3.1 (Sganga & Bauer, 1992) into *Bam*HI−*Xho*I sites of the T7 promoter vector pSP72 (Promega) forming the expression plasmid pSP72::*regA*. This construct was subsequently transformed into the IPTG based T7 RNA polymerase induction strain BL21(DE3)/plysS (Studier et al., 1990).

A plasmid expressing a truncated version of RegB, termed RegB′, was constructed to assay for *in vitro* kinase activity. The RegB′ was constructed by polymerase chain reaction (PCR) based amplification of the carboxy-terminal region of *regB* which lacked the membrane-spanning coding regions (amino acids 1−144) using pCSM9E as a template (Mosley et al., 1994) with an upstream primer of 5′-CCCATATGATCGAATTCGGCTC and a downstream primer of 5′-CCCTCGAGTGTGATCATCAGG. These primers create unique *Nde*I and *Xho*I restriction sites at the 5′ and 3′ end of the *regB′* amplified regions. The amplified RegB′ DNA was subsequently cloned into the *Nde*I and *Sal*I sites of the T7 expression vector T7-7 (Ausubel et al., 1987) forming the construct pT7-7::RegB′. pT7-7::RegB′ was subsequently transformed into strain C600/pGP1-2 (Tabor & Richardson, 1987; Ausubel et al., 1987) for overexpression of RegB′ as described below.

Overexpression and Purification of RegA and RegB′. For RegA purification, the overexpression strain BL21(DE3)/pLysS harboring plasmid pSP72::*RegA* was grown overnight at 37 °C in LB medium containing 100 μg/mL ampicillin. The overnight culture was then diluted 1:100 into 1 L of prewarmed LB-ampicillin medium and grown under the same conditions as described above. When the optical density ($OD_{600\,nm}$) reached 0.4, IPTG was added to a final concentration of 0.5 mM to induce expression of T7 RNA polymerase. After 2−3 h of incubation, the cells were harvested by centrifugation at 7600*g* at 4 °C for 10 min. The cell pellet was then washed twice at 4 °C with 20 mM Tris-HCl (pH 8.0) containing 50 mM NaCl. The pellet was then resuspended in 10 mL of 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM DTT, 0.1 mM EDTA, and 0.05 mM PMSF and frozen overnight at −70 °C. Cells were then freeze−thawed twice, supplemented with a few crystals of RNaseA and DNase I, and briefly sonicated to complete lysis. The lysate was then centrifuged at 17200*g* for 10 min to remove cell debris and further centrifuged at 100000*g* for 90 min using a Ti 50 (Beckman Corp.) rotor. The supernatant was collected, filtered through a 0.2-μm Acrodisc (Gelman), and then applied to a FPLC Mono S 5/5 cation-exchange column (Pharmacia) at room temperature. Protein was eluted at a rate of 0.5 mL/min with a 0−1 M NaCl concave gradient (formula: 0−10 mL = 0% buffer B, 10−20 mL = 15% buffer B, 20−40 mL = 30% buffer B, 40−50 mL = 50% buffer B where buffer A is 50 mM HEPES, pH 7.8, and buffer B is 50 mM HEPES, pH 7.8, 1 M NaCl). Fractions containing RegA, which typically eluted at 250 mM NaCl, were pooled and concentrated to 2 mg of protein/mL using Centricon 3 (Amicon) according to the manufacturer's instructions. Concentrated RegA was then applied to a Superose HR10/30 gel filtration column (Pharmacia) equilibrated and eluted with 50 mM HEPES, pH 7.8, 100 mM NaCl, 0.1 mM EDTA, and 0.05 mM PMSF at a flow rate of 0.3 mL/min. Pure RegA typically eluted at the 15 mL fraction as a maximum 280-nm absorbing peak. All purification steps with the exception of FPLC chromatography were performed at 4 °C. Protein concentration was determined using the Bradford assay (Bio-Rad). Fractions containing RegA were visualized by Coomassie blue staining using a 12% SDS−PAGE.

For purification of RegB′ an overnight culture of *E. coli* strain C600/pGP1-2/pT7-7::RegB′ was diluted 1:40 into 1.0 L of prewarmed (30 °C) LB medium containing 50 μg/mL kanamycin and 50 μg/mL ampicillin. Cells were grown at 30 °C to an optical density of 0.4 at 590 nm at which point the culture was rapidly heated to 42 °C by shaking under hot tap water (~60 °C) and subsequently placed in a 42 °C water bath for 30 min followed by a shift to 30 °C for an additional 3 h. Two to three liters of heat-induced cell cultures were harvested by centrifugation at 4 °C, washed twice with 4 °C lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM β-mercaptoethanol (β-ME)[1]]. The cell pellet was resuspended in 40 mL of 4 °C lysis buffer containing 1 mM PMSF and a small aliquot of DNase I. The cell suspension was lysed by passage twice through a chilled french pressure cell at 18 000 psi and then diluted with an additional 200 mL of 4 °C lysis buffer containing 1 mM PMSF plus a small aliquot of DNase I. The cell lysate was clarified by centrifugation at 10000*g* for 10 min at 4 °C followed by centrifugation at 39000*g* for 60 min. The pellet fraction which contained RegB′ was solubilized by suspension in 4 °C 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 1 mM EDTA, 10 mM β-ME, and 6 M urea to a final protein concentration of 1 mg/mL. After gentle stirring for 1 h at 4 °C, the suspension was centrifuged at 39000*g* for 60 min at 4 °C. The supernatant was then collected and saved on ice while the precipitate was washed with the same 6 M urea buffer as above. The urea-solubilized supernatant fraction from the first wash was subsequently pooled with that of the second urea wash. Activated DEAE Bio-Gel A (Bio-Rad), which was equilibrated with 50 mM Tris-HCl (pH 7.8), 25 mM NaCl, 10 mM β-ME, and 6 M urea, was then added at a ratio of 10% (v/v) to the combined supernatant and gently shaken for 10 s at 10-min intervals for 30 min and then allowed to sit undisturbed at 4 °C for an additional 30 min. After 1 h of incubation, the DEAE Bio-Gel A was collected by centrifugation at 5000*g* for 5 min, poured on Buchner funnel, and washed twice with a 10× volume of 4 °C 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 10 mM β-ME, and 6 M urea. RegB′ was then eluted from the DEAE Bio-Gel A by the addition of 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM β-ME, and 6 M urea with 1.0-mL aliquots being collected. Each fraction was visualized by SDS−PAGE with fractions containing RegB′ concentrated to 1 mg of protein/mL by Centricon-10 membrane (Amicon) centrifugation and stored at −70 °C. At this stage RegB′ was typically 60~70% pure. For further purification, 5 mL of the DEAE Bio-Gel A fraction was applied to a 1 cm × 10 cm hydroxylapatite (Bio-Rad) column which was pre-equilibrated with 20 mM HEPES-NaOH (pH 7.0), 0.1 mM EDTA, 10 mM β-ME, and 6 M urea. The column was washed with several column volumes of the pre-equilibration buffer that also contained 50 mM phosphate buffer (pH 7.0). RegB′ was eluted by 20 mM HEPES-NaOH (pH 7.0), 0.1 mM EDTA, 10 mM β-ME, and 6 M urea containing 80 mM

[1] Abbreviations: β-ME, β-mercapthoethanol.

phosphate buffer (pH 7.0) and concentrated to 1 mg of protein/mL by Centricon-10 (Amicon) and stored −70 °C until use. The protein purity at this stage was more than 90%.

Refolding of RegA and RegB′. For RegA refolding, gel filtration fractions containing RegA were pooled and concentrated to 2 mg/mL protein by Centricon 3 centrifugation (Amicon). The RegA sample was weighed and supplemented with an equal (w/w) amount of guanidine hydrochloride to give a final concentration of 6 M guanidine hydrochloride with the denatured RegA then being stirred (or shook) gently at 4 °C for at least 1 h. Small 100-μL aliquots of unfolded protein were rapidly dispersed by the addition of 5.0 mL (50-fold dilution) of RegA refolding buffer composed of 25 mM HEPES, pH 7.8, 10 mM $MgCl_2$, 40 mM KCl, and 1 mM DTT to give a final protein concentration of approximately 20−30 μg/mL. The diluted RegA was then allowed to reach equilibrium for 2 h at 4 °C and subsequently concentrated by Centricon 3 (Amicon) centrifugation. The concentrated RegA sample was then dialyzed overnight at 4 °C against RegA refolding buffer that was supplemented with 20% glycerol and stored at −70 °C.

For refolding RegB′, the samples were diluted to a final concentration of 0.6 mg of protein/mL in 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 6 M urea. The protein was then diluted 1:1 in 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 300 mM KCl, and 0.1 mM EDTA to give a final urea concentration of 3.0 M. The sample was then placed in a glass tube capped with rubber septa to which pure Ar (Ultra purity high grade, AirCo/Mineweld Co.) was flowed to the sample through septa for at least 1 h at room temperature while gently stirring. Fresh β-ME was then added to the final concentration of 70 mM under Ar flow and further incubated for 30 min at room temperature. The sample was then dialyzed against 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 200 mM KCl, 0.1 mM EDTA, and 70 mM β-ME at room temperature for 6 h. Anaerobic dialysis buffer was prepared by degassing buffer (containing dialysis tubing) that lacked β-ME under vacuum for several min followed by purging with Ar for 15 min. Fresh β-ME was then added followed by an additional 15 min of Ar purging. (The dialysis tubing was also rinsed with anaerobic dialysis buffer prior to use.) After 6 h of dialysis, the buffer was changed to 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 200 mM KCl, and 0.1 mM EDTA, to which fresh DTT (0.1 mM) was added prior to use. The sample was then dialyzed at 4 °C for an additional 12 h. Refolded RegB′ was then kept in an air-tight tube at −70 °C and generally remained active for several weeks. To ensure anaerobic conditions which are optimal for RegB′ activity, all manipulations were performed in an anaerobic chamber (Coy Corp) that contained a gas mixture of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen which was scrubbed free of oxygen with a platinum catalyst.

Phosphorylation Reactions. Phosphorylation reaction mixtures (10 μL) contained 7 μg (20 pmol) of RegB′ protein in 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 200 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, and 45 μM [γ-^{32}P]ATP. The reaction was initiated by mixing 9 μL of a reaction mixture lacking ATP with 1 μL of an [γ-^{32}P]ATP mixture that was prepared by mixing 1 μL of 10 μCi/μL [γ-^{32}P]ATP (Amersham 3000 Ci/mmol) with 9 μL of 500 μM ATP to give a final concentration of 450 μM (2.2 Ci/mmol, 1 Ci = 37GBq). The reaction mixture was incubated at 22 °C at the indicated times and stopped by adding 2× SDS loading dye and frozen in a dry-ice−ethanol bath immediately. For phosphorylation of RegA, RegB′ was phosphorylated as described above for 2 min, followed by the addition of RegA. The reaction mixtures were incubated at room temperature and stopped by adding 2× SDS loading dye. The samples were heated to 55 °C for 5 min and then separated by SDS−PAGE analysis using 4% stacking and 15% separating acrylamide gel concentrations. After electrophoresis the proteins were transferred to a ProBlott membrane (Applied Biosystems), dried, and autoradiographed at −70 °C.

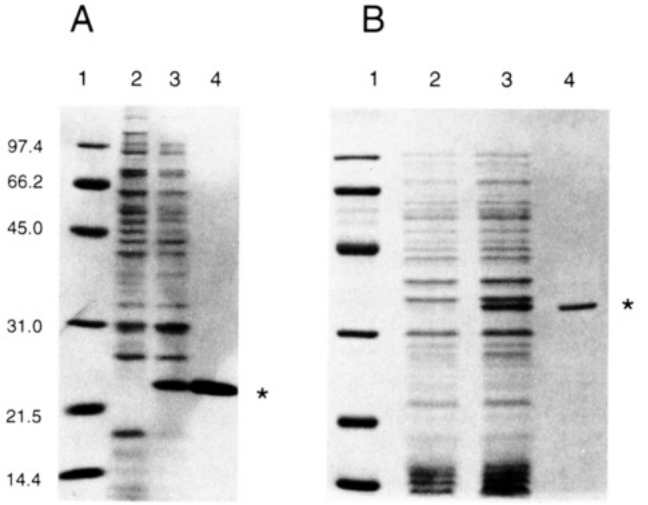

FIGURE 1: Commassie blue stained SDS−PAGE profile of overexpressed RegA and RegB′. (A) (Lane 1) Electrophoretic mobility of polypeptides of known molecular weights. (Lane 2) Whole cell extracts of the inducible T7 RNA polymerase overexpression strain containing the vector T7-7. (Lane 3) As described in lane 2 with the exception that the overexpression strain contains the RegA overexpression plasmid pSP72::*regA*. (Lane 4) Isolated RegA polypeptide. (B) (Lanes 1 and 2) The same polypeptide profiles as indicated in lanes 1 and 2 in gel profile A. (Lane 3) Whole cell extracts of an inducible T7 RNA polymerase overexpression strain that contains the RegB′ overexpression plasmid pT7-7::RegB′. (Lane 4) Isolated RegB′ polypeptide. The asterisk denotes RegA and RegB′.

Chemical Stability of Phosphorylated Proteins. Phosphorylated proteins that were transferred to ProBlott membrane were either untreated or treated for 1 h at 22 °C with 50 mM Tris-HCl (pH 7.8), 3 M NaOH, or 1 M HCl. Strips were neutralized with 50 mM Tris-HCl (pH 7.8), washed with distilled water, dried, and autoradiographed at −70 °C.

RESULTS AND DISCUSSION

Overexpression and Purification of Sensory Transduction Components. An *E. coli* based IPTG inducible T7 RNA polymerase expression system (Studier et al., 1990) was utilized to heterologously overexpress RegA in *E. coli.* As shown in the SDS−PAGE profile of whole cell lysate in Figure 1A, the RegA polypeptide is highly expressed upon IPTG based induction of T7 RNA polymerase. Despite the high level of induction, the overexpressed RegA polypeptide was found to be consistently localized in the soluble fraction of the cell lysate. Thus, after pelleting membrane and ribosomal fractions by high speed centrifugation (100000g for 90 min), a soluble protein fraction was obtained that is highly enriched for RegA (>50% of the soluble protein

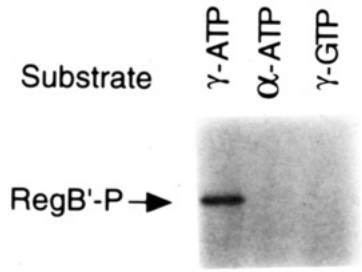

FIGURE 2: Nucleotide and phosphate specificity of RegB′ autophosphorylation. RegB′ was incubated at 22 °C with 45 μM (2.2 Ci/mmol) [γ-^{32}P]ATP, 45 μM (2.2 Ci/mmol) [α-^{32}P]ATP, or 45 μM (2.2 Ci/mmol) [γ-^{32}P]GTP. After 20 min the reaction was stopped by the addition of an SDS dye solution. The polypeptides were then separated by SDS−PAGE and autoradiographed.

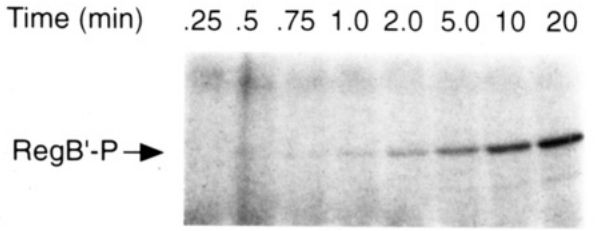

FIGURE 3: RegB′ autophosphorylation. Isolated RegB′ (20 pmol) was incubated at 22 °C with 45 μM (2.2 Ci/mmol) [γ-^{32}P]ATP. Aliquots of the reaction were removed at the indicated times, and the reactions were stopped by the addition of SDS dye solution followed by chilling on ice. The reactions were then separated by SDS−PAGE analysis and autoradiographed.

represents RegA; data not shown). Further purification by cation-exchange and size exclusion chromatography yielded RegA that was essentially purified to homogeneity as judged by SDS−PAGE (Figure 1A) and amino-terminal sequence analysis (data not shown). Despite the observation that overexpressed RegA remains soluble, we have observed that RegA is not biochemically active without prior denaturation in 6 M guanidine hydrochloride followed by renaturation (see Material and Methods). Demonstration of biochemical activity, as measured by acceptance of a phosphate group, is discussed below.

Purification of full length membrane-spanning sensor kinases has proven to be problematic in most cases where such purification has been attempted (Igo & Silhavy, 1988; Iuchi & Lin, 1992; Jin et al., 1990; Winans, 1989). Fortunately, truncated cytosolic versions of many membrane-spanning sensor kinases retain autophosphorylation and subsequent phosphotransfer capabilities (Aiba et al., 1989; Gilles-Gonzalez, 1991; Igo & Silhavy, 1988; Iuchi & Lin, 1992; Jin et al., 1990; Nakashima et al., 1993; Schroder et al., 1994; Uhl & Miler, 1994). To facilitate purification of RegB, we constructed a vector which expressed only the cytosolic portion of RegB (termed RegB′). RegB′ lacks the first 144 amino acids which is the region that contains most of the hydrophobic portions of the polypeptide but does retain the conserved histidine and kinase blocks of the polypeptide (Mosley et al., 1994). The level of RegB′ produced by T7 RNA polymerase based overexpression was observed to be considerably lower than that produced for RegA (Figure 1B). Furthermore, RegB′ was found to be localized to an inclusion body fraction of the cell lysate rather than the soluble fraction as observed for RegA. Solubilization of the washed RegB′ inclusion bodies in 6 M urea followed by ion exchange and hydroxylapatite chromatography typically yielded preparations that are >90% pure as judged by SDS−PAGE (Figure 1B) and amino-terminal sequence analysis (data not shown). Refolding of RegB′ was accomplished by rapid dilution of the urea to an intermediate denaturant condition where RegB′ was incubated to allow potential formation of some secondary structure. Partially renatured RegB′ was subsequently dialyzed to remove the remaining chaotrope to complete refolding as described in Materials and Methods.

Autophosphorylation of RegB′. Incubation of the isolated RegB′ polypeptide with [γ-^{32}P]ATP for 20 min in the presence of 5 mM $MgCl_2$, followed by SDS−PAGE and subsequent autoradiography of the gel, indicated that RegB′ exhibited autophosphorylation activity (Figure 2). Autophosphorylation is specific for the γ phosphate of ATP as demonstrated by the absence of RegB′ phosphorylation in the presence of [α-^{32}P]ATP or [γ-^{32}P]GTP (Figure 2).

A time course of RegB′ autophosphorylation is shown in Figure 3. As indicated by the autoradiograph, there is an increase in phosphate incorporation by RegB′ during a 20-min incubation period at 25 °C. The time course of RegB′ autophosphorylation is similar to that observed during other *in vitro* studies of cytosolic (truncated) versions of membrane-spanning sensor kinases which exhibit an increase in autophosphoryation over a 10−120-min time period (Aiba et al., 1989; Iuchi & Lin, 1992; Lois et al., 1993; Monson et al., 1992; Nakashima et al., 1993; Schroder et al., 1994; Uhl & Miler, 1994). The rate of autophosphorylation with truncated sensor kinases is generally slower than that observed with full length sensor kinases that do not exhibit membrane-spanning domains such as NtrC and CheA which typically reach a plateau of autophosphorylation in a few minutes (Hess et al., 1988a,b; Weiss & Magasanik, 1988). Presumably the slow rate of autophosphorylation observed with truncated membrane-spanning kinases reflect a disruption of proper dimerization of the kinase subunits which is required for phosphorylation (Hess et al., 1988b; Pan et al., 1993; Yang & Inouye, 1991; Parkinson, 1993; Parkinson & Kofoid, 1992).

Phosphotransfer from RegB′∼P to RegA. Prior genetic analyses of photosystem gene expression indicated that RegB and RegA constitute sensor kinase/response regulator partners (respectively) in a sensory transduction cascade (Mosley et al., 1994; Sganga & Bauer, 1992). In order to verify that RegB and RegA are indeed partner components in such a cascade, we performed phosphotransfer experiments to assay whether autophosphorylated RegB′ has the capability of phosphorylating RegA. For these experiments RegB′ was autophosphorylated by preincubation with [γ-^{32}P]ATP for 2 min, at which point RegA was added. Aliquots were withdrawn at indicated time points, and the reactions were stopped by the addition of an SDS dye solution followed by chilling on ice. The RegB′ and RegA components were then separated by SDS−PAGE and assayed for phosphorylation by autoradiography. As indicated by the autoradiograph in Figure 4, incubation of RegB′ with [γ-^{32}P]ATP resulted in autophosphorylation of RegB′ whereas a similar 20-min incubation of RegA with [γ-^{32}P]ATP did not result in the formation of phosphorylated RegA. However, RegA∼P was rapidly formed when RegA was mixed with preformed RegB′∼P. Indeed, the kinetics of phosphate transfer from RegB′∼P to RegA is judged to be very rapid and nearly complete as indicated by most of the phosphate transferring from RegB′∼P to RegA in the manner of a few seconds

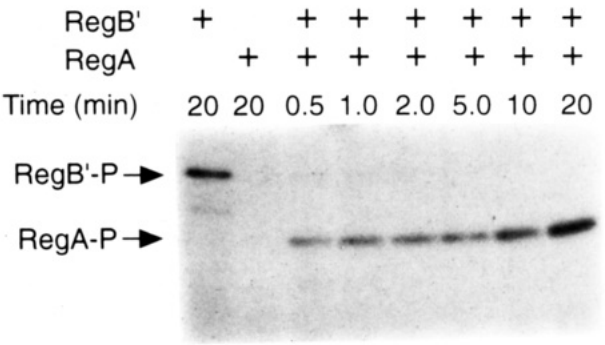

FIGURE 4: Phosphate transfer from RegB′~P to RegA. RegB′~P was formed by preincubation of 2 μM of RegB′ with 45 μM (2.2 Ci/mmol) [γ-^{32}P]ATP at 22 °C. After a 2-min preincubation, 2 μM of RegA was added, and aliquots of the mixture were then removed and stopped by addition of SDS loading dyes followed by chilling on ice. In the control lanes with RegB′ or RegA alone, 2 μM of the respective proteins were incubated with 45 μM (2.2 Ci/mmol) [γ-^{32}P]ATP for 20 min at 22 °C. The reactions were separated by SDS−PAGE analysis and autoradiographed.

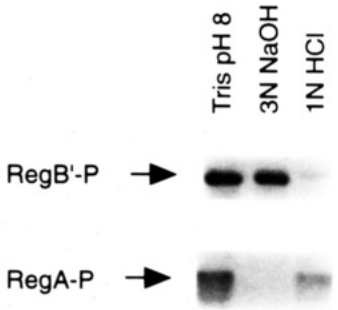

FIGURE 5: Chemical stability of phospholigand to RegB′ and RegA. RegB′~P and RegA~P were formed as indicated in Figure 4 and separated by SDS−PAGE. After Western blot transfer to ProBlott mambrane, the lanes were cut into strips and incubated for 1 h at 22 °C in 50 mM Tris-HCl, pH 8.0, 3.0 M NaOH, or 1.0 M HCl. The strips were then neutralized by washing in 50 mM Tris-HCl, pH 8.0, dried, and autoradiographed.

(<30 s). The level of RegA~P rises over the 20-min incubation period without detection of significant amounts of RegB′~P, a further indication of slow RegB′ autophosphorylation followed by rapid transfer of the phosphate to RegA. A similar rapid rate of phosphotransfer is also observed with CheA~P phosphotransfer to CheY (Borkovich & Simon, 1990; Hess et al., 1988a,b), EnvZ~P phosphotransfer to OmpR (Aiba et al., 1989), and NtrB~P phosphotransfer to NtrC (Weiss et al., 1991).

Chemical Nature of the RegB and RegA Phospholigands. The nature of the phosphotransfer chemistry was also investigated for RegB′ and RegA. In most biochemically studied prokaryotic sensory transduction systems of this kind it has been observed that the sensor kinase autophosphorylates at a conserved histidine residue, which in the case of RegB corresponds to His_{204} (Mosley et al., 1994). The phosphate is then thought to be transferred to a conserved aspartate residue in the response regulator, which in the case of RegA corresponds to Asp_{63} (Sganga & Bauer, 1992). Histidinyl phosphate ligands are known to be stable in the presence of base but highly labile to acid, whereas the inverse occurs for acyl linkage (Hultquist, 1968; Fujitaki & Smith, 1984; Martensen, 1984; Weiss & Magasanik, 1988). As shown in Figure 5, RegB′~P does indeed shows stability when incubated for 1 h in the presence of 3 M NaOH, as well as lability when incubated in the presence of 1 M HCl, a characteristic feature of a histidinyl linkage. On the other hand, the phosphate ligand to RegA is more labile under alkaline conditions then under acidic conditions (Figure 5) which is consistent of an acyl phosphate linkage of an aspartate residue (Fujitaki & Smith, 1984; Martensen, 1984; Weiss & Magasanik, 1988).

CONCLUSION

Prior genetic and molecular genetic analyses have indicated that RegB and RegA constituted cognate sensor kinase and response regulator partners, respectively, in a sensory transduction cascade that control light harvesting and reaction center gene expression in response to anaerobiosis (Mosley et al., 1994; Sganga & Bauer, 1992). The results of this study provides *in vitro* biochemical confirmation that RegB′ indeed has the capability to autophosphorylate and to subsequently transfer the phosphate to the response regulator RegA. In all respects, the autophosphorylation and phosphotransfer capabilities exhibited by RegB′ are typical of that observed with other histidine sensor kinases (Parkinson, 1993; Parkinson & Kofoid, 1992). As discussed more fully in Mosley et al. (1994), there are reasons to believe that the sensory transduction cascade responsible for controlling photosystem gene expression in response to anaerobiosis is more complex than a simple phosphotransfer from RegB to RegA. Mosley et al. (1994) has proposed that there exists additional sensor kinase(s) that feed into this sensory transduction cascade and that there may be a "downstream" DNA binding component that could accept a phosphate from RegA. Continued genetic, molecular genetic and biochemical analysis of potential regulatory factors should continue to shed light on complexities of the regulatory circuit that controls photosystem gene expression in response to alterations in oxygen tension.

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