Identification of a Ubiquinone-binding Site That Affects Autophosphorylation of the Sensor Kinase RegB*

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Rhodobacter capsulatus regulates many metabolic processes in response to the level of environmental oxygen and the energy state of the cell. One of the key global redox regulators of the cell’s metabolic physiology is the sensor kinase RegB that controls the synthesis of numerous energy generation and utilization processes. In this study, we have succeeded in purifying full-length RegB containing six transmembrane-spanning elements. Exogenous addition of excess oxidized coenzyme Q1 is capable of inhibiting RegB autophosphorylation ~6-fold. However, the addition of reduced coenzyme Q1 exhibits no inhibitory effect on kinase activity. A ubiquinone-binding site, as defined by azidoquinone photo affinity cross-linking, was determined to lie within a periplasmic loop between transmembrane helices 3 and 4 that contains a fully conserved heptapeptide sequence GGXXNPF. Mutation of the phenylalanine in this heptapeptide renders RegB constitutively active in vivo, indicating that this domain is responsible for sensing the redox state of the ubiquinone pool and subsequently controlling RegB autophosphorylation.

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Strain, Media, and Growth Conditions—R. capsulatus strain, SB1003 was used as the parent strain for all of the mutants generated for this study. All of the R. capsulatus strains were grown at 34 °C in either RCV or PY, with spectinomycin and gentamycin concentrations of 10 and 1.5 μg/ml, respectively. E. coli strain DH5α was grown at 37 °C in Luria broth for all cloning procedures, and strain BL21 (Ade3) was grown in Terrific broth for protein overexpression, with kanamycin, spectinomycin, and monitor the redox state of a component of the electron transport chain. Specifically, ubiquinones and cytochrome cbb3, oxidase have been suggested components that may interact with RegB and modulate its kinase activity (4, 5). There is also evidence that RegB contains a redox active cysteine residue that when oxidized inhibits kinase activity (6). Neither of these modes of regulation are mutually exclusive. For example, the widely studied global sensor kinase ArcB from Escherichia coli controls a number of cellular processes in response to changes in oxygen tension. It has been proposed that activity of ArcB is regulated by two redox active cysteines that form intermolecular disulfide bonds when oxidized by ubiquinone (7, 8). Because the oxidation-reduction state of the ubiquinone pool fluctuates greatly based on oxygen tension, it has been proposed that an interaction of ArcB with the ubiquinone pool ultimately regulates the activity of this kinase (7, 8).

Given that RegB is a membrane-bound sensor kinase, one can presume that it is in close contact with the ubiquinone pool and thus could potentially monitor the oxidation/reduction state of this membrane soluble electron carrier. In purple bacteria, the oxidation state of the ubiquinone pool is known to dramatically fluctuate, becoming predominantly oxidized under aerobic respiratory growth and predominantly reduced under anaerobic photosynthetic growth conditions (9). Given that purple bacteria have a large ubiquinone pool (10) that undergoes significant changes in redox potential (9), it is apparent that this molecule would be an excellent indicator of changes in environmental oxygen tension.

In this study, we describe the isolation and biochemical characterization of a full-length membrane-spanning version of RegB. We provide in vitro evidence that oxidized ubiquinone is a potent inhibitor of RegB. A ubiquinone-binding site was identified by performing photo affinity cross-linking with a ubiquinone analog that binds to a universally conserved heptapeptide sequence GGXXNPF in the membrane-spanning input domain of RegB. Mutational analysis of this heptapeptide sequence results in dramatic derepression of RegB autophosphorylation in vivo, resulting in elevated expression of photosynthesis genes even in the presence of oxygen.

EXPERIMENTAL PROCEDURES

Strain, Media, and Growth Conditions—R. capsulatus strain, SB1003 was used as the parent strain for all of the mutants generated for this study. All of the R. capsulatus strains were grown at 34 °C in either RCV or PY, with spectinomycin and gentamycin concentrations of 10 and 1.5 μg/ml, respectively. E. coli strain DH5α was grown at 37 °C in Luria broth for all cloning procedures, and strain BL21 (Ade3) was grown in Terrific broth for protein overexpression, with kanamycin, spectin-
omycin, and ampicillin concentrations of 50, 50, and 150 μg/ml, respectively.

**Plasmid Construction**—An expression plasmid for full-length RegB was constructed by PCR of the *regB* gene using primers; RegBFullNcoI (5’-TACATGAGGCGGGTGATCGAGACATTTTC) and RegBFullXhol (5’-ATCCGAGGGCGGGTGATCGAGACATTTTC) with genomic template DNA obtained from wild type cells. The PCR product was cloned into the NcoI and Xhol sites of pET28.

A chromosomal in-frame deletion of *regB* was constructed by first PCR amplifying the entire *regB* gene plus 500 bp upstream and downstream of the *regB* open reading frame using primers RegBdbSacF (5’-GAACGCCCCCTTCGCGC) and a reverse complement of this primer, RegBdbXbaR (5’-TATCTAGACCGCATGATCCAGTCTGTC) for the downstream region with plasmid pCRScriptRegB ligated together they generated an in-frame deletion of RegBdel downstream region. The amplified fragment was cloned into pCRScript SK+ (Stratagene) forming plasmid pCRScriptRegB+ and sequenced to ensure correct amplification. Plasmid pCRScriptRegB+ was then used as a PCR template for construction of the in-frame deletion of *regB* and as a template to generate regB point mutations.

The in-frame *regB* deletion was constructed by PCR amplifying 500-bp segments of DNA flanking the *regB* gene using primers RegBdelUp (5’-ATGATATCCTATCTGCCATGTCG) for the upstream region and RegBdelXbaR (5’-TATCTAGACCGCATGATCCAGTCTGTC) and RegBdelDown (5’-ATGATATCCTGCCATGATCACAATTC) for the downstream region with plasmid pCRScriptRegB+ as a template. EcoRV sites were engineered into the RegBdelUp and RegBdelDown primers, such that when the two PCR products were added to a final concentration of 75 μM of full-length RegB in a buffer comprised of 20 mM Tris, pH 8.0, 20 mM imidazole, 150 mM NaCl, and 10% glycerol and allowed to incubate with 1 ml of settled charged nickel resin (Novagen) for 1 h at room temperature shaking at 40 rpm on an orbital shaker. The nickel resin was then pelleted by centrifugation at 10,000 × g for 5 min at 4 °C. The supernatant was discarded, and the resin was applied to a disposable column and washed with 50 column volumes of wash buffer comprised of 20 mM Tris, pH 8.0, 20 mM imidazole, 150 mM NaCl, and 10% glycerol at 24 °C. When purifying full-length RegB for use with azido-Q3 binding experiments, the wash buffer step was modified by the addition of a second wash step of 50 column volumes of wash buffer containing 1% sodium cholate to remove and replace protein-bound n-dodecyl β-D-maltoside that interferes with azido-Q binding. Full-length RegB was then eluted with 10 ml of elution buffer comprised of 20 mM Tris, pH 8.0, 200 mM imidazole, 150 mM NaCl, and 10% glycerol at 24 °C with 1-ml fractions collected and analyzed for protein content using the Bio-Rad assay. Fractions containing protein were separated by SDS-PAGE to confirm the presence of RegB. Appropriate fractions were pooled and dialyzed against 10 mM Tris, pH 8.0, 150 mM NaCl, 50% glycerol at 4 °C and then stored at −20 °C until the kinase assays were completed.

**Kinase Assays**—The kinase assays were completed as described by Bird et al. (12). For ubiquinone addition experiments, benzoquinone (Sigma catalog number D9150) or coenzyme Q1 (Sigma catalog number C7956) was solubilized in 95% ethanol at a concentration of 167 μM and added to replicate kinase with one assay containing only the solvent (<1% ethanol) and the other reaction containing solvent plus either benzoquinone or coenzyme Q1. In cases where ubiquinone was reduced to ubiquinol, 10 μM dithiothreitol was added to the ubiquinone prior to addition to the kinase assays.

**Affinity Binding of [3H]Azidoquinone**—Tritium-labeled azidoquinone ([3H]azido-Q) was synthesized as described previously (13). Affinity cross-linking of [3H]azido-Q to sodium cholate-purified full-length RegB was performed by mixing 42 mM of [3H]azido-Q that was solubilized in 95% ethanol with 42 μM of full-length RegB in a buffer comprised of 20 mM Tris, pH 8.0, 150 mM NaCl, and 30% glycerol. The final concentration of ethanol in the mixture was kept lower than 5% to prevent denaturation of the protein. After incubation for 60 min at 4 °C in the dark, the protein and [3H]azido-Q was placed in a 2-mm light path quartz cuvette and exposed to long wavelength UV light (Spectroline EN-14, 365-nm-long wavelength, 23 watts) at 0 °C for 20 min at a distance of 4 cm from the light source. Unbound [3H]azido-Q was then separated from UV cross-linked [3H]azido-Q-RegB by paper chromatography using chloroform:methanol (2:1, v/v) as a solvent system. [3H]Azido-Q-RegB remained at the paper origin, whereas unbound [3H]azido-Q migrated at the solvent front. Chromatographically purified [3H]azido-Q-RegB was then solubilized from the paper matrix by the addition of elution buffer comprised of 30 mM Tris-Cl, pH 7.4, 0.5% SDS, 1 mM urea and then subjected to protease K digestion at 37 °C for 6 h to

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2 Z. Jiang and C. Bauer, unpublished data.

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3 The abbreviations used are: azido-Q, azidoquinone; HPLC, high pressure liquid chromatography; TM, transmembrane domain; PAS, Per Amt Sim.
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FIGURE 1. SDS-PAGE analysis and autophosphorylation assays of RegB. A, solubilized and purified RegB separated by SDS-PAGE. Lane 1 contains the molecular weight mass followed by the wash fraction and the elution fraction after the addition of imidazole. B, autoradiograph of purified monomer RegB using γ-32P-labeled ATP as a tracer. The aliquots were removed at 0.25, 0.5, 0.75, 1, 2, 4, 10, and 15 min after the addition of ATP, and the reaction was quenched with SDS-PAGE loading buffer before being separated by SDS-PAGE. The graph below the autoradiograph represents the arbitrary units of 32P incorporation derived from phosphorimaging data analysis.

using a protease K:RegB ratio of 1:50 (w/w). The protease K-digested sample was then subjected to HPLC separation on a Supelcosil™ LC-308 (C8, 5-µm particles, 300-Å inner diameter, 25-cm length) using a gradient formed from 0.1% trifluoroacetic acid and 90% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 0.8 ml/min with 0.4-ml fractions collected. The fractions recovered from the HPLC chromatograms were analyzed for peptide patterns by UV absorbance recorded with a Waters 996 diode array detector and the presence of [3H] by scintillation counting in a Packard Tri-Carb 1900CA scintillation analyzer. Fractions from several independent HPLC chromatograms that contained [3H] protein profiles were then subjected to amino-terminal sequence analysis at the Molecular Biology Resource Facility of the Saint Francis Hospital of Tulsa Medical Research Institute, University of Oklahoma Health Science Center, under the supervision of Dr. Ken Jackson.

Spectroscopy and Northern Blot Analysis—Spectral analysis of R. capsulatus cell membranes were performed as previously published (6). For Northern blot analysis, cellular RNA was isolated using a RNAeasy Mini kit (Qiagen). The RNA content was assayed by absorbance at 280 nm and then normalized for ribosomal RNA content after electrophoretic separation and visualization of 15 S RNA on a formaldehyde gel. The Northern blot analysis was performed as described previously (14). The RNA blot was probed with a digoxigenin-labeled (Roche Applied Science catalog number 1-636-090) puf or puc probe for 16 h at 65 °C. The digoxigenin-labeled puf and puc probes were generated by PCR from genomic template using primers PufQup (5′-TGCGTAGCTCTACCCTGGTACAGC), PufAdown (5′-GCTCCCAAGCTTCTGGTGTCAGC), PucUP (5′-CTCCCCATATGCTGCTCAGC), and PucDown (5′-TTACTGAGGCCGCCAAGCAGC), respectively. Detection of the digoxigenin-labeled probe was carried out as recommended by the manufacturer of the Dig luminescent detection kit (Roche Applied Science catalog number 1-636-514) with hybridization levels visualized using radiographic film and quantitated using a Fluorchem 5500 imaging system from Alpha Innotech. The 15 S ribosomal RNA was also quantitated and used to normalize Northern blot hybridization levels.

RESULTS

Purification of Full-length Membrane-spanning RegB—A specific involvement of the membrane-spanning domain of RegB in redox sensing has not been extensively explored beyond the construction of a few mutations in the membrane-spanning region, several of which affect phosphorylation (15). To biochemically explore whether the membrane-spanning domain controls RegB activity, we expressed His6-tagged full-length RegB in E. coli and isolated full-length protein from crude membrane fractions after solubilization with the detergent n-dodecyl β-d-maltoside (see “Experimental Procedures”). Detergent solubilized full-length RegB was then purified by nickel ion chromatography and visualized by SDS-PAGE (Fig. 1A). The most prominent band on the gel corresponds to a RegB monomer at 47 kDa and a RegB dimer at 98 kDa, which is close to the calculated monomer and dimer size for RegB at 50.1 and 100.2 kDa. Western blot analysis using an anti-His tag antibody (Santa Cruz Biotechnology catalog number SC803) verified the identity of these bands as His-tagged RegB (data not shown).

Autophosphorylation assays were performed on full-length RegB in the presence of γ-32P-ATP with aliquots being removed at intervals ranging from 0.25 to 15 min (Fig. 1B). The autoradiograph in Fig. 1B demonstrates that purified full-length RegB exhibits excellent autophosphorylation with activity detected as early as 0.25 min, linear within the first 2 min, and reaching a plateau in ~10 min. Similar phosphorylation was observed with a soluble truncated form of RegB (RegBΔ), which does not contain the membrane-spanning domain (6) as well as the full-length RegB homolog from Rhodobacter sphaeroides (16). This indicates that the mild detergent, n-dodecyl β-d-maltoside does not compromise the structural integrity of RegB nor significantly impact the level of activity of the protein.

RegB Autophosphorylation Is Regulated By The Redox State of Ubiquinone—To address the role of ubiquinone in controlling kinase activity, we added a 50-fold molar excess of coenzyme Q1, which is a close analog of ubiquinone containing only one isoprenoid side chain unit. When an oxidized form of this soluble quinone analog was allowed to incubate with RegB for 20 min prior to the initiation of autophosphorylation, there was a 5.4-fold decrease in autophosphorylation levels after the reactions reached plateau (Fig. 2A). Half-maximal kinase inhibition was achieved at a 20-fold molar excess of ubiquinone to RegB (data not shown). Note that there is a vast excess of quinones over that of the molar amount of RegB/cell (10), so it is not surprising that a 20-fold excess of coenzyme Q1 is needed to observe half-maximal inhibition. A similar inhibitory affect on RegB activity was observed with the addition of oxidized benzoquinone, which contains only the polar head group of coenzyme Q1, suggesting that the redox active head group of ubiquinone possesses the inhibitory signal (data not shown). Finally, kinase assays in the presence of a 50-fold excess of reduced coenzyme Q1 (ubiquinol) does not affect the autophosphorylation activity of full-length RegB (Fig. 2B). Thus, only the presence of oxidized quinone has an inhibitory affect on RegB kinase activity.

Involvement of Cys265—We previously demonstrated that RegB contains a fully conserved redox active cysteine (Cys265), located in the cytoplasmic domain between the H-box site of phosphorylation and the
ATP-binding cassette (6). In vitro evidence indicated that oxidation of Cys265 leads to significant inhibition of kinase activity of the truncated cytosolic form of RegB (RegBs) (6). However, mutational analysis of Cys265 in full-length RegB only partially abrogated redox control in vivo, suggesting that additional factors beyond that of Cys265 were involved in controlling RegB kinase activity (6). Thus, redox regulation of full-

Prior to the addition of $[^{32}\text{P}]$ATP. Aliquots were removed at 1, 2, 4, and 8 min and quenched with SDS-PAGE loading buffer before being separated by SDS-PAGE. C, autophosphorylation assays of C65SSA mutant RegB in the presence and absence of ubiquinone (Q$_{1}$) at a 50-fold molar excess to RegB. Ethanol was added to the RegB sample lacking ubiquinone to account for the ethanol concentration of the ubiquinone treated sample. The two reactions were incubated for 20 min at 37 °C before the addition of $[^{32}\text{P}]$ATP. Aliquots were removed at 0.5, 1, 2, 4, and 10 min and quenched with SDS-PAGE loading buffer before being separated by SDS-PAGE. All of the autophosphorylation assays were visualized and quantitated using the Typhoon phosphorimaging system (Amersham Biosciences).
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length RegB autophosphorylation could be a result of altering the oxidation state of a bound ubiquinone, the redox state of Cys265, or both.

To differentiate these possibilities, we isolated a full-length version of RegB that contained a cysteine to alanine mutation at amino acid 265 (C265A) and then assayed this mutant form of full-length RegB for redox-mediated changes in autophosphorylation in vitro. The results of these assays demonstrate that the addition of oxidized coenzyme Q1 to full-length C265A RegB still results in inhibition of autophosphorylation (Fig. 2C). Thus, oxidized coenzyme Q1 is not simply affecting the redox state of Cys265. This indicates that redox control of wild type full-length RegB most likely involves both an alteration of the redox state of Cys265 as well as direct inhibition of activity by oxidized ubiquinone.

Identification of the Ubiquinone-binding Site—A tritium-labeled photo affinity analog of ubiquinone, 3-azido-2-methyl-5-methoxy-[3H]6-decyl-1,4-benzoquinone (azido-Q) was used to identify a ubiquinone-binding site in RegB. This azidoubiquinone derivative and related analogs have been widely used to identify Q-binding domains present in NADH-Q oxidoreductase (17), disulfide bond protein B (18), succinate-Q oxidoreductases (19–21), cytochrome bc1 complexes (13, 22–26), and plastoquinone-binding sites in the cytochrome bf complex from spinach chloroplasts (27). The binding of this quinone analog was performed by equilibrating purified full-length RegB with 20-fold molar excess of azido-Q for 1 h at 0 °C in the dark. The RegB-quinone analog complex was then illuminated with long wavelength UV light (365 nm) for 20 min to initiate photoaffinity cross-linking. Electrophoretically and chromatographically separated azido-Q-treated RegB demonstrates good incorporation of tritium, indicating that azido-Q undergoes efficient cross-linking to RegB (data not shown).

Kinase assays indicate that the activity of RegB is decreased >95% after photoaffinity cross-linking in the presence of azido-Q (Fig. 3A). Inactivation is not due to the inhibition of RegB by photolyzed products of azido-Q, because no inhibition of activity is observed when azido-Q is first photolyzed in the absence of RegB and then mixed with the enzyme. Inactivation is also not due to protein damage by UV radiation because illumination of the enzyme alone does not affect activity. To further establish that inactivation of RegB activity is a direct result of covalent linkage of azido-Q, we performed kinase assays on RegB-azido-Q complexes that had undergone different periods of illumination. As shown in Fig. 3A, when RegB is treated with 20-fold molar excess of azido-Q and then illuminated for different time periods, activity decreases as the time of illumination increases. Moreover, the amount of azido-Q incorporated into RegB parallels the extent of inactivation, until maximum incorporation and maximum inactivation is reached at 20 min of illumination. Although illumination for longer than 20 min causes no further decrease in activity, azido-Q uptake continues with a slower rate, indicating that further incorporation is due to nonspecific binding of
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In Vivo Characterization of Ubiquinone-binding Site Mutants—We addressed the physiological significance of the azido-Q binding heptapeptide sequence GGXXNPF by constructing alanine substitution mutations of the Asn and Phe residues that convert the GGXXNPF heptapeptide sequence to GGXXAPF (mutation N110A) and GGXXNPA (mutation F112A). These mutations were chosen on the supposition that the heptapeptide forms a ubiquinone-binding pocket with Asn^110^ providing hydrogen bond interactions, and Phe^112^ providing π–π interactions with the ubiquinone polar head group. regB constructs containing these mutations were cloned into a suicide vector and then recombined into the chromosome of an R. capsulatus strain that contains a chromosomal deletion of regB. As a control, we also recombined a wild type copy of the regB gene into the same regB deletion strain. Because the RegB-RegA regulon controls expression of a large number of aerobic and anaerobic processes (4), we attempted to grow wild type and mutant recombinants under a variety of conditions. In comparison with the wild type control that grew in all tested conditions, the N110A alanine substitution mutants were only viable when grown anaerobically (photosynthetically) on agar-solidified RCV minimal medium. Under this condition, colonies of the N110A strain were significantly darker in appearance than wild type recombinants (supplemental Fig. S1), which is significant given that RegA is a potent anaerobic activator of photosynthesis gene expression when phosphorylated by RegB. Because the N110A mutation would not grow on rich medium or aerobically on minimal medium, this strain was not further characterized.

In contrast to poor viability of the N110A mutation, the F112A recombinants were viable under several growth conditions. When grown on RCV minimal medium, the F112A mutant grew well photo-synthetically, producing large colonies after 3 days of growth. Under this condition, colonies of the F112A strain were significantly smaller colonies. In addition, the F112A mutant exhibited elevated photosynthesis gene expression when phosphorylated by RegB. regB constructs containing these mutations were cloned into a suicide vector and then recombined into the chromosome of an R. capsulatus strain that contains a chromosomal deletion of regB. As a control, we also recombined a wild type copy of the regB gene into the same regB deletion strain. Because the RegB-RegA regulon controls expression of a large number of aerobic and anaerobic processes (4), we attempted to grow wild type and mutant recombinants under a variety of conditions. In comparison with the wild type control that grew in all tested conditions, the N110A alanine substitution mutants were only viable when grown anaerobically (photosynthetically) on agar-solidified RCV minimal medium. Under this condition, colonies of the N110A strain were significantly darker in appearance than wild type recombinants (supplemental Fig. S1), which is significant given that RegA is a potent anaerobic activator of photosynthesis gene expression when phosphorylated by RegB. Because the N110A mutation would not grow on rich medium or aerobically on minimal medium, this strain was not further characterized.

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FIGURE 6. Northern blot analysis of puf and puc operon expression. The puf (A) and puc (B) Northern blot autoradiograph of RNA isolated from aerobic and anaerobic grown wild type (WT) and the F112A mutant (F-A). The RNA was probed using a digoxigenin-labeled probe and visualized using x-ray film. The data were then plotted as a bar graph. RNA transcript for puf and puc were normalized using 16S ribosomal RNA levels as a control. Northern blots were carried out three times independently with three different RNA preparations. The data were consistent between all three replicates.

that this mutation results in 94, 61, and 14% increases in photosystem production under aerobic, semi-aerobic, and photosynthetic growth conditions relative to the wild type recombinant strain, respectively (Fig. 5, A–C). The dramatic elevation in aerobic photosystem production of the F112A mutation is significant given that wild type RegB kinase activity is normally low under this growth condition. Thus, the F112A mutation appears to disrupt the ability of oxidized ubiquinone to repress RegB autophosphorylation in vivo.

The RegB-RegA regulon is known to directly control expression of the puf operon that encodes for the light harvesting I and reaction center proteins, as well as the puc operon that encodes the light harvesting II polypeptides. To show a direct effect on gene expression, we performed Northern blot analysis of puf and puc mRNA extracted from both the F112A mutant and the wild type recombinant strains grown under both aerobic and anaerobic growth conditions. In agreement with the spectral data, mRNA levels of puf and puc operons were highly elevated under aerobic growth conditions in the F112A mutant, with puf expression elevated 2.5-fold and puc expression elevated 6.5-fold (Fig. 6). Interestingly, puf and puc expression were also elevated 1.6- and 1.9-fold under photosynthetic growth conditions, respectively, indicating that wild type RegB is not fully derepressed under photosynthetic growth conditions (Fig. 6). This suggests that the ubiquinone pool is not fully reduced even under the tested anaerobic growth conditions.

DISCUSSION

The RegB/RegA signal transduction system regulates the expression of multiple cellular processes such as respiration, photosynthesis, hydrogen utilization, carbon fixation, and nitrogen fixation (4). Many of these processes use redox chemistry to catalyze specific reactions and thus affect the energy state of the cell. The common link for all RegB controlled processes is the membrane-bound electron/proton carrier, ubiquinone. The ubiquinone derivative found in Rhodobacter sphaeroides species is ubiquinone-10, which is produced at extremely high levels with the pool localized to the cytoplasmic/intracytoplasmic membrane, which is also the site of RegB localization (9). Furthermore, the redox balance of the ubiquinone pool is known to dramatically shift in photosynthetic bacteria from a predominantly oxidized state under aerobic conditions to being predominantly reduced under photosynthetic (anaerobic) conditions (9). The abundance and dramatic changes in the oxidation/reduction state of the ubiquinone pool makes it an extremely attractive sensor molecule for RegB to monitor the energy status of the cell and subsequently regulate synthesis of the photosystem, components of the respiratory chain and energy utilizing fixation pathways.

This study reveals that the conserved motif GGXXNPF, located between transmembrane 3 and 4 of RegB (Fig. 4), constitutes a ubiquinone-binding site. A previously reported Pho fusion study on RegB places the ubiquinone-binding domain at the junction of the second periplasmic loop and the beginning of the membrane interface of TM4 (28). This placement is in close agreement with TopPred, which predicts that the ubiquinone-binding domain is housed within the bacterial membrane at the beginning of TM4. The membrane location of the predicted RegB ubiquinone-binding site is also consistent with that of other ubiquinone-binding domains that are typically located within or very near the membrane. Out in vitro photoaffinity cross-linking results with [3H]azido-Q, and in vivo mutational analysis of this region are also supported by Oh et al. (15), who used multiple (five consecutive) Ala substitution analyses to identify a region extending from TM3 to TM4 as being potentially involved in the redox responsive behavior of RegB in R. sphaeroides (15). Our study confirms that this region is involved in redox sensing and has further assigned this region as a highly conserved ubiquinone-binding site.

Models of Quinone Inactivation of RegB—One can envision several ways that ubiquinone may affect the kinase activity of RegB. Binding of this cofactor could be promoting an allosteric modification of the tertiary structure of RegB ultimately inhibiting RegB autophosphorylation. Alternatively, ubiquinone could be promoting physical oxidation of one or more residues in RegB. Neither mechanism is mutually exclusive, leaving open the possibility that ubiquinone could regulate kinase activity via both allosteric and redox mechanisms. In regards to an allosteric mechanism, one can envision that the aromatic side group of Phe112 could stack with the para-hydroxybenzoate ring of ubiquinone via stabilizing π-π interactions between these rings. Asn110 could also act as a hydrogen bond donor to the oxidized ubiquinone, stabilizing ubiquinone-protein interactions. Indeed, a hydrogen bond interaction of Asn110 to ubiquinone could be a switch that leads to allosteric modification of RegB structure. Under anaerobic conditions, when the ubiquinone pool is shifted toward the doubly protonated ubiquinol state, hydrogen bond interactions between Asn110 and the ubiquinone could be lost. This could lead to rearrangement of hydrogen bonds in RegB, resulting in a change in structure that favors autophosphorylation.
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The results of our previous study on a truncated version of RegB indicated that alterations of the redox state of Cys\(^{265}\) also affected autophosphorylation in vitro (6). Interestingly, mutational analysis of Cys\(^{265}\) showed attenuated, but not absent, redox control of RegB activity in vivo, suggesting that there are additional redox inputs controlling RegB activity (6). The results of this study indicate that the redox state of the ubiquinone pool constitutes an additional mechanism of controlling activity of RegB in response to changes in cellular redox.

The predicted membrane topology of RegB places the GGXXNPF quinone-binding domain in a short periplasmic loop near TM4, which is contrasted by Cys\(^{265}\) that is located in the cytosolic domain between the site of phosphorylation and the kinase domain (6). This would indicate that ubiquinone may not be directly affecting the redox state of Cys\(^{265}\) and that Cys\(^{265}\) may instead be a separate redox input to control the activity of RegB perhaps by direct oxidation of Cys\(^{265}\) via oxygen.

Even though continued studies will be needed to reveal the underlying mechanism by which ubiquinone and Cys\(^{265}\) together inhibit RegB autophosphorylation, the relation between ubiquinone and redox active cysteine residues is not unprecedented. In fact, two such examples are well described in the scientific literature. The disulfide bond-generating protein, DsbB has the ability to interact with ubiquinone and use the oxidative power of that ubiquinone to induce a disulfide bond in adjacent cysteine residues (29). Recently, the sensor kinase, ArcB from \(E. coli\) was also shown to use the oxidation power of ubiquinone to generate an intermolecular disulfide bond that diminished kinase activity (7). These two studies, in concert with the data presented in this manuscript, provide a compelling argument that the interaction between cysteine residues and ubiquinone may be a common theme for redox-sensing and -responding mechanisms in biology.

In addition to ArcB, the sensor kinases BvgS and EvgS from \(Bordetella pertussis\) and \(E. coli\), respectively also exhibit decreased autophosphorylation in the presence of ubiquinone in vitro (30). These two kinases have a domain structure that is very similar to that of ArcB with two transmembrane-spanning helices followed by a cytoplasmic Per Arnt Sim (PAS) domain and then a kinase domain. In ArcB, the PAS domain contains two cysteine residues that form intermolecular disulfide bonds in response to the addition of ubiquinone (7). It has been inferred that the PAS domain is responsible for ubiquinone binding; however, direct evidence for binding of ubiquinone to the ArcB PAS domain has not been provided. In addition, mutations in putative ubiquinone-binding residues of the BvgS PAS domain had little effect on ubiquinone inhibition of kinase activity (30). Interestingly, all of the published in vitro analysis on ArcB, BvgS, and EvgS has been performed using truncated forms of these protein that lack their transmembrane-spanning domains (7, 8, 30). Key to the success of this study was the purification of full-length RegB that contains six transmembrane-spanning helices. The utilization of ubiquinone as a cofactor to regulate RegB activity is not surprising given that an interaction of RegB with the ubiquinone pool would clearly provide a mechanism by which this sensor kinase could control expression of downstream genes that ultimately affect the redox state of the cell. As is the case with other sensor kinases, it remains to be determined how binding of this cofactor ultimately leads to inhibition of kinase activity.

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