

Signal transduction by the global regulator RegB is mediated by a redox-active cysteine

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All living organisms alter their physiology in response to changes in oxygen tension. The photosynthetic bacterium uses the RegB–RegA signal transduction cascade to control a wide variety of oxygen-responding processes such as respiration, photosynthesis, carbon fixation and nitrogen fixation. We demonstrate that a highly conserved cysteine has a role in controlling the activity of the sensor kinase, RegB. *In vitro* studies indicate that exposure of RegB to oxidizing conditions results in the formation of an intermolecular disulfide bond and that disulfide bond formation is metal-dependent, with the metal fulfilling a structural role. Formation of a disulfide bond *in vitro* is also shown to convert the kinase from an active dimer into an inactive tetramer state. Mutational analysis indicates that a cysteine residue flanked by cationic amino acids is involved in redox sensing *in vitro* and *in vivo*. These residues appear to constitute a novel ‘redox-box’ that is present in sensor kinases from diverse species of bacteria.

Keywords: histidine kinase/photosystem gene expression/redox regulation/*Rhodobacter capsulatus*

Introduction

Oxygen is a highly reactive oxidant used by a majority of living organisms as a terminal electron acceptor. As respiratory-driven electron transport generates ATP by oxidative phosphorylation, it is extremely beneficial for cells to closely monitor environmental oxygen levels and to quickly respond to changes in oxygen tension. In a wide range of proteobacteria, the sensor kinase RegB plays a major role in the redox control of many diverse cellular processes. Under anaerobic conditions, RegB autophosphorylates at a conserved histidine residue and subsequently transduces a signal to its cognate response regulator, RegA by phosphoryl group transfer to an aspartate residue (Inoue *et al.*, 1995; Bird *et al.*, 1999). RegA~P then regulates transcription of genes whose products are directly involved in photosynthesis (Sganga and Bauer, 1992; Mosley *et al.*, 1995), respiration (Swem *et al.*, 2001), carbon fixation (Vichivanives *et al.*, 2000), nitrogen fixation (Elsen *et al.*, 2000), hydrogen utilization

(Elsen *et al.*, 2000), cytochrome biosynthesis (Swem *et al.*, 2001) and aerotaxis (Romagnoli *et al.*, 2002).

There are numerous histidine kinase/aspartate response regulator systems present in bacteria (West and Stock, 2001), with a few also observed in eukaryotic cells (Saito, 2001). However, mechanisms by which sensor kinases perceive the environment and control kinase activity are widely unknown. In fact, a detailed understanding of a mechanism for sensing oxygen is known for only one other histidine sensor kinase, FixL, which can directly sense oxygen using a bound heme (Miyatake *et al.*, 2000). There is also a report that the activity of the redox-responding global histidine kinase ArcB is affected by the oxidation state of quinones (Georgellis *et al.*, 2001). However, it remains to be determined how oxidized quinone inhibits ArcB activity. In this paper, we describe the mechanism by which a truncated version of RegB (RegB^s) from *Rhodobacter capsulatus* can sense environmental redox potential by demonstrating that RegB^s is capable of forming a metal-dependent, intermolecular disulfide bond that acts as a molecular switch for controlling kinase activity *in vitro*. Therefore, RegB may be mechanistically similar to OxyR, CrtJ and RsrA that contain redox-reactive cysteines (Aslund *et al.*, 1999; Paget *et al.*, 2001; Kim *et al.*, 2002; Masuda *et al.*, 2002). Genome analysis indicates that the redox-reactive cysteine residue is highly conserved in histidine kinases from a broad distribution of eubacteria.

Results

Metal is a required cofactor for redox control in vitro

Like many other sensor kinases, RegB is a membrane spanning protein. However, RegB is atypical in that it contains very small periplasmic and cytoplasmic loops between its transmembrane regions (Ouchane and Kaplan, 1999; Chen *et al.*, 2000). This led us to inquire whether a soluble truncated version of RegB that encodes the conserved cytosolic histidine phosphorylation and kinase domains may contain redox-sensing capabilities. For this analysis, a truncated form of RegB (RegB^s) that lacks all membrane spanning domains was overexpressed in *Escherichia coli* and purified by anion exchange chromatography. Our initial kinase assays indicated that purified RegB^s was capable of autophosphorylating at a rate that was indistinguishable from that of full-length purified RegB (Potter *et al.*, 2002). Furthermore, RegB^s exhibited a slight increase in autophosphorylation under reduced conditions (data not shown). This latter result suggested that overexpressed RegB^s could be largely depleted of a cofactor needed for redox-dependent autophosphorylation.

One hint as to the nature of a required redox cofactor was obtained from the highly conserved *regB-senC-regA*

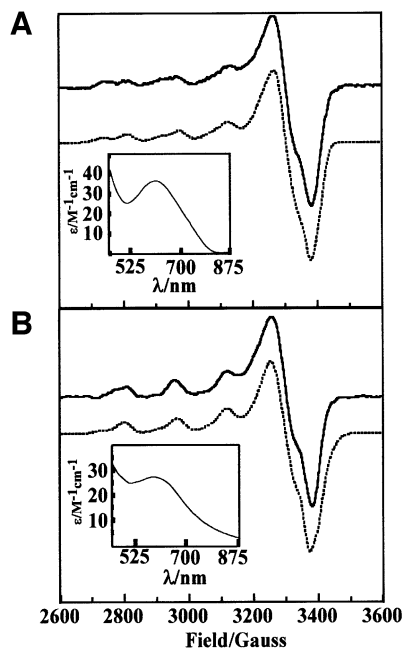


Fig. 1. Electron paramagnetic resonance (EPR) analysis of the RegB^s copper center. (A) X-band EPR spectra of wild-type RegB^s and (B) C265A RegB^s collected at 77 K in 10 mM Tris pH 8.0, 300 mM NaCl and 50% glycerol. Top spectrum (solid line) is experimental data and the bottom trace (dotted) is simulated data for both (A) and (B). Insets: electronic absorption spectra of the visible region for wild-type RegB^s and C265A RegB^s collected at 298 K in buffer mentioned in Materials and methods.

genome organization that is found in six different genera of α -proteobacteria (Masuda *et al.*, 1999). In these species, the *regB* gene is divergently transcribed from the *senC* gene (Buggy and Bauer, 1995), with transcription of both genes co-regulated by the phosphorylation state of RegA (Du *et al.*, 1999). Previous *in vivo* mutational analysis indicates that RegB is unable to properly sense oxygen when *senC* is deleted from the chromosome, suggesting an involvement of SenC in the redox-sensing capabilities of RegB (Eraso and Kaplan, 2000). Interestingly, SenC exhibits significant sequence similarity to the mitochondrial inner membrane protein Sco1, which is thought to function as a mitochondrial copper chaperone (Nittis *et al.*, 2001). Both SenC and Sco1 have been shown to bind stoichiometric amounts of copper *in vitro*, indicating that they may have analogous roles (Beers *et al.*, 2002; McEwan *et al.*, 2002). This suggests that the redox-sensing activity of RegB may be dependent on the putative copper chaperone activity of SenC, raising the possibility that RegB may bind copper.

Copper-binding proteins that are highly overexpressed in *E. coli* are devoid of significant amounts of copper, owing to the fact that *E. coli* cells contain little or no free copper ions (Silver, 1996) and that *E. coli* actively inhibits transport of excess copper into the cytoplasm from the growth medium (Cervantes and Gutierrez-Corona, 1994). However, isolated copper-binding apoproteins can be reconstituted by the addition of exogenous CuCl₂, followed by extensive dialysis (Lamb *et al.*, 2000). To test whether RegB^s is able to bind copper, 300 μ M CuCl₂ was slowly added to 100 μ M RegB^s and then dialyzed

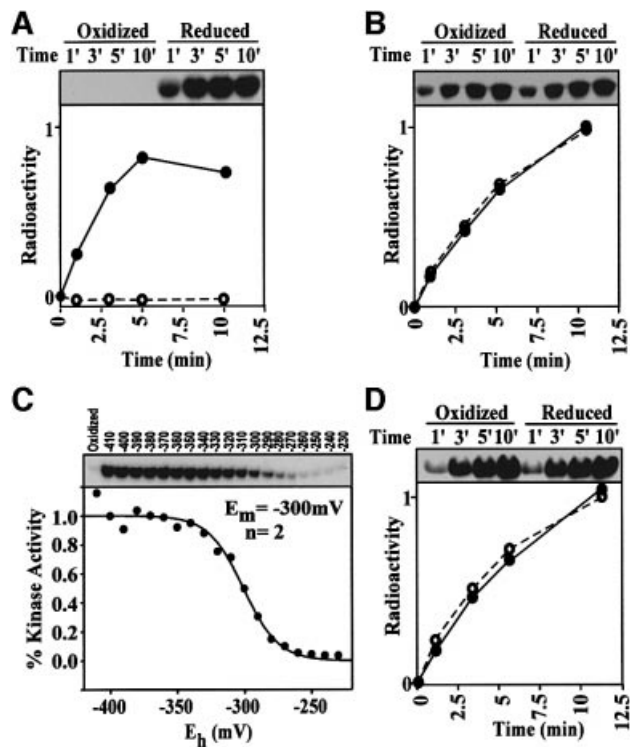


Fig. 2. Involvement of copper and Cys265 in redox-regulated activity of RegB^s. (A) Autophosphorylation of oxidized (open circles) and reduced (filled circles) copper-reconstituted RegB^s for defined periods after addition of [γ -³²P]ATP. (B) Autophosphorylation of oxidized (open circles) and reduced (closed circles) EDTA-treated RegB^s. (C) Kinase activity redox titration where RegB^s samples were placed at defined ambient redox potentials for 2 h before autophosphorylation assays were initiated with the addition of [γ -³²P]ATP. Each kinase assay was allowed to proceed for 10 min, quenched by the addition of a reducing 0.1% SDS dye solution and then separated by gel electrophoresis. The level of autophosphorylation was visually assayed for phosphate incorporation by exposure to film, and was also quantitated using a phosphorImager. Percent phosphorylation was plotted against potential, with the data fitted to a two-electron Nernst equation. (D) Autophosphorylation of oxidized (open circles) and reduced (closed circles) copper-reconstituted C265A mutant RegB^s under oxidizing (open circles) and reducing (closed circles) conditions. Each lane contains 10 μ mol of RegB^s.

extensively. Three independent preparations yielded bound copper-to-RegB^s molar ratios of 0.9, 0.9 and 0.8 (Brenner and Harris, 1995). As a control, similar treatment of lysozyme with CuCl₂, followed by dialysis, yielded no detectable bound Cu²⁺. These results indicate that RegB^s contains a single copper-binding site per monomer.

The electronic absorption spectrum of copper-replete RegB^s exhibits two features not observed in copper-depleted protein: a broad peak at 615 nm (Figure 1A, inset) and a shoulder at 315 nm (data not shown). The broad feature centered at 615 nm can be attributed to ligand field transitions within the d-orbitals of Cu²⁺ (Figure 1A, inset). For typical nitrogen/oxygen coordination, the dd-absorption band energy is too high to be assigned to a trigonal bipyramidal geometry, and too low to be consistent with a square coplanar structure (Lever, 1984; Hathaway, 1987). However, the energy of the absorption band does fall within the energy range typically observed for either a five-coordinate square-based pyramid or a six-coordinate tetragonally elongated octahedron with mixed nitrogen/oxygen ligands (Lever, 1984; Hathaway, 1987; Astley

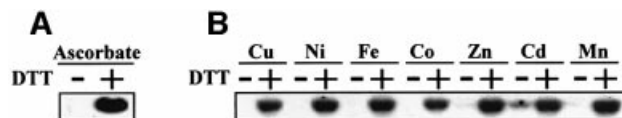


Fig. 3. A structural role for the metal center. (A) Copper-replete RegB^s was pre-reduced with ascorbate at pH 8.0 for 1 h, or first with ascorbate for 30 min and then with dithiothreitol (DTT) for 30 min at pH 8.0, before the kinase assay was initiated with the addition of [γ -³²P]ATP. After 10 min incubation, the samples were quenched with the addition of a reducing 0.1% SDS dye solution and then separated by gel electrophoresis. (B) RegB^s apoprotein was reconstituted with different metals as indicated. Kinase assays were performed under air-oxidizing (no DTT) or reducing conditions (+10 mM DTT). The reactions were initiated by the addition of [γ -³²P]ATP and quenched after 10 min incubation followed by gel electrophoresis.

et al., 1995). The higher energy shoulder observed at 315 nm has a broad tail extending into the visible region of the spectrum. Given the absence of other chromophores associated with RegB^s, these high energy features are consistent with the energies of copper-His ligand-to-metal charge transfer transitions (Fawcett *et al.*, 1980; Bernarducci *et al.*, 1981; Innes *et al.*, 1988).

Analysis of the electron paramagnetic resonance (EPR) spectrum of oxidized RegB^s revealed the presence of two distinct d⁹ Cu²⁺ centers in approximately equimolar amounts (Figure 1A). Since RegB^s binds copper with a 1:1 stoichiometry, it appears that ~50% of RegB sites contain a type *a* copper center (Cu^a) and ~50% contain a type *b* copper center (Cu^b). The EPR copper *g*-values indicate that there are two independent, low symmetry Cu²⁺ sites, with both sites comprised of mixed nitrogen and oxygen coordination (Peisach and Blumberg, 1974; Hathaway, 1987; McLachlan *et al.*, 1995; Hemmert *et al.*, 1999; Huang *et al.*, 2000). The *A*-values suggest that these centers are likely six-coordinate (*A_z^a*) in the Cu^a site and five-coordinate or weakly six-coordinate (*A_z^b*) at the Cu^b site (Peisach and Blumberg, 1974; Hathaway, 1987). Nitrogen-only coordination is precluded by the amino acid composition, as there are only three completely conserved histidine residues in the truncated form of RegB.

To determine whether the presence of copper influences kinase activity, autophosphorylation assays (Bird *et al.*, 1999) were performed in the presence and absence of a reducing agent with aliquots removed at 1, 3, 5 and 10 min. Remarkably, there is barely detectable autophosphorylation of copper-replete RegB^s under air-oxidizing conditions, whereas under reducing (2 mM dithiothreitol, DTT) conditions there is a high level of autophosphorylation (Figure 2A). Measurement of ³²P incorporation indicates that there is a 10⁴-fold increase in autophosphorylation when copper-replete RegB^s is converted from an air-oxidized to a reduced form using DTT. Redox regulation is a reversible process, since reduced RegB^s can be oxidized with potassium permanganate to turn off kinase activity and then again treated with a reductant to regain activity (data not shown).

To further show that metal is needed for redox control, copper-replete RegB^s protein was treated with the metal-chelating agent EDTA (ethylenediaminetetraacetic acid), dialyzed and then assayed for activity. After EDTA treatment, which removed assayable copper, the RegB^s preparation no longer responded to redox equivalents.

Instead, both oxidized and reduced copper-depleted RegB^s phosphorylated to levels that are very similar to those observed with reduced copper-replete RegB^s (Figure 2B), indicating that copper is crucial for oxidation-dependent inhibition of kinase activity.

Kinase activity of copper-replete RegB^s was measured at defined ambient redox potentials to determine the oxidation-reduction midpoint potential of the 'redox switch' in RegB^s. Individual autophosphorylation assays were performed using samples equilibrated at different defined ambient redox potentials ranging from -230 to -410 mV, in 10 mV increments, prior to kinase activity measurements (Hirasawa *et al.*, 1998; Krimm *et al.*, 1998; Setterdahl *et al.*, 2000). As shown in the plot of percentage activity against ambient redox potential (Figure 2C), the data give an excellent fit to the Nernst equation for a two-electron process. The calculated average midpoint potential (*E_m*) was -300 mV (± 10 mV based on six replicates) at pH 8.0. The *E_m* value was independent of incubation time (2, 4 or 18 h) and the concentration of the reducing agent in the buffer, as is expected for true equilibrium titrations.

Metal reduction does not stimulate kinase activity

Given that reduction of copper from Cu²⁺ to Cu⁺ would only require one electron, a 'fit' of the activity redox plot to a two-electron event indicates that reduction of copper may not be involved in converting RegB^s into an active state. To further clarify this point, we assayed the effect of adding ascorbate to RegB^s, which is a poor reductant for disulfide bonds but is known to reduce nitrogen/oxygen-coordinated Cu²⁺. Electronic absorption and EPR analysis of ascorbate-treated RegB^s revealed a loss of the absorption feature at 615 nm and the EPR signal in the *g*~2 region, indicating the reduction of the RegB^s copper center (data not shown). However, despite the reduction of copper, ascorbate-treated RegB^s exhibited no significant autophosphorylation (Figure 3A). The fact that ascorbate with an *E_m* value of 58 mV can reduce the copper center, but is not capable of stimulating kinase activity which has a measured *E_m* value of -300 mV (Figure 2C), clearly reveals that reduction of copper is independent of the stimulation of autophosphorylation. This suggests that the copper ion has another role, such as placing RegB^s into a conformation that allows the true redox sensor to function properly. As a control, we observed that kinase activity was restored when ascorbate-treated RegB^s was subsequently treated with 10 mM DTT (*E_m* = -330 mV) (Figure 3A), indicating that ascorbate is not simply inactivating or denaturing the protein, but is instead failing to reduce the actual redox sensor that is needed to stimulate kinase activity.

To test for metal specificity, we first treated RegB^s with EDTA to remove copper and then reconstituted the metal-depleted apoprotein with the divalent metals, nickel, iron, cobalt, zinc, cadmium or manganese. After metal reconstitution, kinase assays were performed on each sample under both air-oxidizing and reducing (2 mM DTT) conditions. This analysis demonstrated that redox-responsive autophosphorylation of RegB^s occurs with each of the tested metals (Figure 3B). The fact that Zn²⁺ does not undergo oxidation/reduction at the tested potentials, coupled with the observation that zinc-reconstituted

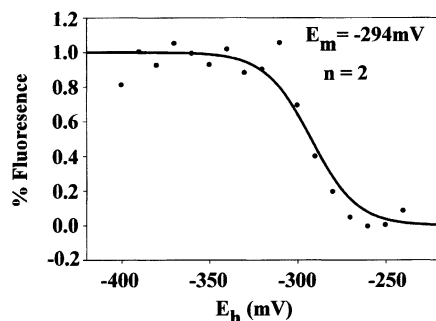


Fig. 4. Monobromobimane (mBBr) titration of RegB^s disulfide bond. RegB^s samples were placed at given potentials as indicated for 2 h at room temperature using ratios of oxidized and reduced dithiothreitol as the redox mediator before mBBr was incubated with each sample. The fluorescence of each sample was then converted to percent fluorescence and plotted against redox potential. The data were fitted to a two-electron Nernst equation yielding a midpoint potential of -294 mV.

RegB^s shows excellent redox control, clearly demonstrates that the metal plays a structural rather than a redox role.

RegB kinase activity is regulated by intermolecular disulfide bond formation *in vitro*

Since the metal center in RegB^s does not play a redox role, we used the fluorescent probe monobromobimane (mBBr) that forms a covalent adduct with the free sulfhydryl group of reduced cysteine to determine whether a redox-active disulfide existed in RegB^s. Although mBBr is itself weakly fluorescent, a covalent adduct of mBBr with cysteine thiols shows significant fluorescence. Thus, disulfide bond breakage/formation can be directly assayed by measuring the amount of mBBr that covalently binds to RegB^s under defined redox conditions (Hirasawa *et al.*, 1998; Krimm *et al.*, 1998; Setterdahl *et al.*, 2000). To measure free thiols in copper-replete RegB^s, aliquots of the protein were first incubated for 2 h at potentials ranging from -230 to -400 mV, in 10 mV increments, and then allowed to react with mBBr. A plot of the fluorescence of the mBBr-protein adduct against ambient redox potential (Figure 4) demonstrates that a significant increase in mBBr-protein fluorescence occurs as the ambient redox potential becomes more reducing. As is the case for the activity-based redox titrations, the mBBr data fit to a Nernst two-electron equation. The observed E_m value for the mBBr fluorescence titration was -294 mV (± 10 mV) at pH 8.0 (based on four replicate titrations), which is within experimental error of the E_m value derived from activity-based redox titrations (-300 mV). These data suggest that a reversible two-electron disulfide/dithiol redox couple may be the actual redox-active site that affects RegB^s kinase activity.

Analysis of the amino acid sequence of RegB^s reveals only a single cysteine residue (Cys265) in the over-expressed cytosolic domain of RegB. To test whether Cys265 is indeed involved in redox sensing, a cysteine-to-alanine mutation was constructed (C265A^s) as described in Materials and methods, with mutant C265A^s protein purified, reconstituted with copper and assayed for kinase activity. The results of this analysis (Figure 2D) indicate that C265A^s exhibits constitutively high activity under both oxidizing and reducing conditions. Indeed, the level of phosphorylation exhibited by reduced and oxidized

C265A^s protein is very similar to that observed in reduced wild-type RegB^s. An mBBr-based redox titration of C265A^s protein also demonstrated constitutively low fluorescence levels that were independent of the ambient potential imposed on the samples (data not shown), indicating an absence of redox-active thiols in the mutant RegB^s.

As C265A^s and copper-deplete wild-type RegB^s were both constitutively active under both oxidizing and reducing conditions, there is a possibility that C265A^s is incapable of binding copper. However, metal analysis on copper-reconstituted C265A^s protein shows that the mutant protein bound stoichiometric amounts of copper (1:1). EPR analysis of oxidized C265A^s also revealed the presence of a strong Cu²⁺ EPR signal deriving from two distinct Cu²⁺ centers that are comparable to those observed with wild-type RegB^s (Figure 1B), thereby indicating that Cys265 does not directly bind copper. However, the Cu^a and Cu^b stoichiometry exist in a $\sim 5:1$ ratio in the mutant rather than the $\sim 1:1$ stoichiometry observed with wild-type RegB^s. The fact that the mutant and wild-type proteins both contain Cu^a and Cu^b EPR features also indicates that the Cys265 sulfhydryl is not providing a coordinating ligand to Cu²⁺. This correlates well with the g -values, which indicate that both Cu²⁺ sites contain primarily mixed nitrogen/oxygen coordination precluding a sulfur ligand.

In addition to alterations in the Cu^a:Cu^b ratio, the C264A RegB^s protein exhibits a 20 nm blue-shift in the Cu²⁺ ligand field absorption band, from 615 nm in wild-type RegB^s to 595 nm in C265A^s (Figure 1B, inset). In the light of the 5:1 Cu^a:Cu^b ratio, and the EPR parameter for Cu^a, this spectral shift indicates that the mixed nitrogen/oxygen tetragonal Cu^a center contains more tightly coordinated nitrogen-donor ligands than does the Cu^b site. These results also indicate that the inability of the C265A^s mutant to form a disulfide bridge does not significantly affect the geometry of the copper coordinating ligands but does affect the structure of the copper centers.

Disulfide bond formation affects oligomerization of RegB *in vitro*

To obtain a better understanding of the role of disulfide bond formation on controlling RegB^s activity, we performed size-exclusion chromatography of copper-replete RegB^s under oxidizing and reducing conditions. The estimated molecular mass of copper-replete RegB^s was determined to be ~ 70 kDa under reducing conditions (Figure 5A and C). Since RegB^s has a calculated mass of 36 kDa, this indicates that reduced wild-type RegB^s exists as a dimer in solution, which is in good agreement with numerous studies that have shown that sensor kinases autophosphorylate *in vivo* as dimers (West and Stock, 2001). In contrast, oxidized wild-type RegB^s, which is inactive as a kinase, elutes with a molecular mass of 140 kDa, which corresponds closely to the calculated mass for a tetramer of RegB^s (Figure 5A and C). Interestingly, constitutively active C265A^s predominately exists as a dimer under both oxidizing and reducing conditions (Figure 5B and C). Interconversion of RegB^s between dimer and tetrameric states appears to be an inherent property of the protein, given that oxidized C265A^s,

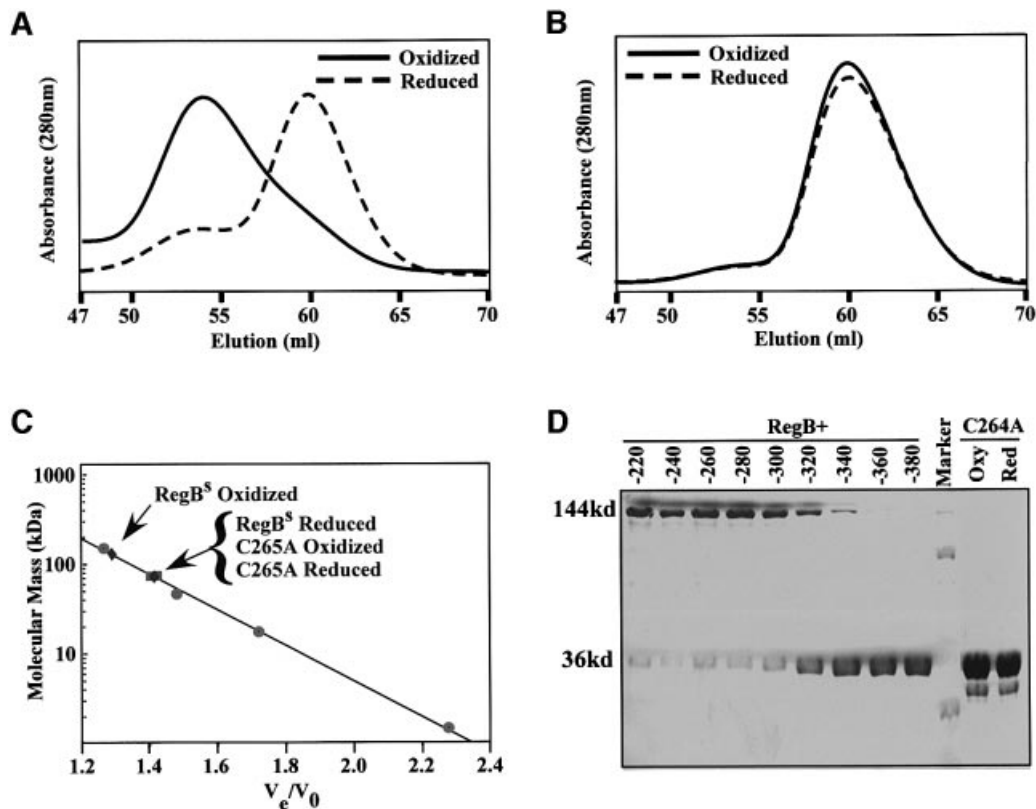


Fig. 5. Size-exclusion chromatography of RegB^s and C265A^s. (A) Elution profile of Superose 12 eluted wild-type RegB^s and (B) C265A mutant RegB^s under oxidizing (solid line) and reducing (dashed line) conditions. (C) Standard curve drawn according to the peak elution volumes (V_e , elution volume; V_0 , void volume) of the molecular weight standards: 158, 44, 17 and 1.35 kDa (green circles) as detected by absorption at 280 nm. The elution position of oxidized and reduced wild-type RegB^s (blue diamonds) and C265A (red open squares) were then applied to the plot based upon their elution position. (D) SDS-PAGE of RegB^s after equilibration at defined redox potentials.

reduced C265A^s and reduced wild-type RegB^s all contain some tetrameric form of the protein (~15–20%). Conversely, oxidized wild-type RegB^s exists primarily as a tetramer, but also contains some dimer form (~15%) (Figure 5). Thus, the role of the disulfide appears to be to affect the equilibrium between these states in favor of the tetrameric form.

We also performed SDS-polyacrylamide gel electrophoresis (PAGE) of RegB^s that was pre-equilibrated in buffers designed to poise the protein at different defined ambient redox potentials. In this assay, we observed that RegB^s formed a tetramer that was stable in the presence of SDS under oxidizing conditions ($E_h \geq -220$ mV) (Figure 5D). In contrast, under highly reducing conditions ($E_h = -380$ mV), RegB^s migrated as a monomer during electrophoresis, which we attribute to SDS-mediated dissociation of the dimer complex. The amount of tetrameric RegB^s was decreased by half when the protein was incubated at an ambient potential of ~ -310 mV (i.e. at an E_h value where the disulfide would be expected to be $\sim 50\%$ converted to the dithiol form) for 2 h before being subjected to electrophoresis. These oligomerization-based redox titration data are in good agreement with midpoint potentials that were calculated for kinase activity and mBBr labeling. Gel electrophoresis also indicated that the C265A^s protein was also largely depleted of tetramers under oxidizing and reducing conditions, thereby confirming that Cys265 is crucial for oligomerization (data not shown).

From these data, we conclude that the off state of oxidized RegB^s corresponds to a tetramer, which is promoted by the formation of an intermolecular disulfide bond between Cys265 residues.

Cys265 also regulates RegB kinase activity in vivo

To ascertain the involvement of Cys265 *in vivo*, we placed the C265A *regB* point mutation into the chromosome of *R. capsulatus* using allelic replacement. Inspection of colony pigmentation indicated that the C265A *regB* mutant exhibited a significantly darker pigmentation than that of the wild-type strain when grown under aerobic conditions. Since the RegB-RegA signal transduction system activates photosynthesis gene expression, one would expect that a constitutively active RegB protein would lead to an increase in photosystem pigments under aerobic growth conditions. To quantitatively assess this phenotype, the wild-type and the *regB* mutant strains were grown semi-aerobically to the same cell density, lysed by sonication and spectrally scanned from 400 to 900 nm. The results of this analysis demonstrate that the *regB* mutant strain made 73% more photopigments than wild-type cells grown under the same conditions (Figure 6A). A similar increase in pigment production was also observed during aerobic and photosynthetic growth conditions (data not shown).

To measure the *in vivo* effect of the C265A *regB* mutation on photosynthesis gene expression, we

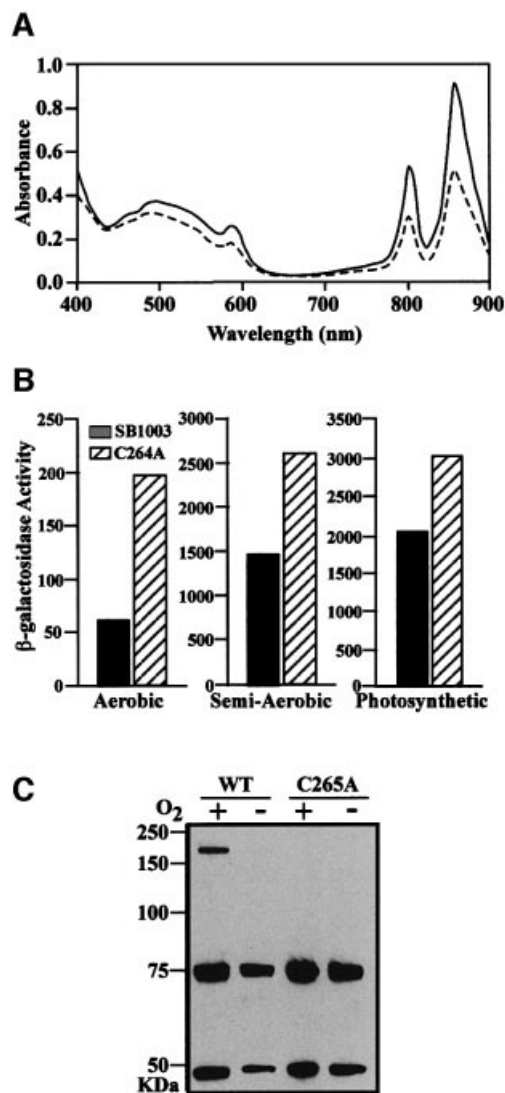


Fig. 6. *In vivo* importance of Cys265 in RegB^s. (A) A spectral scan of crude cell extracts from semi-aerobically grown wild-type (dashed line) and C264A mutant (solid line) RegB^s strains. (B) β -Galactosidase activity of *puf* promoter in wild-type and C264A mutant RegB strains grown aerobically, semi-aerobically and photosynthetically. Activity units represent micromoles of ONPG hydrolyzed/min/mg protein. (C) Western blot analysis of wild-type and C265A RegB-flag tagged strains of *R. capsulatus* grown aerobically or anaerobically. The 75 kDa band is a cross-reacting protein used as a loading standard, whereas the 47 kDa band is the RegB monomer and the 200 kDa band in the wild-type oxidized lane is the RegB tetramer.

performed β -galactosidase assays on strains containing the *puf* promoter plasmid pCB532 Ω (Bauer *et al.*, 1988). The *puf* operon, which encodes structural components for light harvesting and reaction center complexes, is anaerobically activated by the RegB–RegA signal transduction cascade, as well as aerobically repressed by the transcription factor AerR (Dong *et al.*, 2002). The results of this analysis indicate that expression of the *puf* operon is elevated 3-fold in C265A *regB* mutant strain under aerobic conditions and 1.7- and 1.5-fold under semi-aerobic and anaerobic conditions, respectively, relative to wild-type *R. capsulatus* (Figure 6B).

To investigate whether full-length RegB is capable of undergoing changes in oligomeric state *in vivo*, we

constructed chromosomally encoded C-terminal flag-tag variants of wild-type and C265A *regB* that allowed detection of full-length RegB protein by non-denaturing western blot analysis. Western blot analysis of aerobically and anaerobically grown wild-type *R. capsulatus* cell extracts that do not contain a flag-tagged RegB indicated that there is a 75 kDa protein that strongly interacts with the anti-flag antibody (data not shown). Because this cross-hybridizing protein was constitutively present, it was used as a loading control. Extracts from anaerobically grown wild-type RegB-flag cells yielded a single 47 kDa band that corresponds to the size of full-length RegB monomer. In contrast, when the cells were grown aerobically, a significant portion of RegB exists as a 250 kDa tetramer (Figure 6C). Also consistent with *in vitro* observations, the C265A-flag *regB* mutant strain did not contain any tetramer when grown aerobically or anaerobically, indicating that the cysteine residue is indeed needed for tetramerization both *in vitro* and *in vivo* (Figure 6C). RegB dimer was not observed on the gel because the SDS disrupts the hydrophobic interactions that hold the dimer together. However, when the disulfide bond is formed between dimer pairs, they become resistant to SDS degradation and run as a tetramer. These data indicate that Cys265 is not only crucial for the *in vitro* redox sensing but is also functional *in vivo*.

Discussion

Our data indicate that the redox-regulated activity of the truncated cytosolic portion of RegB (RegB^s) is mediated *in vitro* by a redox-reactive cysteine that is capable of undergoing metal-dependent formation of a disulfide bond. It is evident from our redox and metal substitution studies that the metal in RegB^s plays a structural role, as oxidation or reduction of the metal does not affect disulfide bond formation. Presumably, the metal is needed to place RegB^s in a conformation that will allow disulfide bond formation to occur under oxidizing conditions. Given that a mutation in the putative copper chaperone SenC renders RegB constitutively active (Eraso and Kaplan, 2000), we suspect that the metal associated with RegB *in vivo* is copper, although purification of RegB from *R. capsulatus* will ultimately be needed to address this question. This conclusion is supported by the finding that mutations in the putative copper transport proteins RdxI, RdxH and RdxS result in derepression of the RegB–RegA pathway in *Rhodobacter sphaeroides* (Roh and Kaplan, 2000). However, our *in vitro* results clearly indicate that other metals can fulfill this role.

Although the *in vitro* data suggest that disulfide bond formation may be responsible for the redox-sensing capability of RegB, it cannot be discounted that other oxidized cysteine derivatives may be occurring *in vivo* that affect the autophosphorylation activity of full-length RegB. Indeed, it has been recently discovered that the redox-responsive transcriptional regulator OxyR can alter DNA-binding ability in response to different modifications of a highly conserved cysteine residue (Kim *et al.*, 2002). Specifically, a highly reactive cysteine residue in OxyR can undergo sulfur-hydroxylation, sulfur-nitrosylation and sulfur-glutathionylation *in vivo*, with each modification differently affecting the DNA-binding activity of OxyR.

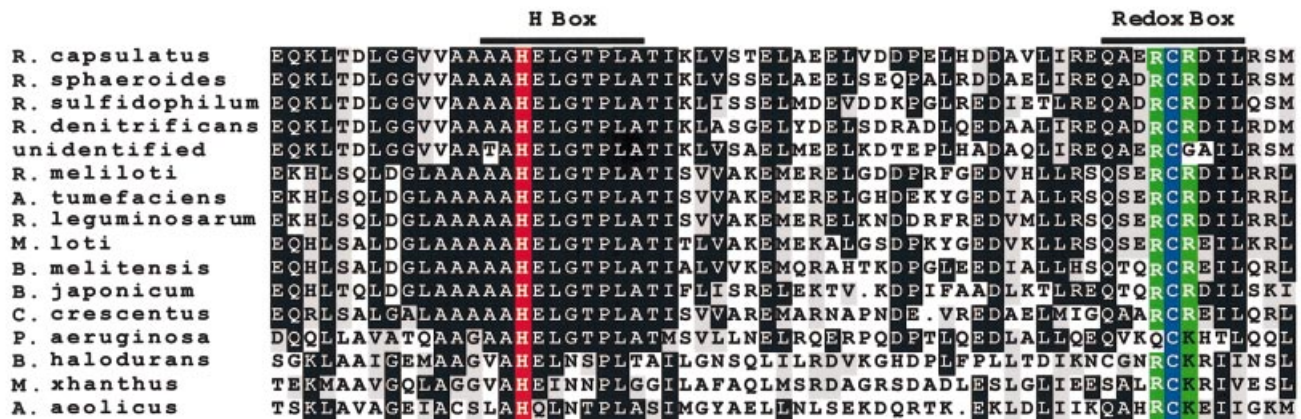


Fig. 7. Sequence alignment of several RegB homologs. Alignment of sensor kinases that exhibit sequence similarity to the region of RegB^s from the site of phosphorylation through Cys265. The H-box represents the site of phosphorylation, and the redox-box represents the reactive cysteine residue flanked by cationic amino acids.

While these modifications may facilitate eventual disulfide bond formation, they are all stable intermediates *in vivo*. A similar scenario may apply to Cys265 of RegB where modifications of the cysteine may modulate kinase activity. Cysteine residues residing within a basic pocket will have a significantly lower pK_a value, resulting in deprotonation of the sulfhydryl group at physiological pH. Interestingly, all RegB homologs contain fully conserved basic residues that flank Cys265, which may decrease Cys265 pK_a , causing deprotonation. Deprotonated cysteine is a highly reactive species that can react spontaneously with H₂O₂ (a byproduct of respiration) to form SOH (sulfenic acid). Sulfenic acid is also a known intermediate step toward disulfide bond formation, and since <20% of RegB forms a disulfide bond *in vivo* when grown under aerobic conditions (Figure 6C), it is quite possible that the remainder of air-oxidized RegB may exist as an SOH. This conclusion is supported by our analysis that shows that Cys265 of RegB has the capability to undergo disulfide bond formation *in vivo* and *in vitro*, classifying it as a truly redox-active residue. Our western blot data also demonstrate that mutational disruption of Cys265 abolishes all oligomerization (Figure 6C), in contrast to a Cys68 mutation that is still capable of forming oligomers (data not shown). Thus, only Cys265 appears to have a role in the redox-sensing mechanism of RegB *in vivo* and *in vitro*.

Although it is generally assumed that most sulfhydryls remain in a reduced state in the cytosol of prokaryotes, the presence of a redox-active cysteine in the cytosolic domain of RegB is not unprecedented. We recently demonstrated that the DNA-binding activity of the aerobic repressor CrtJ in *R. capsulatus* is enhanced by oxygen-mediated formation of an intramolecular disulfide bond (Masuda *et al.*, 2002). Thus, RegB constitutes a second *R. capsulatus* transcription factor that utilizes a redox-active cysteine as a mechanism of controlling gene expression in response to alteration in oxygen tension.

It is also apparent that many bacterial species are frequently faced with the need to respond to sudden changes in ambient oxygen tension. Inspection of available genome databases indicates that the highly conserved

RegB–RegA regulon is present in a diverse number of α -proteobacterial species. An alignment of various RegB homologs indicates that they each contain a highly conserved H-box with a histidine residue that undergoes phosphorylation. In addition, there is an area of conservation centered around Cys265, which we have designated the ‘redox domain’, that exists in a linker region between the H-box and the downstream ATP binding kinase domain (Figure 7). On both sides of Cys265 there are highly conserved cationic amino acids such as Arg and Lys that are thought to stimulate reactive formation of disulfide bonds or sulfenic acid derivatives (Jones, 2002). In addition to the α -proteobacterial RegB homologs, there are a number of other additional HPK 3e type sensor kinases (Grebe and Stock, 1999) that also contain the same redox-box (Figure 7). This includes histidine kinases from such diverse species as *Myxococcus xanthus* (δ -proteobacteria), *Pseudomonas aeruginosa* (γ -proteobacteria), *Bacillus halodurans* (gram positive) and *Aquifex aeolicus* (aquificales). Although it remains to be established that these sensor kinases autophosphorylate in a redox-dependent manner, it is quite possible that this region constitutes a domain that controls a redox-responsive sensory transduction cascade in these diverse bacterial species.

Materials and methods

Strains, media and growth conditions

R. capsulatus strain SB1003 was used as the parent strain for all RegB mutational analysis. BL21(DE3) was utilized for the expression of RegB^s and C265A^s. *R. capsulatus* conjugation was performed using S17-1 λ pir. Terrific broth/Luria broth and PY medium were used for agar solidified plates and liquid cultures of *E. coli* and *R. capsulatus*, respectively. Kanamycin and gentamycin was used at a concentration of 50 and 10 μ g/ml for *E. coli* and 10 and 1.5 μ g/ml for *R. capsulatus*, respectively.

Generating RegB point mutation

Exchanging the RegB^s region with a KmR cartridge interrupted the *R. capsulatus* regB gene, and the mutant strain was named SM01 (S. Masuda and C.E. Bauer, unpublished strain construction). The cysteine residue at position 265 in RegB was changed to alanine by PCR mutagenesis using Pfu DNA polymerase as described previously (Wang and Malcolm, 1999), and the fragment was cloned into the Gm^R-suicide

vector pZJD29A (J.Jiang and C.E.Bauer, unpublished plasmid construction) that has a *sacB* gene encoding the lavansucrase of *Bacillus subtilis*. The resulting plasmid was then transferred to the *R.capsulatus* strain SM01 by S17-1 λ pir based conjugation. Gm^R cells were selected for and subsequently grown in the presence of 5% sucrose to select double-crossover candidates. The resulting colonies were checked for Km and Gm sensitivity, before confirming chromosomal replacement of the C265A point mutation in *regB*, by PCR and sequence analysis.

Construction of expression vectors

A truncated version of RegB that starts at amino acid M196 (RegB^s) was constructed by PCR amplifying the C-terminal region of *regB* with primers 5'-CCCATGGCGGATGCGCTTTTCGC and 5'-CCTCGAGAACGATTGTGATCATCAGGC. The C265A^s (RegB^s mutant) was first generated in the chromosome of *R.capsulatus* and then PCR amplified from the chromosome with the primers stated above. The DNA segment was cloned into *NcoI* and *XhoI* sites of pET29(+)(Novagen) to construct an in-frame fusion of *regB* at M196 with an N-terminus S-tag that is present in the overexpression vector.

Protein overexpression and purification

The expression plasmids pET29RegB^s and pET29C265A^s were transformed into BL21 (DE3) and selected with kanamycin. RegB^s and C265A^s was overexpressed in the *E.coli* strain BL21(DE3) by growing to an OD of 0.5 and then inducing expression with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 4 h at 37°C. The cells were harvested by centrifugation and lysed in 10 mM Tris-HCl pH 8.0, 150 mM NaCl by three passes through a French press cell. After removal of cell debris by centrifugation at 27 000 g for 30 min, and the supernatant was then passed through a Hitrap Q (HP) column (Pharmacia) and washed with 10 mM Tris-HCl pH 8.0. A salt gradient from 0–500 mM NaCl in 10 mM Tris-HCl pH 8.0 was passed through the column, with RegB^s eluting at ~300 mM NaCl. The resulting RegB^s was treated with 300 μ M divalent cation and allowed to incubate on ice for 30 min before extensive dialysis against 10 mM Tris pH 8.0 and 300 mM NaCl. The RegB^s was ultimately dialyzed against 10 mM Tris pH 8.0, 300 mM NaCl and 50% glycerol for storage at -20°C.

Construction of the *R.capsulatus* strain carrying flag-tagged RegB

The C-terminus of *regB* was PCR amplified from *R.capsulatus* genomic DNA with primers RegBflagHindIII 5'-CCAAGCTTATCTGCGGTCGATGGGGC and RegBflagPstI 5'-TTCTGCAGGGCGGTGATCGGAACATTC. The PCR product was then force cloned into the HindIII, PstI site of pJM21, placing *regB* in frame with the flag M2 epitope sequence. The *regB*-flag tag sequence was then subcloned into the suicide vector pZJD3 using the enzymes *SpeI* and *HindIII* and mated into wild-type and C265A *R.capsulatus* strains using S17-1 λ pir. Single recombination events were selected by gentamycin resistance encoded by the pZJD3 vector.

Autophosphorylation assays

Phosphorylation assays were in 1 \times kinase buffer (20 mM Tris pH 8.0, 10 mM MgCl₂, 1 mM CaCl₂, 100 mM KCl and 300 mM NaCl) and either no DTT for oxidized or 10 mM DTT for reduced conditions. The reactions were started at $t = 0$ by adding a one-tenth volume of ATP mix (0.5 μ Ci [γ -³²P]ATP, 10 mM cold ATP) and then quenched by removing aliquots and mixing with SDS-PAGE loading dye containing 25 mM DTT. The samples were then separated by SDS-PAGE, visualized with autoradiographic film and quantitated with a phosphorImager.

Autophosphorylation redox potential assays

Different redox potentials were generated using ratios of oxidized and reduced DTT. Aliquots of copper-replete RegB^s were equilibrated to a given potential by incubation in 1 \times kinase buffer at specific potentials for 2, 4 or 18 h before phosphorylation assays were started as described previously. Samples were allowed to phosphorylate 10 min before being quenched with SDS-PAGE loading dye and separated by electrophoresis. The level of autophosphorylation was assayed by exposure to autoradiographic film as well as quantified for percent activity using a phosphorImager. The percent activity was plotted against the redox potential of each assay, with data points best fit by a Nernst two-electron line equation.

mBBR redox titration

The fluorescent probe mBBR (Calbiochem) was used to determine whether a redox-active disulfide existed in RegB^s. To measure free

sulfhydryls in copper-replete RegB^s, aliquots of the protein were first incubated for 2 h at potentials ranging from -230 mV to -400 mV, in 10 mV increments, before being allowed to react with 300 mM mBBR for 20 min. The protein was then precipitated by the addition of 100 μ l of 20% trichloroacetic acid, incubated on ice for 30 min, pelleted by centrifugation at 12 000 g and resuspended in 100 mM Tris pH 8.0, 1% SDS, before scanning for fluorescence on an Aminco-Bowman Series 2 Luminescence spectrometer with excitation at 380 nm and emission at 480 nm. The percent fluorescence was plotted against redox potential, and then best fit by a Nernst two-electron line equation.

EPR analysis

The RegB^s and C265A^s proteins were concentrated to 1.5 mM and then dialyzed against 10 mM Tris pH 8.0, 300 mM NaCl and 50% glycerol. All EPR spectra were recorded at 77 K at X-band (9.5 GHz) on an ESP 300 Bruker instrument. Typical EPR conditions: microwave power, 10 mW; modulation amplitude, 2–20 G; modulation frequency, 100 kHz; receiver gain; (2–5) $\times 10^4$. EPR spectra were simulated using a Monte Carlo method for the copper signals (Gaffney and Silverstone, 1993; Neese, 1995).

Spectroscopy

All UV/Vis spectroscopy was performed on a Beckman BU 640 spectrophotometer. Extinction coefficients were determined for the copper dd-bands using the copper concentration within the RegB^s samples and the following equation: Abs = ϵ cell path (cm) concentration (M). For *R.capsulatus* spectral scans, *R.capsulatus* wild-type and C265A strains were grown aerobically, semi-aerobically and photosynthetically to 50, 90 and 80 Klett units, respectively. Then, 10 ml of each culture was pelleted by centrifugation at 6000 g for 10 min and resuspended in 1 ml of 10 mM Tris pH 8.0. The samples were sonicated to lyse the cells and then centrifuged at 12 000 g to remove cell debris before being spectrally scanned from 900 to 400 nm.

Gel filtration chromatography

RegB^s and C265A^s were fractionated on a Superose 12 XK 16 column (Pharmacia Biotech) equilibrated with 10 mM Tris pH 8.0, 300 mM NaCl and either no DTT for oxidizing or 10 mM DTT for reducing conditions. The column was size calibrated with commercial gel filtration standards (Bio-Rad).

Western blot analysis

R.capsulatus RegB flag-tag strains were grown aerobically in 1 l baffled flasks shaking at 400 r.p.m to a density of 35 Klett units and pelleted. Pellets were resuspended in 1 ml of cold aerated 10 mM Tris pH 8.0 and sonicated. Sample (15 μ l) was added to non-reducing loading dye just prior to heating to 55°C. Anaerobic cultures were grown in screw top tubes in a jar with anaerobic mix to 85 Klett units, and then 10 ml was transferred to ice cold tubes and centrifuged at 6000 g for 5 min. Pellets were resuspended in 1 ml of degassed cold 10 mM Tris pH 8.0 and sonicated in an anaerobic chamber. Samples (10.5 μ l) were added to non-reducing loading dye and then heated to 55°C before being separated by non-reducing SDS-PAGE electrophoresis. Western blot analysis was achieved using a 1:750 dilution mouse anti-flag M2 HRP conjugate antibody (US Biological) in 8 ml of TBST and 2 ml of 5% milk for 45–60 min. Membrane was washed with 200 ml of 1 \times TBST and visualized with 1.5 ml of Super Signal West Dura Extended Duration Substrate illuminator (Pierce), prior to exposure to autoradiographic film for 2–10 min.

β -galactosidase activity assays

R.capsulatus wild-type and C265A strains harboring the *puf::lacZ* fusion plasmid (pCB532 Ω Spec) (Bauer and Marrs, 1988) were grown aerobically, semi-aerobically and photosynthetically to 50, 90 and 80 Klett units, respectively. The β -galactosidase assays were performed as described by Swem *et al.* (2001).

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