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Protein–protein interactions between CbbR and RegA (PrrA), transcriptional regulators of the *cbb* operons of *Rhodobacter sphaeroides*

Andrew W. Dangel and F. Robert Tabita*
Department of Microbiology and Plant Molecular Biology/Biotechnology Program, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210-1292, USA.

Summary

CbbR and RegA (PrrA) are transcriptional regulators of the *cbb*$_I$ and *cbb*$_{II}$ (Calvin–Benson–Bassham CO_2 fixation pathway) operons of *Rhodobacter sphaeroides*. Both proteins interact specifically with promoter sequences of the *cbb* operons. RegA has four DNA binding sites within the *cbb*$_I$ promoter region, with the CbbR binding site and RegA binding site 1 overlapping each other. This study demonstrated that CbbR and RegA interact and form a discrete complex *in vitro*, as illustrated by gel mobility shift experiments, direct isolation of the proteins from DNA complexes, and chemical cross-linking analyses. For CbbR/RegA interactions to occur, CbbR must be bound to the DNA, with the ability of CbbR to bind the *cbb*$_I$ promoter enhanced by RegA. Conversely, interactions with CbbR did not require RegA to bind the *cbb*$_I$ promoter. RegA itself formed incrementally larger multimeric complexes with DNA as the concentration of RegA increased. The presence of RegA binding sites 1, 2 and 3 promoted RegA/DNA binding at significantly lower concentrations of RegA than when RegA binding site 3 was not present in the *cbb*$_I$ promoter. These studies support the premise that both CbbR and RegA are necessary for optimal transcription of the *cbb*$_I$ operon genes of *R. sphaeroides*.

Introduction

CbbR and RegA (PrrA) are members of different, but well-studied families of transcriptional regulators that each contribute to maximize expression of the two major CO_2 fixation (*cbb*$_I$ and *cbb*$_{II}$) operons of *Rhodobacter sphaeroides*. CbbR is a LysR type transcriptional regulator (LTTR), which is the most commonly utilized protein family for gene regulation in prokaryotes (Schell, 1993). RegA is part of a two-component signal transduction system also involving the membrane-bound histidine kinase, RegB (PrrB) (Sganga and Bauer, 1992; Mosely *et al.*, 1994; Robinson *et al.*, 2000). CbbR is absolutely required to activate transcription of the *cbb*$_I$ operon, with the *cbbR* gene divergently transcribed from the genes of this operon (Gibson and Tabita, 1993). A hallmark of almost all LTTRs is the requirement of a co-inducer (often a metabolite of the regulated pathway) to control expression of the operon (Schell, 1993). *In vivo* and *in vitro* studies of CbbR demonstrate that ribulose 1,5-bisphosphate (RuBP) is a co-inducer for CbbR (Smith and Tabita, 2002; Tichi and Tabita, 2002; Dubbs *et al.*, 2004; Dangel *et al.*, 2005). Most investigations into the multimeric state of LTTRs indicate that LTTRs function as tetramers when bound to its DNA binding site, and like most LTTRs (Hryniewicz and Kredich, 1994; Akakura and Winans, 2002; Muraoka *et al.*, 2003; Smirnova *et al.*, 2004; Ezezika *et al.*, 2007) footprint analysis suggests that CbbR binds to the *cbb*$_I$ promoter as a tetramer (Dubbs *et al.*, 2000).

The RegA/RegB two-component system maintains global regulatory control over redox-affected operons in non-sulphur purple bacteria, especially during aerobic to anaerobic growth transitions (Bauer *et al.*, 1998; Swem *et al.*, 2001; Dubbs and Tabita, 2004; Elsen *et al.*, 2004). Among the operons under control of this two-component system are the *cbb*$_I$ and *cbb*$_{II}$ carbon dioxide fixation operons of *R. sphaeroides* (Qian and Tabita, 1996; Dubbs *et al.*, 2000; Dubbs and Tabita, 2003) and *R. capsulatus* (Vichivanives *et al.*, 2000; Dubbs *et al.*, 2004). Systems that are regulated by RegA and contain promoter loci with RegA binding sites include the *cbb*, nitrogen fixation (*nif*), photosystem biosynthesis, electron transport, and other energy-related operons (Dubbs and Tabita, 2004; Elsen *et al.*, 2004). RegA from *R. sphaeroides* is thought to become activated to regulate certain promoters after phosphorylation of residue Asp-63 (RegA~P), catalysed by RegB (Inoue *et al.*, 1995; Emmerich *et al.*, 1999;

Accepted 18 November, 2008. *For correspondence. E-mail tabita.1@osu.edu; Tel. (+1) 614 292 4297; Fax (+1) 614 292 6337.

Comolli *et al*., 2002). Recent studies suggest that RegA forms a dimer upon phosphorylation (Laguri *et al*., 2003; 2006). Currently, there is no conclusive data on the multimeric state of RegA when bound to DNA. With the exception of RegB, only NtrX from *R. capsulatus*, a response regulator of nitrogen fixation genes, has been shown to interact with RegA (Gregor *et al*., 2007). RNA polymerase is thought to interact with RegA by virtue of the fact that RegA can stimulate *in vitro* transcription of the cytochrome c_2 and *hemA* genes in the presence of RNA polymerase (Comolli *et al*., 2002; Ranson-Olson *et al*., 2006).

The majority of promoters affected by RegA contain two or more binding sites (Dubbs and Tabita, 2004; Elsen *et al*., 2004); for example, the cbb_{II} promoter from *R. sphaeroides* contains six RegA binding sites discernible by footprinting (Dubbs and Tabita, 2003). With respect to the cbb_I promoter of *R. sphaeroides*, there are four RegA binding sites, with binding to site 3 required for optimal activation of the cbb_I promoter *in vivo* (Dubbs *et al*., 2000).

It is intriguing that DNA binding sites for both CbbR and RegA are found within the regulatory regions of the cbb_I and cbb_{II} CO_2 fixation pathway operons of *R. sphaeroides*; in some cases these regions overlap (Dubbs and Tabita, 1998; 2003; Dubbs *et al*., 2000). Specifically, RegA binding site 1 overlaps the CbbR binding site just upstream of the transcription start site of the cbb_I operon in *R. sphaeroides*, as determined by prior footprint analyses. Certainly, protein–protein interactions between CbbR and RegA are plausible considering the proximity of the two proteins when bound to the cbb_I promoter. Inasmuch as both proteins play important roles in controlling cbb_I operon gene expression (Joshi and Tabita, 1996; Qian and Tabita, 1996; Dangel *et al*., 2005), it would be extremely important to understand if these proteins interact when bound to the promoter. This interaction could be crucial and contribute to the rather complicated regulation of the *cbb* operons under the phototrophic and chemotrophic growth strategies employed by *R. sphaeroides*. While the earlier studies were highly suggestive of potential formation of CbbR/RegA protein complexes at the sites where these proteins bind the DNA (Dubbs *et al*., 2000), actual complex formation between these one-component and two-component regulators has not been previously demonstrated.

In this study, it is shown that previously proposed protein–protein interactions and complex formation between CbbR and RegA does in fact occur and such complexes may be isolated and characterized *in vitro*. Moreover, in the course of these studies it was shown not only that RegA can bind DNA with high affinity, but that RegA forms incrementally larger multimeric complexes with DNA (reduced electrophoretic mobility) as the RegA concentration increased. Furthermore, the simultaneous presence of RegA binding sites 1, 2 and 3 allowed RegA to form complexes with cbb_I-specific DNA at significantly lower concentrations than when the individual binding sites were supplied.

Results

RegA binds to the cbb_I *promoter and forms incrementally larger complexes with DNA*

The Reg system regulates transcription of many diverse operons. In this study, six different cbb_I promoter probes from the cbb_I promoter region from *R. sphaeroides* were used in gel mobility shift experiments to examine specific interactions of RegA and CbbR with cbb_I promoter sequences (Fig. 1A). In all instances, binding reactions between RegA and DNA or RegA, CbbR and DNA were performed under the same salt conditions and concentrations to eliminate changes in mobility of protein/DNA complexes due to variations in ionic strength. An example of the specific interaction of RegA with probe-1/2/3 is illustrated (Fig. 1B), with similar results obtained with other probes. In all cases, as shown for probe-1/2/3, competition experiments demonstrated that an excess of unlabelled probe (50 nM) competed with ^{32}P-labelled probe (0.1 nM) for RegA interactions (Fig. 1B).

Subsequent gel mobility shift studies showed that RegA possessed unusual DNA binding characteristics upon varying the concentration of RegA. First, a certain threshold concentration of RegA was required before DNA binding to probes-1/2/3 or -1/2/3/4 occurred. Thus, at 20 nM RegA, no discernible binding was observed, yet at 40 nM strong binding was noted (Fig. 2A and B). Second, RegA formed incrementally larger multimeric complexes with DNA as the concentration of RegA increased (Fig. 2A–D), with only one RegA/DNA complex species observed at any given RegA concentration. Indeed, oligomerization of RegA with probe-1/2/3 was observed at RegA concentrations as high as 1040 nM (Fig. 2C). Oligomerization of RegA appeared to be independent of probe concentration, as the presence of excess probe (50 nM of labelled probe per reaction) did not affect RegA binding (Fig. 2D). The possibility that oligomerization of RegA was caused by some non-specific interaction with any protein, i.e. due to some charged amino acid side chains or some other reason, was ruled out because holding the total protein concentration constant at 1040 nM using bovine serum albumin, while increasing the RegA concentration, did not influence oligomerization of RegA when probe-1/2/3/4 was used (Fig. 2E). Thus, oligomerization appeared specific to RegA.

The relative mobility of each RegA/DNA complex, presumably caused by an incremental change in molecular

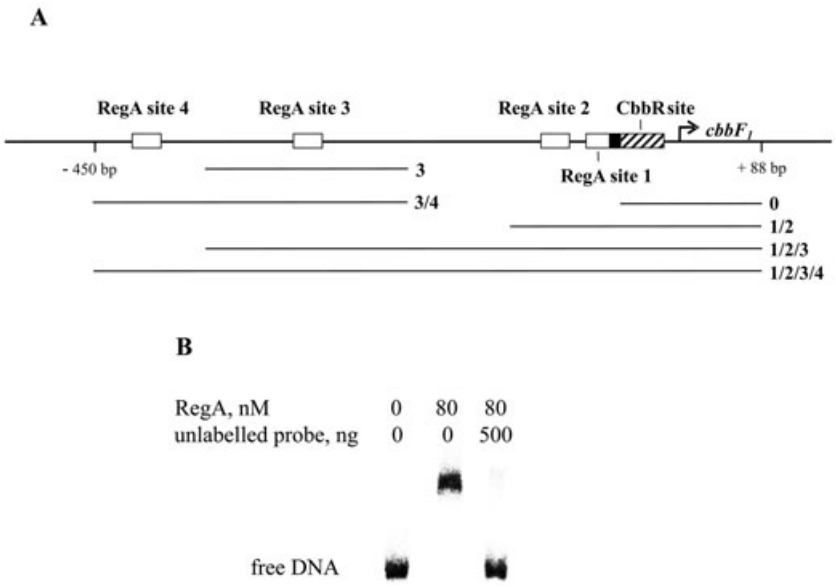

Fig. 1. Promoter region of the *cbb*$_I$ operon from *R. sphaeroides* illustrating the location of the CbbR and RegA binding sites (A). The relative positions of the various DNA probes used in gel mobility shift analyses are indicated. The black box depicts the overlap region between RegA binding site 1 (clear box) and the CbbR binding site (striped box). Probes are named after the RegA site(s) contained in each length of sequence. The arrow indicates the transcriptional start of *cbbF1*, the first gene of the operon. (B) Gel mobility shift assay demonstrating the binding of RegA to [^{32}P]-labelled probe-1/2/3 (0.1 nM). Controls were run with no added RegA and the presence of an excess of unlabelled probe-1/2/3 (50 nM) in the presence of labelled probe-1/2/3 (0.1 nM).

size upon increased RegA addition, suggested that there was a fixed quantity of RegA present in each complex. The molecular size of a RegA monomer and a CbbR (his-tagged) monomer is 21 kDa and 34 kDa respectively. LTTR proteins like CbbR bind to DNA as tetramers (Dubbs *et al*., 2000; Dangel *et al*., 2005). Thus, using CbbR as a size marker and assigning 136 kDa as the approximate molecular weight of a CbbR tetramer, the smallest RegA/DNA complex formed and bound to probes-1/2/3 or -1/2/3/4 at 40 nM RegA was suggested to be a tetramer of 84 kDa (Fig. 2A and B). To the extent that using the CbbR–DNA complex as a relative size marker provides some indication of the molecular weight of DNA–protein complexes, it would appear that each 20 nM incremental increase in RegA resulted in the addition of one more monomer of RegA to each RegA–DNA complex formed (Fig. 2A and B). From these analyses, the complex in lane 6 of Fig. 2A and B would contain a heptamer of RegA (of 149 kDa), as this complex runs slightly slower than the CbbR tetramer (of 136 kDa). Likewise the complexes in lane 5 would contain a RegA hexamer (of 126 kDa) which runs somewhat faster than the CbbR tetramer. Interestingly, the same relative mobility was obtained whether probe-1/2/3 or probe-1/2/3/4 was used in the binding reactions (Fig. 2A and B). Finally, it was apparent that RegA need not be phosphorylated in order to oligomerize and form the different complexes with its cognate DNA binding sites because a RegA-D63A mutant protein, with its specific phosphoacceptor residue, Asp-63, changed to an alanine, bound probe-1/2/3/4 similar to the wild-type RegA protein (Fig. 2F). Partially phosphorylated RegA also appeared to bind in a similar fashion (results not shown).

Promoter sequence-dependent formation of RegA/DNA complexes

Oligomerization of RegA occurred when other combinations of RegA *cbb*$_I$ promoter binding sites were provided, for example, RegA complexes were formed with probe-1/2, probe-3 and probe-3/4 (Fig. 3A–C). Clearly, no particular RegA binding site or combination of sites was necessary for oligomerization.

Previously, it had been shown that the interaction of RegA to binding sites 3 and 4, along with sites 1 and 2, substantially enhanced *in vivo* transcription of the *cbb*$_I$ operon compared with the level of transcription using only RegA binding sites 1 and 2. (Dubbs *et al*., 2000). However, the mechanism by which this enhancement occurred had not been delineated. In the present study, it is clear that the concentration of RegA at which RegA/DNA complexes first appeared was dependent on the probe used in the DNA binding reactions. With probe-1/2/3 or probe-1/2/3/4, as little as 40 nM RegA formed an observable complex with this promoter sequence (Fig. 2A and B, Table 1). However, with probe-1/2, the lowest concentration of RegA that formed a definitive complex was 240 nM (Fig. 3A), with a somewhat diffuse reaction at 160 nM RegA, indicative of a complex just starting to be formed at this concentration of RegA. With probes-3 and -3/4, a discernible complex was formed at 80–160 nM RegA (Fig. 3B and C respectively). Further studies with additional concentrations of RegA indicated that the lowest concentrations of RegA that formed discernible complexes was 160 nM using probes-1/2 and -3, and 80–100 nM with probe-3/4 (Fig. 3D and E, Table 1). It was found that the relative mobility of each of the RegA/DNA

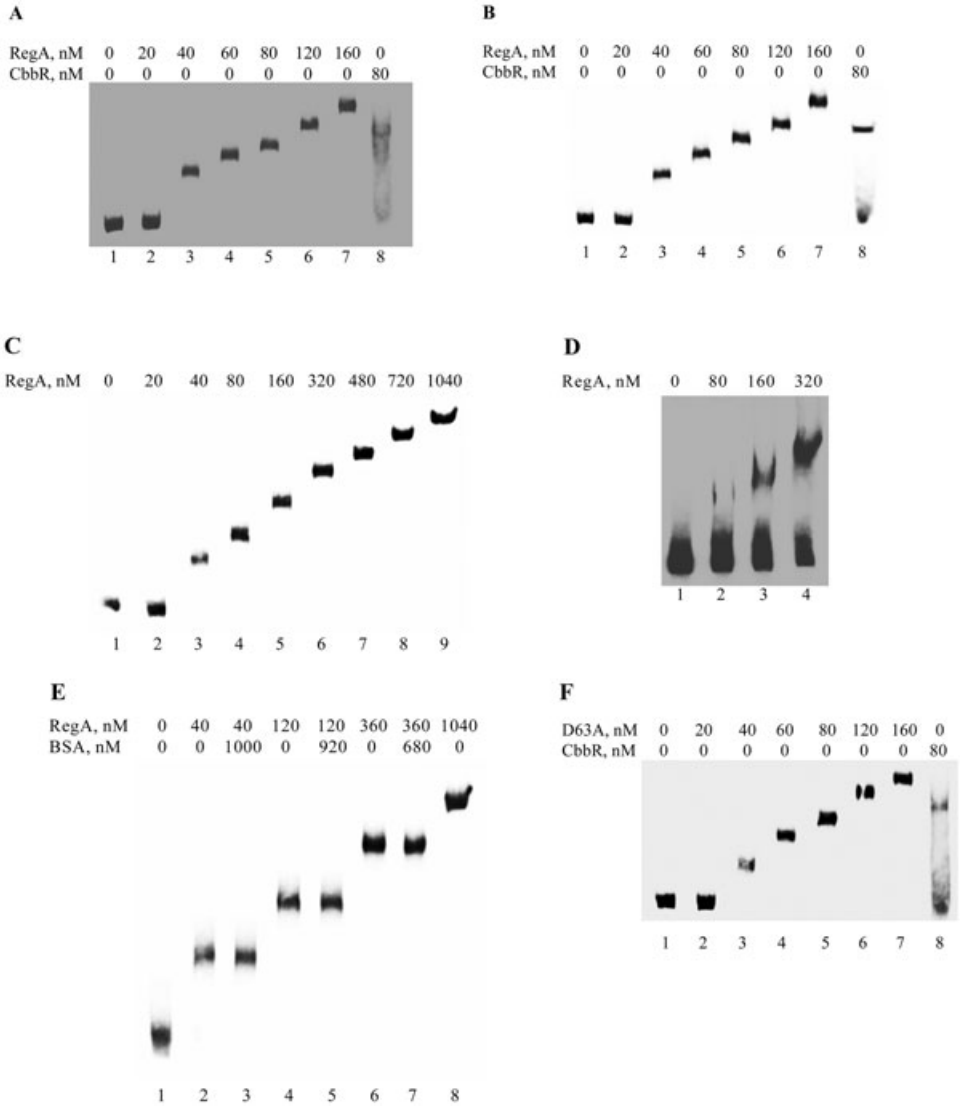

Fig. 2. Phosphorimages of gel mobility shift assays showing (A) the concentration dependence of RegA binding to probe-1/2/3 (0.1 nM), (B) the concentration dependence of RegA binding to probe-1/2/3/4 (0.1 nM), (C) the effect of high concentrations of RegA on the binding to probe-1/2/3, (D) RegA binding in the presence of excess probe-1/2/3 (50 nM of labelled probe per reaction), (E) concentration dependence of RegA binding to probe-1/2/3/4 when the total protein level is held constant using bovine serum albumin, and (F) concentration dependence of RegA-D63A binding to probe-1/2/3/4.

complexes obtained with each probe could be effectively compared using the mobility of the CbbR/probe-1/2 complex as a standard or marker. Such comparisons indicated reduced relative mobility of the RegA/DNA complexes formed with probes-1/2, probe-3 and probe-3/4 compared with the mobility obtained with probe-1/2/3 or probe-1/2/3/4 (results not shown). Finally, RegA bound to probe-1/2/3 or probe-1/2/3/4 gave a much more regular (incremental) response when the concentration of RegA was increased by 20 nM amounts compared with probes-1/2 or probe-3/4 (Fig. 2A and B; Fig. 3D and E). In fact, probe-1/2 and probe-3/4 did not show the regular incremental changes in oligomerization patterns at 20 nM

Table 1. Lowest concentration of RegA that will generate a RegA/DNA complex.

Probe/RegA site(s)[a]	[RegA]	Mobility of RegA/DNA complex[b]
1/2	160 nM	++
3	160 nM	++
3/4	80–100 nM	++
1/2/3	40 nM	+++
1/2/3/4	40 nM	+++

a. Probes-1/2/3 and -1/2/3/4 are at 0.1 nM, probes-1/2 and -3/4 are at 0.2 nM, and probe-3 is at 0.3 nM for each binding reaction with RegA.
b. ++ represents less distance travelled, +++ represents more distance travelled in 6% non-denaturing polyacrylamide gels.

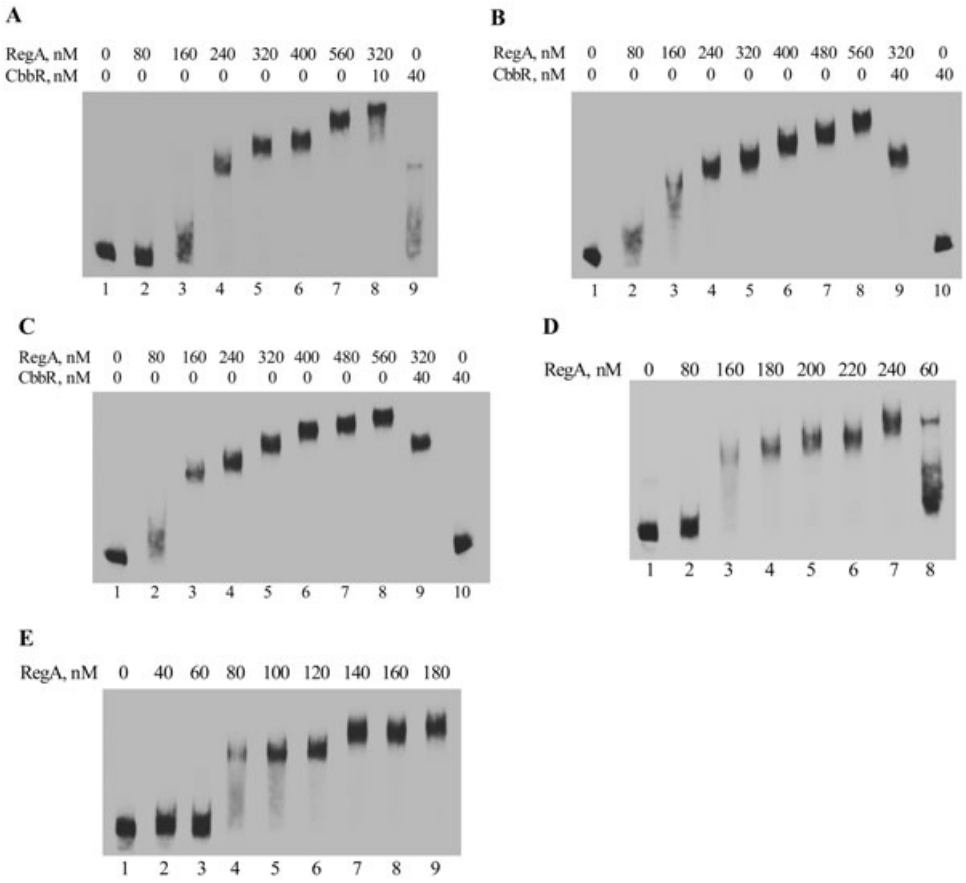

Fig. 3. Phosphorimages of gel mobility shift assays showing (A) the concentration dependence of RegA binding to probe-1/2 (0.2 nM) and the simultaneous binding of CbbR and RegA to probe-1/2, (B) concentration dependence of RegA binding to probe-3 (0.3 nM) and inability of CbbR to bind probe-3 in the presence of RegA, (C) concentration dependence of RegA binding to probe-3/4 (0.2 nM), and inability of CbbR to bind probe-3/4 in the presence of RegA, (D) concentration dependence of RegA binding to probe-1/2 (0.2 nM), and (E) concentration dependence of RegA binding to probe-3/4 (0.2 nM).

increases in RegA concentration (Fig. 3D and E). Thus, in all instances, the most sensitive response to changes in RegA concentration occurred when probe-1/2/3 or probe-1/2/3/4 were used, in keeping with the known enhanced transcription of the *cbb*$_I$ promoter when RegA binding sites 1, 2, 3 and 4 are all present (Dubbs *et al.*, 2000).

CbbR and RegA interact and CbbR must be bound to DNA for this protein–protein interaction to occur

RegA and CbbR were able to bind probe-1/2/3 simultaneously (Fig. 4A). CbbR did not interfere with the oligomerization of RegA (Fig. 4A, lanes 6–8). Indeed, CbbR joined the RegA/DNA complex and increased the size of the complex by a fixed quantity, independent of RegA concentration. CbbR was also able to bind to a RegA/probe-1/2 complex, containing only RegA binding sites 1 and 2 and the CbbR site, demonstrating that a CbbR/RegA/DNA complex formed in the absence of RegA binding site 3. These results also confirmed that CbbR and RegA simultaneously bind regions of DNA that are adjacent or overlap (Fig. 3A, lane 8). The possibility that CbbR and RegA interact independent of specific RegA binding sites was also investigated. For these studies, a *cbb*$_I$ promoter probe containing only the CbbR binding site and no RegA binding sites (probe-0) was used. Binding reactions between RegA and probe-0 demonstrated that RegA cannot bind probe-0 (Fig. 4B, lanes 2–4). In the presence of both CbbR and RegA, complexes with probe-0 were formed that were larger than the CbbR/probe-0 complex (Fig. 4*B*, lanes 5–8). This 'super-shift' demonstrated a specific interaction between CbbR and RegA. The generation of larger complexes with probe 0 as the concentration of RegA increased also was characteristic of the presence of RegA in the complex (Fig. 4B, lanes 5–7). Clearly, RegA oligomerized using either CbbR or specific RegA DNA binding sites as a platform.

While binding reactions using probe-0 empirically showed that RegA did not need to be bound to DNA in order to interact with CbbR (Fig. 4B), by contrast it was

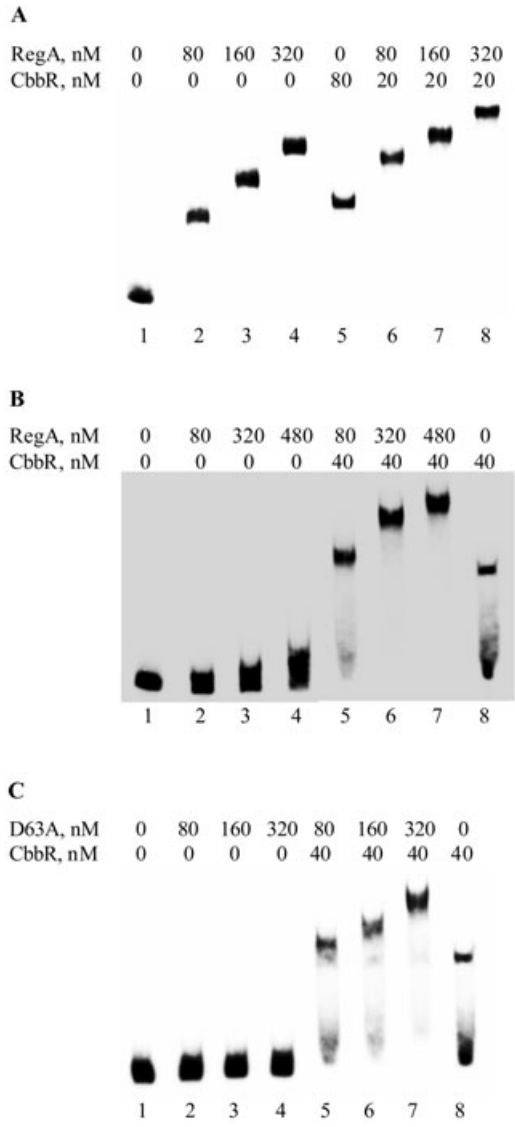

Fig. 4. Phosphorimages of gel mobility shifts showing (A) the simultaneous binding of CbbR and RegA to probe-1/2/3 (0.1 nM), (B) CbbR binding to probe-0 (0.3 nM) and interaction of CbbR and RegA, and (C) CbbR binding to probe-0 (0.3 nM) and interaction of CbbR with RegA-D63A.

shown that CbbR must be bound to the *cbb*$_I$ promoter in order for interactions to occur with RegA. This was illustrated using probe-3 or probe-3/4, which contain no CbbR binding site. Clearly, CbbR could not bind to RegA/DNA complexes in the absence of the CbbR binding site (compare Fig. 3B and C, lanes 5 and 9, where no 'supershift' was observed). It is apparent that *cbb*$_I$ promoter probes lacking either RegA or CbbR binding sites made it feasible to determine the necessity for CbbR to be bound to DNA in order for CbbR/RegA interactions to occur.

The D63A mutant of RegA (with impaired phosphorylation) was analysed to determine if the aspartic acid residue that is phosphorylated in RegA is important for interactions with CbbR. Binding reactions between RegA-D63A and probe-0 demonstrated that RegA-D63A could not bind probe-0 (Fig. 4C, lanes 2–4). When both CbbR and RegA-D63A were used in binding reactions with probe-0, complexes were formed that were larger than the CbbR/probe-0 complex (Fig. 4C, lanes 5–8). This 'supershift' demonstrated an interaction between CbbR and RegA-D63A and that the RegA phosphorylation site was not directly associated with RegA/CbbR interactions. The generation of larger complexes as the concentration of RegA-D63A increased, as before, also indicated the presence of RegA-D63A in the complex (Fig. 4C, lanes 5–7). RegA-D63A can thus oligomerize using CbbR as a platform (Fig. 4C) as well as using DNA as a platform (Fig. 2F) and, insofar as their interactions *in vitro*, RegA-D63A performed quite similar to RegA with CbbR.

Direct evidence of RegA/CbbR interactions and the presence of both proteins in specific RegA/CbbR complexes was obtained after isolating and identifying each protein from such complexes (Fig. 5). In this experiment, RegA/CbbR/probe-0 complexes were assayed via a gel mobility shift; RegA/CbbR/probe-0 complexes containing unlabelled probe-0 were then extracted from polyacrylamide gels as described in *Experimental procedures* (Fig. 5A). As positive controls, RegA and CbbR were extracted and isolated from polyacrylamide gels from RegA/probe-1/2/3 complexes and CbbR/probe-0 complexes respectively (Fig. 5B and C). The extracted proteins were then denatured and separated on SDS-PAGE (12% polyacrylamide) gels and subjected to immunoblot analysis using either specific anti-RegA or anti-His-tagged antibodies (detecting his-tagged CbbR) (Fig. 5D and E respectively). From these experiments, it was clear that the immunoblots detected the presence of CbbR and RegA in the appropriate complexes and confirmed that CbbR binds probe-0 and RegA binds the CbbR/probe-0 complex. Immunoblotting also confirmed that RegA binds and forms a complex with probe-1/2/3. As negative controls, RegA and/or CbbR was electrophoresed in the absence of specific promoter sequences; then material was extracted and separated from gels at the location where the corresponding protein/probe complexes would be expected to migrate. In no case was either CbbR or RegA found at these positions in the gel (Fig. 5D and E), reiterating the need for specific DNA/protein complexes to be formed.

CbbR and RegA can be chemically cross-linked in vitro

Chemical cross-linking of RegA and CbbR using dimethylpimelimidate (DMP) provided further support for specific interactions of the two proteins. DMP contains two imidoester groups (separated by 9.2 Å) that react with primary amines. CbbR (120 nM) was cross-linked to RegA

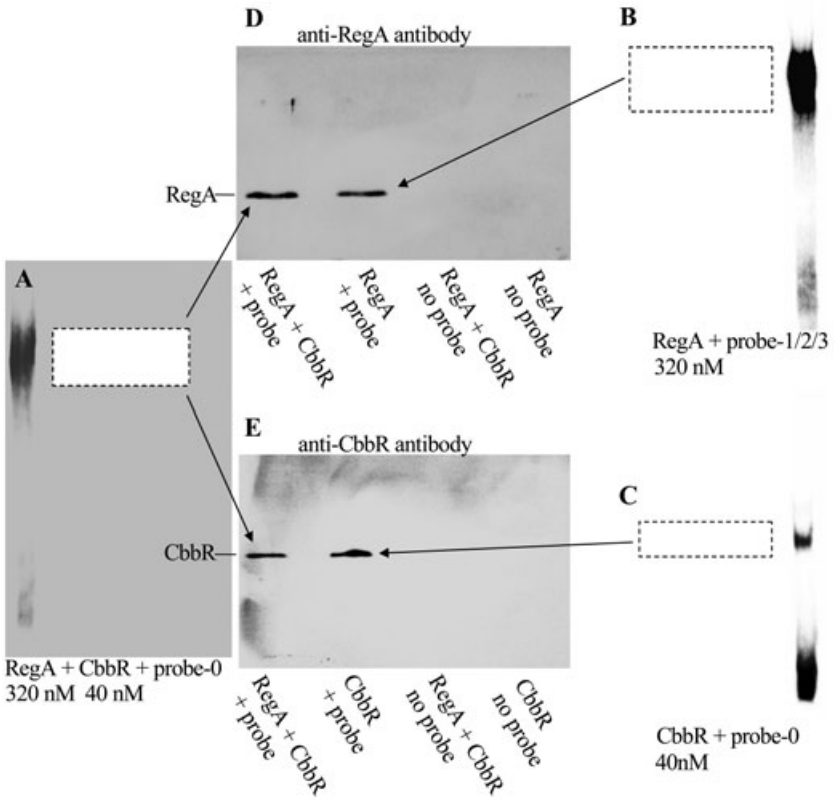

Fig. 5. Immunoblots demonstrating the presence of CbbR and RegA isolated from CbbR/RegA/*cbb*$_I$ promoter complexes separated on non-denaturing polyacrylamide gels. The respective excised gel fragments are represented by dashed-lined boxes; protein was extracted and isolated from the polyacrylamide as described in *Experimental procedures*. Pictured are gel mobility shifts of protein/DNA complexes from: (A) the CbbR/RegA/probe-0 complex; (B) the RegA/probe-1/2/3 complex; and (C) the CbbR/probe-0 complex. RegA and/or CbbR proteins from A–C were extracted, isolated, and then detected using immunoblots (D) with specific anti-RegA antibodies and (E) penta-His monoclonal antibodies to detect His-tagged CbbR. As negative controls, RegA and/or CbbR were electrophoresed in the absence of specific DNA probes and proteins extracted and isolated from polyacrylamide gels corresponding to locations where the protein/probe complexes would be expected to run (D) and (E) in the 'no probe' lanes. Probe-0 is at 0.3 nM and probe-1/2/3 is at 0.1 nM.

(120 nM) with DMP (10 mM) in the presence of probe-0 (20 nM). Cross-linked proteins were then denatured and subjected to SDS-PAGE (9% polyacrylamide) and immunoblot analysis. A monomer of his-tagged CbbR (34 kDa) was cross-linked to a monomer of RegA (21 kDa) to form a 55 kDa species, as detected using antibodies against His-tagged CbbR and RegA (Fig. 6A and B respectively). The 55 kDa complex was the only detectable cross-linked species between CbbR and RegA. Oligomeric species of CbbR (dimers, trimers and tetramers) and RegA (dimers, trimers, tetramers and hexamers) were also observed as cross-linked complexes (Fig. 6A and B respectively).

*The presence of RegA increases the quantity of CbbR/*cbb$_I$ *promoter complexes that are formed: recruitment of CbbR*

Protein–protein interactions often have positive effects and may lead to enhanced regulation or other functions, such as increased enzymatic activity or DNA (RNA) binding. With respect to RegA and CbbR, it was observed that RegA greatly facilitated the binding of CbbR to probe-0 (Fig. 7A and B, compare lanes 7 versus 8, lanes 6 versus 9, and lanes 5 versus 10). For convenient comparison, Fig. 7B is an overexposure of the gel mobility shift assay shown in Fig. 7A. Quantification of band intensity of the CbbR/probe-0 complex compared with the RegA/CbbR/probe-0 complex at the same concentration of CbbR indicated, on average, that the presence of RegA generated a sevenfold enhancement of CbbR binding to probe-0. The relative amount of enhancement was dependent on the concentration of CbbR. In addition to increasing DNA binding of CbbR, the presence of RegA allowed CbbR to bind to the *cbb*$_I$ promoter at a lower concentration than in the absence of RegA. CbbR/probe-0 complexes were not observed until 4 nM CbbR was employed, yet RegA/CbbR/probe-0 complexes were observed at 0.4 nM CbbR (Fig. 7B, lanes 5 and 9). Unlike RegA, increasing the concentration of CbbR did not increase the size of the RegA/CbbR/probe-0 complex (as shown by a decrease in mobility); however, the quantity of the complex did increase (Fig. 7A and B, lanes 3–7).

Discussion

Gel mobility shift analyses showed that RegA can bind specific sequences from the *cbb*$_I$ promoter region of *R. sphaeroides*; however, as the concentration of RegA increased, this protein possessed the unusual propensity to oligomerize and form large RegA/DNA complexes. The oligomerization of RegA was shown to occur with all *cbb*$_I$ promoter probes containing at least one RegA binding site, whether RegA was bound to DNA or to CbbR. While the physiological significance of forming RegA/CbbR

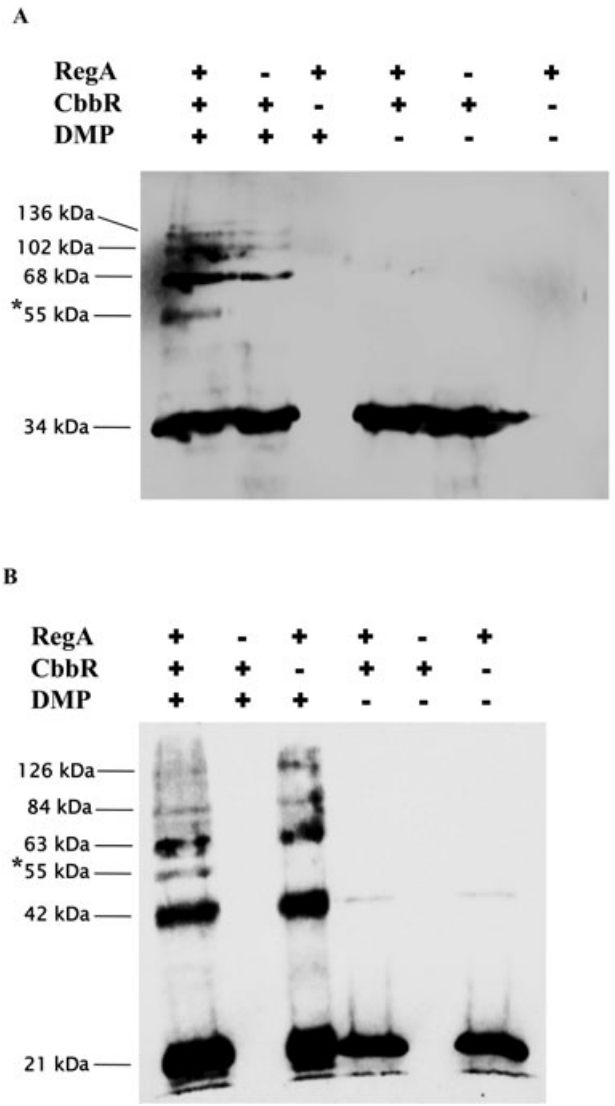

Fig. 6. Chemical cross-linking of CbbR (120 nM) to RegA (120 nM) using DMP as the cross-linker. Pictured are immunoblots of CbbR and RegA cross-linked during binding reactions in the presence of probe-0 (20 nM), illustrating CbbR/RegA cross-linking, as well as CbbR/CbbR and RegA/RegA cross-linkings.
A. Immunoblot analysis using antibodies against His-tagged CbbR (Penta His-tagged monoclonal antibody) detecting a CbbR/RegA complex (*55 kDa), as well as CbbR monomers (34 kDa), dimers (68 kDa), trimers (102 kDa) and tetramers (136 kDa).
B. Immunoblot analysis using antibodies against RegA, detecting a CbbR/RegA complex (*55 kDa), as well as RegA monomers (21 kDa), dimers (42 kDa), trimers (63 kDa), tetramers (84 kDa) and hexamers (126 kDa).

complexes may be apparent (see below), the significance of RegA oligomerization is unclear because oligomerization of RegA occurred at concentrations much higher than that found *in vivo*. NtrC, a bacterial enhancer-binding protein, also demonstrates the ability to oligomerize on the *glnA* promoter of *Salmonella typhimurium* (Wyman *et al*., 1997). Interestingly, phosphorylated NtrC activates *in vitro* transcription only when large oligomers are formed at high concentrations. Possibly, RegA could increase its concentration locally at promoters with multiple RegA binding sites and enhance transcription by oligomerization, similar to NtrC.

RegA binding sites 3 and 4 were shown to be required for optimal transcription of the cbb_I genes *in vivo* (Dubbs *et al*., 2000). *In vitro*, the presence of RegA binding sites 1, 2 and 3 is necessary to form a RegA tetramer when bound to DNA. These are also the smallest RegA/DNA complexes observed, and are probably the size of RegA/DNA complexes found *in vivo*. No other combination of cbb_I promoter probe can form such a small complex with RegA. Moreover, the presence of RegA binding sites 1, 2 and 3 also allowed for the formation of RegA/DNA complexes at lower concentrations of RegA compared with other combinations of RegA binding sites. These observations clearly demonstrated that there is cooperativity between RegA binding site 3 and site 1/2. The presence of RegA binding sites 1, 2 and 3 thus allowed RegA to bind the cbb_I promoter at physiologically relevant RegA concentrations and oligomeric states.

The evidence for cooperativity between RegA binding site 3 and site 1/2, and the spacing between RegA binding sites 3 and 1/2 suggests that a DNA loop may form between RegA binding site 3 and RegA binding sites 1/2, facilitated by RegA. Inspection of gel mobility shifts with RegA binding sites 1, 2 and 3 (Fig. 2A–C) indicated that a

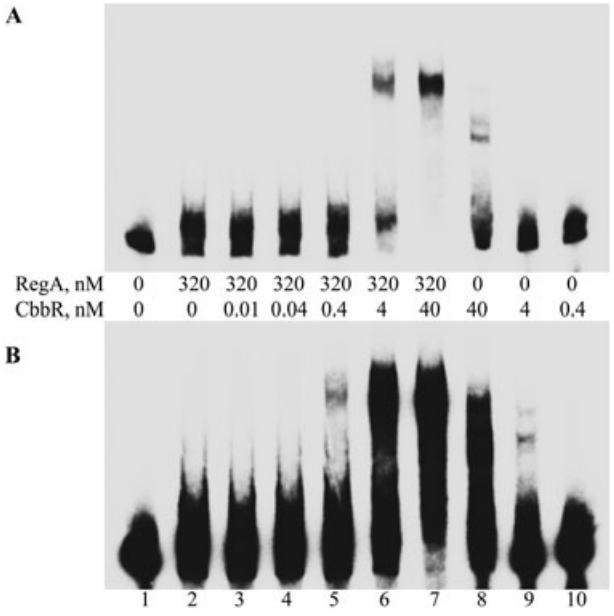

Fig. 7. The presence of RegA increases the amount of CbbR that binds to cbb_I promoter DNA. Phosphorimages of gel mobility shifts illustrating: (A) concentration dependence of CbbR binding to probe-0 (0.3 nM) in the presence (320 nM) or absence of RegA; and (B) overexposure of the data in A to facilitate observation of less prominent CbbR/RegA/probe-0 complexes.

DNA loop was most likely not formed between RegA binding sites 3 and 1/2 when the complexes were separated on non-denaturing polyacrylamide gels. DNA loops have very low mobility in polyacrylamide gels (Kramer *et al*., 1987), and the mobility of the RegA/probe-1/2/3 complex was quite high, as would be expected for linear DNA bound to RegA. This mobility correlated well with a CbbR/DNA standard run on the same gel, enabling estimates of the molecular size of such RegA/DNA complexes to be made. Perhaps a DNA loop might be an intermediate state in the process of transferring RegA bound at site 3 to RegA bound at site 1/2, that is, once RegA at site 3 binds RegA at site 1/2, RegA releases site 3 and the DNA becomes linear again. This model would explain why only probe-1/2/3 or probe-1/2/3/4 could form RegA tetramers. The transfer of RegA bound at site 3 to RegA bound at site 1/2 may allow for the correct conformation of RegA to form a RegA tetramer. Additionally, reducing the distance between RegA binding site 3 and site 1/2 compromises the ability of RegA to bind the cbb_I promoter at lower concentrations, thereby reducing the affinity of RegA for the cbb_I promoter (data not shown).

There are some examples whereby LTTR family proteins have previously been shown to bind to other proteins. Thus, CatR, CysB, CrgA, GcvA, NahR and OxyR, have all been shown to bind the alpha subunit of RNA polymerase, and GcvA also interacts with the sigma-70 subunit (Tao *et al*., 1993; McFall *et al*., 1998; Park *et al*., 2002; Deghmane *et al*., 2004; Lochowska *et al*., 2004; Stauffer and Stauffer, 2005). AphB and GcvA were even shown to interact with other transcription regulators, with AphB interacting with AphA to activate the *tcpPH* promoter in *Vibrio cholerae* (Kovacikova *et al*., 2004) and GcvA interacting with GcvR to repress the *gcv* promoter in *Escherichia coli* (Ghrist *et al*., 2001). In this study, direct evidence for the interaction of the two major transcription factors involved in controlling cbb_I transcription, CbbR and RegA, was demonstrated by gel mobility shift assays and chemical cross-linking studies. This was further confirmed after immunoblot analyses actually showed the presence of CbbR and RegA in isolated RegA/CbbR DNA complexes. Such studies provide a firm framework for previous indications and hypotheses that considered the probable interaction of these proteins at the promoter region of the cbb_I operon (Dubbs *et al*., 2000). The interaction of CbbR and RegA is most likely necessary for regulation of cbb_I transcription. While CbbR/RegA protein–protein interactions are not necessary for RegA/cbb_I promoter complex formation, CbbR must be bound to the cbb_I promoter to interact with RegA. Such a situation might prevent unnecessary interactions between the two proteins, which would not occur unless the proteins were bound to the cbb_I promoter. NtrX is the only other transcriptional regulator that is known to interact with RegA, and NtrX may also bind RegA only when bound to the *puf* promoter from *R. capsulatus* (Gregor *et al*., 2007). RegA is known to be a global regulator of many operons, and the aforementioned restriction on CbbR/RegA interactions may allow the RegA/RegB two component system to function efficiently, ensuring that RegA only binds transcriptional regulators such as CbbR at the appropriate promoter site. Furthermore, RegA is not required to bind DNA to interact with CbbR, possibly indicating that RegA changes conformation or is not bound to DNA once interaction with CbbR occurs. This suggests RegA may change its position to initiate transcription. The fact that RegA increased the level of CbbR/cbb_I promoter complexes by at least sevenfold, therefore increasing the affinity of CbbR for the cbb_I promoter, indicated that RegA could lower the activation energy required for specific CbbR/DNA interactions, or RegA might increase the stability of the CbbR/cbb_I promoter complex. RegA also lowered the concentration of CbbR necessary to bind the cbb_I promoter, lending further support to RegA as a stabilizer of CbbR/cbb_I complexes. RegA could thus function to efficiently control CbbR/RegA complex formation on the cbb_I promoter, thereby contributing to transcriptional control.

Recent studies utilizing nuclear magnetic resonance and line broadening techniques have demonstrated that a phosphorylated RegA (RegA~P) dimer binds and activates the *cycAP2* promoter from *R. sphaeroides*; however, these studies did not directly consider the oligomeric state of unphosphorylated RegA bound to the *cycAP2* promoter (Laguri *et al*., 2006). From the available results, a model for CbbR and RegA complex formation on the cbb_I promoter may be proposed, and the role of such interactions influencing transcription should be considered (Fig. 8). This model includes a potential DNA loop intermediate, as well as a switch from RegA to RegA~P and an increase in RuBP concentration, signalling a change from chemoheterotrophic growth to autotrophic growth. RuBP is a necessary co-inducer for CbbR to positively regulate the *cbb* operons (Smith and Tabita, 2002; Tichi and Tabita, 2002; Dangel *et al*., 2005), and as a global regulator, phosphorylated RegA is necessary to activate transcription (Inoue *et al*., 1995; Emmerich *et al*., 1999; Comolli *et al*., 2002; Ranson-Olson *et al*., 2006). In this model both the presence of RuBP and the phosphorylation of RegA are crucial for the transcription of cbb_I. Possibly, the binding of RuBP to CbbR and the phosphorylation of RegA produces a conformational change and influences the CbbR/RegA interaction, allowing RNA polymerase to bind to the complex and initiate transcription.

Finally, protein–protein interactions between CbbR and RegA raise the possibility that other LTTRs and transcriptional regulator proteins could potentially interact with

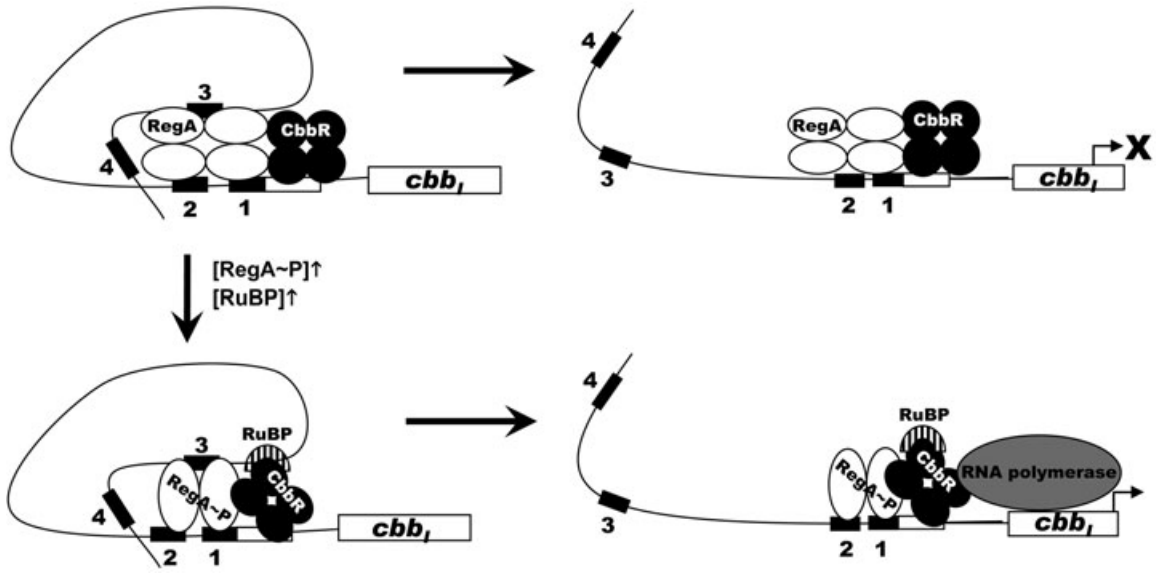

Fig. 8. Model depicting complex formation between RegA (or RegA~P), CbbR and the *cbb*$_I$ promoter from *R. sphaeroides*, leading to regulation of the *cbb*$_I$ operon. Small black boxes represent RegA binding sites, and the white box represents the CbbR binding site. In this model, RegA refers to the unphosphorylated protein while RegA~P is the phosphorylated form of RegA. The *cbb*$_I$ box depicts the transcriptional start site of the *cbb*$_I$ operon. CbbR tetramers, RegA tetramers and RegA~P dimers are illustrated. An intermediate loop structure generated by RegA or RegA~P is shown. The striped semicircle represents RuBP bound to CbbR. Increases in RegA~P and RuBP concentrations, denoted as [RegA~P]↑ and [RuBP]↑, are a result of changing from chemoheterotrophic to photoautotrophic or chemoautotrophic growth conditions. This in turn is proposed to result in conformational changes in the CbbR/RegA(RegA~P) complex (top portion of model) that allows recruitment of RNA polymerase and activation of transcription and subsequent CO_2 fixation (bottom portion of model). X indicates no recruitment of RNA polymerase to the *cbb*$_I$ promoter, thus no *cbb*$_I$ gene transcription and no CO_2 fixation under these growth conditions.

RegA. Certainly, as the LTTR family proteins possess a generally conserved structure, one might envision that other such proteins might interact with the global regulator RegA. Perhaps such interactions might insure enhanced transcriptional regulation above what LTTRs can provide alone.

Experimental procedures

Strains and plasmids

Escherichia coli strains BL21(DE3) and ER2566 were used for the production of recombinant CbbR and RegA respectively. The pG-Tf2 plasmid (Cm^r), which overexpresses the GroEL protein, was found to improve the isolation of soluble CbbR, and this plasmid was incorporated into strains used for recombinant protein production. For CbbR isolation, the pHisCbbR plasmid (Kn^r) was created by cloning the region containing *cbbR* from *R. sphaeroides* as a 1045 bp NdeI/BamHI fragment from pET11R-11 (Dubbs and Tabita, 1998), into NdeI/BamHI-digested pET28a. This His_6-tagged vector allows production of an N-terminal His_6-tagged CbbR protein. Plasmid pJC407 (Ap^r) contains the *regA* coding region from *R. sphaeroides* cloned into the intein/chitin-binding fusion vector, pTYB4, as previously described, producing a C-terminal intein-tagged RegA protein (Comolli *et al.*, 2002). Plasmid pJC417 (Ap^r) contains the *regA* coding region (with an A to C change at position 187 in *regA* to generate an aspartic acid to alanine substitution at amino acid 63) from *R. sphaeroides* cloned into the intein/chitin-binding fusion vector, pTYB4, as previously described. This produces a C-terminal intein-tagged RegA-D63A protein (Comolli *et al.*, 2002). Plasmid pKC1-5 (Kn^r) contains the *cbb*$_I$ promoter region (719 bp fragment) from *R. sphaeroides*, suitable for use as a template for PCR amplification to generate the gel mobility shift probes (Dubbs and Tabita, 1998).

Synthesis and purification of CbbR and RegA

CbbR is poorly soluble. However, a modification of the previously employed purification protocol (Dangel *et al.*, 2005) was used to prepare purified and soluble CbbR. A 2 l culture of *E. coli* BL21(DE3), carrying pG-Tf2 and pHisCbbR, was grown aerobically in Luria–Bertani medium supplemented with kanamycin (25 μg ml^{-1}), chlorampenicol (12.5 μg ml^{-1}) and tetracycline (0.01 μg ml^{-1}) at 37°C to an OD_{600} of 0.4. Expression of *cbbR* was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside followed by shaking at 25°C for an additional 16 h. The cells were resuspended in 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0 and lysed using a French press. After centrifugation at 25 000 *g* for 20 min, the cleared supernatant was mixed gently with Ni^{+2}-NTA agarose for 1 h, placed in a column, washed with 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, pH 8.0, and eluted with 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0. The purified His-CbbR was then denatured in 6 M guanidine-HCl, 10 mM Tris pH

8.0 and 1 mM DTT and dialysed against successively lower concentrations of guanidine (Dangel *et al*., 2005). To refold CbbR, final dialysis of His-CbbR was against 300 mM K^+ glutamate, 10 mM Tris, pH 8.5, 1 mM DTT and 30% glycerol; the protein was then stored in this buffer at –80°C.

RegA and RegA-D63A were purified as previously described (Comolli *et al*., 2002), with the following modifications:. The intein–chitin binding/RegA fusion protein was produced in ER2566 cells carrying the pJC407 plasmid (pJC417 for RegA-D63A) and passed through a French pressure chamber for lysis. Cleared supernatant was gently mixed with chitin beads for 1 h before washing and cleavage of the intein fusion peptide. Eluted RegA and RegA-D63A were dialysed against 50 mM HEPES, pH 7.8, 264 mM NaCl, 11 mM KCl, 5 mM $MgCl_2$, 2.2 mM $CaCl_2$, 300 mM sucrose and 30 mM DTT. Final dialysis was against 50 mM HEPES, pH 7.8, 200 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 35% glycerol before storage at –80°C.

Gel mobility shift assays

Gel mobility shift assays were performed as previously described (Dangel *et al*., 2005), with the following modifications. The pKC1-5 plasmid contains the *cbb*$_I$ promoter region and was used to PCR amplify the probes for the gel mobility shift assays. The following is a list of oligonucleotide pairs used to generate the probes.

Probe-0 (165 bp), 5′-GATTGGATCCACCATTTCCAAATTCCCGAACAG-3′
5′-GATTGGATCCGGTCCATCACGTCCTGCAACTC-3′

Probe-1/2 (237 bp), 5′-GATTGGATCCAGCGAGGCGCTGCCCGCCACCG-3′
5′-GATTGGATCCGGTCCATCACGTCCTGCAACTC-3′

Probe-1/2/3 (485 bp), 5′-GATTGGATCCTCGAGACCACACCCAGCGTCACC-3′
5′-GATTGGATCCGGTCCATCACGTCCTGCAACTC-3′

Probe-1/2/3/4 (551 bp), 5′-GATTGGATCCGATTCGGATCTCGGGGCAGGCGA-3′
5′-GATTGGATCCGGTCCATCACGTCCTGCAACTC-3′

Probe-3 (185 bp), 5′-GATTGGATCCTCGAGACCACACCCAGCGTCACC-3′
5′-GATTGGATCCAGGAAGCCTTCGGTCGTGCCGCT-3′

Probe-3/4 (255 bp), 5′-GATTGGATCCGATTCGGATCTCGGGGCAGGCGA-3′
5′-GATTGGATCCAGGAAGCCTTCGGTCGTGCCGCT-3′

All probes have a BamHI site incorporated at their 5′ and 3′ ends. Probes were digested with BamHI before labelling with ^{32}P-dCTP via an end-filling reaction using Klenow DNA polymerase. Each binding reaction between CbbR or RegA and DNA contained 0.1 nM of labelled probe, unless otherwise indicated.

Extraction of protein/DNA complexes from polyacrylamide gels

CbbR/promoter, RegA/promoter or CbbR/RegA/promoter complexes were excised from native polyacrylamide gels with a razor blade. The excised polyacrylamide was placed in dialysis tubing and electrophoresis was performed in a horizontal gel apparatus for 1 h using 50 mM Tris pH 8.0, 380 mM glycine, 2 mM EDTA (gel mobility shift running buffer) to electroelute the protein/DNA complexes from the polyacrylamide. The supernatant containing the extracted CbbR and/or RegA was subsequently used for immunoblot analysis.

Immunoblot analysis

Extracted CbbR and/or RegA were subjected to SDS-PAGE and transferred to Immunobilon-P membranes (Millipore, Billerica, MA). The Penta-His monoclonal antibody (Qiagen, Valencia, CA) was used to detect the presence of His-tagged CbbR in immunoblot assays. Anti-RegA polyclonal antibodies were used to detect the presence of RegA in immunoblot assays (a kind gift from Dr Tim Donohue, University of Wisconsin) (Comolli *et al*., 2002). The immunoblots were developed as previously described (Dangel *et al*., 2005), and analysed with a Storm 840 imaging system (Molecular Dynamics, Sunnyvale, CA).

Chemical cross-linking

CbbR and RegA were cross-linked using DMP. The DMP cross-linking procedure was previously described (DiBella *et al*., 2001) and was modified for this study. Briefly, CbbR (120 nM) and RegA (120 nM) in 40 µl of binding buffer (30 mM K^+ glutamate, 10 mM Tris, pH 8.5, 1 mM DTT, and 30% glycerol) containing 200 ng of probe 0 were incubated for 20 min at 25°C. Triethanolamine-HCl (pH 8.2) was added to a final concentration of 100 mM. DMP was added to a final concentration of 10 mM and the mixture incubated for 1 h at 25°C. CbbR alone and RegA alone were also cross-linked using DMP. The cross-linked reaction mixtures were then subjected to SDS-PAGE and transferred to Immunobilon-P membranes. The membranes were subjected to immunoblot analysis, using either the Penta-His monoclonal antibody or anti-RegA polyclonal antibodies to detect cross-linked complexes of CbbR/RegA.

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