Regulatory Twist and Synergistic Role of Metabolic Coinducer and Response Regulator-Mediated CbbR-cbb\textsubscript{I} Interactions in *Rhodopseudomonas palustris* CGA010

Gauri S. Joshi,\textsuperscript{a} Michael Zianni,\textsuperscript{b} Cedric E. Bobst,\textsuperscript{a, c} F. Robert Tabita\textsuperscript{a}

Department of Microbiology, The Ohio State University, Columbus, Ohio, USA;\textsuperscript{a} Plant-Microbe Genomics Facility, The Ohio State University, Columbus, Ohio, USA;\textsuperscript{b} Department of Chemistry, University of Massachusetts Amherst, Amherst, Massachusetts, USA;\textsuperscript{c} F. Robert Tabita, Tabita.1@osu.edu

*Rhodopseudomonas palustris* assimilates CO\textsubscript{2} by the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway. Most genes required for a functional CBB pathway are clustered into the *cbb* \textsubscript{p} and *cbb* \textsubscript{H} operons, with the *cbb* \textsubscript{p} operon subject to control by a LysR transcriptional activator, CbbR, encoded by *cbbR*, which is divergently transcribed from the *cbbLS* genes (encoding form I RubisCO) of the *cbb* \textsubscript{p} operon. Juxtaposed between the genes encoding CbbR and CbbLS are genes that encode a three-protein two-component system (CbbRRS system) that functions to modify the ability of CbbR to regulate *cbbLS* expression. Previous studies indicated that the response regulators, as well as various coinducers (effectors), specifically influence CbbR-promoter interactions. In the current study, it was shown via several experimental approaches that the response regulators and coinducers act synergistically on CbbR to influence *cbbLS* transcription. Synergistic effects on the formation of specific CbbR-DNA complexes were quantified using surface plasmon resonance (SPR) procedures. Gel mobility shift and DNA footprint analyses further indicated structural changes in the DNA arising from the presence of response regulators and coinducer molecules binding to CbbR. Based on previous studies, and especially emphasized by the current investigation, it is clear that protein complexes influence promoter activity and the *cbbLS* transcription machinery.

\textit{Rhodopseudomonas palustris} assimilates CO\textsubscript{2} by the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway. Most of the genes required for the CBB pathway are clustered into the *cbb* \textsubscript{p} and *cbb* \textsubscript{H} operons. In addition to the CbbRRS system encoded by the *cbbRRS* genes, the *cbb* \textsubscript{H} region also contains the *cbbIL* genes encoding Form I RubisCO and a divergently transcribed master regulator gene, *cbbR*, encoding CbbR, a LysR regulator (LTTR) (1, 2). Form I RubisCO synthesis is specifically dependent on the presence of an intact and functional *cbbR* gene (1, 3). CbbR plays an important role in regulating the expression of *cbb* genes in all proteobacteria (4–12). However, unlike other nonsulfur purple photosynthetic bacteria, *R. palustris* makes use of a redox-regulated and dedicated two-component system (CbbbRS), whose genes are juxtaposed between *cbbR* and *cbbLS*. The CbbbRRS two-component system contributes to the regulation of *cbbLS* (form I RubisCO) transcription (1, 13), but functionality is specific to photoautotrophic (PA) growth conditions (1, 3). Moreover, the CbbbRRS system is clearly an atypical two-component system consisting of a hybrid sensor kinase (CbbbSR) and two response regulators (CbbbRR1 and CbbbRR2). Additional work suggested that both response regulators, with no ability to bind DNA on their own, modify and influence the ability of CbbR to interact at the *cbbLS* promoter. Gel mobility shift assays showed that CbbbRR1 increased the binding affinity of CbbR at the *cbbLS* regulatory region 3- to 5-fold whereas CbbbRR2 appeared to interact with CbbR and stabilize its binding to the promoter (14).

The characteristic features of regulators belonging to the LTTR family include a conserved helix-turn-helix (HTH) DNA binding domain at the N terminus and a C terminus that is usually involved in multimerization and coinducer recognition. Coinducer binding may alter the promoter binding properties of the LTTR and often favors contact with RNA polymerase (RNAP) for productive expression of the target genes. The coinducer is usually an intermediate or end product of the pathway regulated by the LTTR (15–17). Previous studies with different CbbR proteins and their coinducers suggested that the coinducer responses are organism specific (5, 7, 11, 18). In most cases, the presence or absence of coinducer determines the degree of DNA bending or relaxation, also observed by changes in the appearance of hypersensitive sites and DNA protection.

Like most other LTTRs, *R. palustris* CbbR binds the *cbbLS* promoter within a region containing the LTTR consensus motif (16, 17, 19, 20). The DNA binding ability of *R. palustris* CbbR is coinducer independent; however, RuBP, ATP, FBP, and NADPH enhanced DNA binding of CbbR at physiologically relevant concentrations (20). Interestingly, the presence of each of these metabolites, at the concentrations tested, likely reflects favorable intracellular conditions conducive for the CBB cycle to proceed. The greatest binding occurred in the presence of RuBP, ATP, FBP, and NADPH at a concentration of 500 \textmu M. RuBP and NADPH (500 \textmu M) improved the CbbR footprint on the *cbbLS* regulatory region, while ATP and FBP (500 \textmu M) induced significant hypersensitivity, indicating changes in the DNA structure; however, neither coinducer changed the size of the footprint (20).

In light of the above-described findings and since the response regulators and coinducers individually influence CbbR-DNA in-
and metabolic effectors. R. palustris with the involvement of specific protein complexes operation of a complex system to regulate cbbSR pGSJSP Intergenic region between cbbLS NADPH) and CbbRR1 together increased CbbR binding at the suggested that the coinducers (RuBP, ATP, and FBP, but not influence CbbR-mediated transcription. Results of gel mobility interactions (14, 20), it was incumbent on us to determine whether the response regulators and coinducers might act synergistically to influence CbbR-mediated transcription. Results of gel mobility shift assays and surface plasmon resonance (SPR) experiments suggested that the coinducers (RuBP, ATP, and FBP, but not NADPH) and CbbRR1 together increased CbbR binding at the cbbLS promoter. Each coinducer (RuBP, ATP, FBP, and NADPH) in combination with CbbRR2 restored but did not enhance CbbR binding at the cbbLS regulatory region. These results indicate the operation of a complex system to regulate cbbLS transcription in R. palustris with the involvement of specific protein complexes and metabolic effectors.

MATERIALS AND METHODS

Plasmids. The plasmids used in this study are listed in Table 1.

DNA manipulations. All DNA manipulations such as cloning, restriction enzyme digestion, DNA ligation, and agarose gel electrophoresis were performed in accordance with standard protocols as previously described (3, 14, 20).

Synthesis and purification of CbbR and CbbRR1 and CbbRR2. N-terminal His6-tagged recombinant CbbR was synthesized in Escherichia coli BL21(DE3) and purified by nickel affinity chromatography as previously described (14, 20). CbbRR1 and CbbRR2 were purified as previously described (14).

Gel mobility shift assays. Gel mobility shift assays were performed as previously described (5, 14, 20). The pGSJS90 plasmid, which contained the intergenic region between cbbSR and cbbL, was used for PCR amplification of the target DNA, probe SP90. The primers for PCR amplification of SP90 were 5'-GATCCAGATCGCCGCTGCCTGCAGGATATAAG-3' and 5'-GATCCAGATCGCCGCTGCCTGCAGGATATAAG-3' (underlining indicates restriction sites). The target DNA region was labeled with [32P]CTP in an end-filling reaction using Klenow DNA polymerase. Each binding reaction mixture contained 0.3 nM labeled target. Binding reaction mixtures (50 μl) were set up with CbbR (25 nM) alone or with CbbRR1 and/or CbbRR2 (400 nM each) or coinducers, radiolabeled target (~10,000 cpm), and 1.83 μg/μl of poly(dl-dC) in a buffer containing 30 mM potassium glutamate, 10 mM Tris-Cl (pH 8.5), 5 mM MgCl2, and 1 mM DTT. CbbR was incubated in the presence of competitor poly(dl-dC) DNA for 5 min at room temperature prior to addition of the radiolabeled target DNA. Coinciders were included in the reaction mixture to reach a final concentration ranging from 5 to 500 μM and were added prior to the addition of the target. Controls containing similar ionic strengths of inert salts were routinely employed and run beside the experimental reaction mixtures. The reaction mixture was incubated for 6 min at room temperature after addition of the target. The samples were separated on a Tris-glycine buffer system (6). Following drying, the gel was analyzed by autoradiography and visualized with a Storm 840 Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

DNase I footprinting. To map the binding site(s) of CbbR within the cbbLS promoter region in the presence of response regulators CbbRR1 and CbbRR2, DNase I footprinting was performed using fluorescently labeled DNA and an automated fluorescent DNA analyzer (9, 21). Briefly, plasmid pGSJFP, which contains the intergenic region between cbbSR and cbbL (199 bp) and which extends 216 bp into cbbSR and 44 bp into cbbL, was used as the template to generate the 459-bp probe (FP). The probe was generated by PCR amplification with the primers Fpcbb1-VIC (5'-VIC-AAGCTGATCAGGAGGCG-3') and Fpbb2-FAM (5'-VIC-AAGCTGATCAGGAGGCG-3') from Applied Biosystems. CbbR (100 nM) and CbbRR1 or CbbRR2 (400 nM) were incubated with 1.83 μg of poly(dl-dC) for 10 min at room temperature in binding buffer (30 mM potassium glutamate, 1 mM DTT, 5 mM magnesium acetate, 2 mM CaCl2, 0.125 mg/ml bovine serum albumin [BSA], 30% glycerol, 10 mM Tris-Cl [pH 8.5]). After this incubation, 100 to 120 ng of fluorescently labeled probe was added to the reaction mixture (final volume, 20 μl) and incubated for 20 min at room temperature. DNase I (Worthington Biochemicals, Lakewood, NJ) digestion was performed with 0.125 μg of DNase I per 20-μl reaction mixture for 5 min at room temperature. The reaction was then stopped by incubation at 75°C for 10 min. Control digestions with the probe were performed in the absence of the protein or in the presence of BSA. The samples were analyzed as previously described (20).

Surface plasmon resonance detection of protein-DNA interactions. CbbR-DNA interactions were determined as described previously (14, 20). CbbR-DNA interactions and the effects of response regulators and coinducers on CbbR binding were determined with a fully automated BIAcore T100 system (Uppsala, Sweden). For binding, the biotinylated DNA was injected (Hanks balanced salt solution [HBSS]–0.5 M NaCl) at a flow rate of 5 μl/min for 540 s. This resulted in a response of 270 response units (RU). CbbR was purified as described previously (14). For testing the interaction of CbbR with DNA, a solution consisting of 50 mM Tris-Cl (pH 7.5), 200 mM NaCl, 5 mM MgCl2, and 1 mM EDTA was used as the running buffer. To determine the effect of the response regulators and coinducers on each CbbR binding, a 61-μl sample of CbbR (0.065 nM) containing the coinducer and CbbRR1 (5 nM) and/or CbbRR2 (5 nM) in running buffer was injected over the sensor surface. Kinetic data analysis of CbbR binding reactions at the cbb promoter was done with BIAevaluation software v. 4.0 using a 1:1 kinetic binding model. This model describes a 1:1 interaction at the sensor surface: A + B ↔ AB. Here A is the concentration of the analyte (CbbR), B is the total ligand (DNA) on the sensor surface, and AB is the initial binding response. The parameters reported by the model include the association rate constant (kd; M−1 s−1), the dissociation rate constant (kd s−1), and the affinity constant (Kd = Ks Kd; nM).

RESULTS

DNase I protection assays to probe the response regulator-mediated effect on CbbR binding. In addition to the major transcriptional regulator, CbbR, the control of cbbLS operon transcription in R. palustris appears to be complex, involving cellular metabolites (metabolic effectors or coinducers) as well as additional protein regulatory factors (14, 20). The CbbR binding region within the cbb operon mapped to two sites with classic LTTR

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Table 1 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQE80</td>
<td>cis-repressed, IPTG-inducible, N-terminal His6-tagged recombinant protein expression vector; Amp</td>
<td>Qiagen Laboratory stock</td>
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<tr>
<td>pQE160</td>
<td>cbbR coding region cloned into the BamHI-HindIII sites of pQE80; Amp</td>
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<tr>
<td>pQE8027</td>
<td>cbbR1 coding region cloned into the BamHI-HindIII sites of pQE80; Amp</td>
<td>1</td>
</tr>
<tr>
<td>pQE8024</td>
<td>cbbRR2 coding region cloned into the BamHI-HindIII sites of pQE80; Amp</td>
<td>1</td>
</tr>
<tr>
<td>pGSI3P</td>
<td>Intergenic region between cbbSR and cbbL (199 bp) in pCR-Blunt</td>
<td>14</td>
</tr>
<tr>
<td>pGSI3P90</td>
<td>Intergenic region between cbbSR and cbbL (90 bp) in pCR-Blunt</td>
<td>14</td>
</tr>
<tr>
<td>pGSJFP</td>
<td>Intergenic region between cbbSR and cbbL (199 bp) extending 216 bp 5' into cbbSR and 44 bp 3' into cbbL</td>
<td>14</td>
</tr>
</tbody>
</table>

* Amp, ampicillin; IPTG, isopropyl-β-D-thiogalactopyranoside.
motifs in the cbbSR-cbbL intergenic region (20). DNase I footprinting was subsequently performed to examine any changes in CbbR binding in the presence of the response regulators CbbRR1 and CbbRR2 (Fig. 1). The CbbR protected region is over 55 bp in length relative to the ATG translation start of cbbLS, with distinct sites susceptible to DNase I cleavage (hypersensitive) (20). The DNase I hypersensitivity response indicated that CbbR bends the DNA upon binding. There were no significant changes in the size of the footprint in the presence of CbbR and CbbRR1 compared to CbbR alone (Fig. 1C), except for the appearance of three additional hypersensitive sites at positions 160, 138 (Fig. 1Band E). The base at position 160, originally strongly protected in the presence of CbbR binding, was moderately hypersensitive in the presence of CbbRR1 and CbbRR2. The bases at positions 138 and 142 were protected bases at positions relative to the ATG translation start site (ATG) of cbbL. RFU, relative fluorescent units. (C to E) DNase I protection results seen in the presence of CbbR (C), CbbRR1 (D), and CbbRR2 (E) are summarized. Results are compared to a BSA control. Putative CbbR binding sites are indicated in bold. The putative –35 and –10 regions are underlined.

Synergistic role of CbbRRS system response regulators and coinducers in CbbR binding. CbbR binding was greatly enhanced in gel mobility shift assays by the presence of biosynthetic intermediates or end products of the CBB pathway, including RuBP (1.6-fold enhancement), ATP (2.5-fold), FBP (2-fold), and NADPH (4-fold) (20). Since it was apparent that the metabolic intermediates/coinducers and the response regulators individually influenced CbbR-promoter interactions, we determined whether these factors might act cooperatively on CbbR. Such studies would facilitate understanding and lead to a better appreciation of the mechanisms underlying CbbR-mediated transcriptional regulation. Indeed, it may be envisioned that a synergistic scenario would likely be encountered by CbbR in the cell. Thus, the effect of each coinducer (ATP, NADPH, RuBP, and FBP, all at 500 µM) was tested in tandem with that of each response regulator (CbbRR1 and CbbRR2, each at 400 nM) on the promoter binding ability of CbbR (25 nM). The results of gel mobility shift assays suggested that the coinducer and CbbRR1 together increased CbbR binding at the cbbLS promoter over that seen in the presence

### FIG 1

(A and B) DNase I footprinting of the CbbR binding sites within the cbbLS region of *R. palustris* in the presence of response regulator (A) CbbRR1 or (B) CbbRR2. The DNase I-digested reaction mixtures were prepared and analyzed by capillary electrophoresis with a 3730 DNA analyzer (Applied Biosystems). The difference in peak height of CbbR plus CbbRR1/CbbRR2 and the negative control (BSA) was calculated for each nucleotide position after digestion of the probe incubated with either CbbR plus CbbRR1/CbbRR2 or BSA as a negative control. Protection is represented by negative peak heights, while positive peaks are signs of hypersensitivity. Nucletide positions refer to the region of CbbR binding (202 to 120) on the probe DNA relative to the translation start site (ATG) of cbbL, respectively, in gray. The putative –35 and –10 regions are underlined.

(A) DNase I footprinting of the CbbR binding sites within the cbbLS region of *R. palustris* (20). DNase I footprinting was subsequently performed to examine any changes in CbbR binding in the presence of the response regulators CbbRR1 and CbbRR2 (Fig. 1). The CbbR protected region is over 55 bp in length relative to the ATG translation start of cbbLS, with distinct sites susceptible to DNase I cleavage (hypersensitive) (20). The DNase I hypersensitivity response indicated that CbbR bends the DNA upon binding. There were no significant changes in the size of the footprint in the presence of CbbR and CbbRR1 compared to CbbR alone (Fig. 1C), except for the appearance of three additional hypersensitive sites at positions –168, –160, and –135 (relative to the ATG translation start of cbbL) (Fig. 1A and D). The bases at positions –168 and –160, moderately hypersensitive in the presence of CbbR alone, had become strongly hypersensitive. Compared to CbbR alone, there was a loss of protection at bases –174, –161, and –124, while two new bases at positions –192 and –180 were moderately protected.

The binding of CbbR in the presence of CbbRR2 resulted in the appearance of new and strongly hypersensitive sites at positions –177, –160, and –138 (Fig. 1B and E). The base at –138 was originally protected by CbbR binding alone or in combination with CbbRR1. There was additional protection at position –184 and loss of protection at –153, exclusive to the presence of CbbRR2 alone. New bases at –187 and –136 were moderately protected and that at –161, originally strongly protected in the presence of CbbR alone, had become moderately protected. Common to CbbR alone and CbbR plus CbbRR1 or CbbR plus CbbRR2 were protected bases at positions –178, –154, –147, and –132 and a common strong hypersensitive site at –142. The appearance with CbbRR2 of hypersensitive and protected sites, some distinct from those seen in the presence of CbbRR1, indicated subtle changes or an altered protein-DNA conformation due to formation of a complex of the two proteins.

### Regulatory Twist

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of coinducer alone (Fig. 2A). This was observed as an increased intensity of a single CbbR-DNA complex and seemed to be an additive effect of two positive effectors acting synergistically to increase CbbR binding. This additive effect was especially evident in the presence of RuBP (3-to-4-fold-enhanced intensity), ATP (4-fold), and FBP (4-fold) (Fig. 2A) but not in the presence of NADPH (4-fold), where there does not appear to be an additive effect with CbbRR1 (Fig. 2B). The observed synergy was also dependent on the coinducer concentration; a 10- or 100-fold-lower concentration of coinducer did not elicit the same response (see Fig. S1A, B, and C in the supplemental material). Interestingly, synergy was also not evident in the presence of all coinducers and CbbRR1, under which conditions CbbR binding increased between 3-fold (in the presence of coinducers) and 3.5-fold (in the presence of coinducers and CbbRR1) (see Fig. S2 in the supplemental material).

The combination of CbbRR2 and coinducer reduced CbbR binding at the cbbLS regulatory region (Fig. 3). Under these conditions, neither coinducer seemed to have any effect on CbbR binding, as was seen previously (20) or in combination with CbbRR1 (Fig. 2). In fact, the combination of CbbRR2 and all coinducers (unlike that of CbbRR1 and all coinducers) completely abolished CbbR binding (see Fig. S3 in the supplemental material). Interestingly, synergy was also not evident in the presence of all coinducers and CbbRR1, under which conditions CbbR binding increased between 3-fold (in the presence of coinducers) and 3.5-fold (in the presence of coinducers and CbbRR1) (see Fig. S2 in the supplemental material).

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The results of these experiments in which the response regulator proteins and the coinducers were added together were surprising. The results indicated that the coinducers in general caused a structural change in CbbR that either enhanced (CbbRR1 plus coinducer) or restored but did not enhance (CbbRR2 plus coinducer) binding of CbbR at the promoter (Fig. 2 and 3). From these results, it was clear that the two response regulators exhibited distinct effects when added in combination with known coinducer metabolites.

SPR analysis of synergistic effects of CbbRRS system response regulators and coinducers on CbbR binding. From previous SPR experiments, it is clear that CbbR interacts with CbbRR1 and CbbRR2, as manifested by an increase in resonance signal over that for CbbR alone (20). Comparisons were thus made to the control binding responses of CbbR alone or CbbR in combination with the coinducers or response regulators. First, CbbR, in combination with CbbRR1 and coinducer, was passed over the chip sensor surface containing the immobilized cbbLS promoter DNA. The resultant response was compared to that seen with CbbR alone, CbbR plus coinducer, or CbbR plus CbbRR1. Previous results indicated that CbbR in the presence of RuBP (500 μM) bound the DNA with a 3-to-4-fold-higher association rate \( k_a = 14 \pm 2.7 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) than CbbR alone (20). In the presence of both CbbRR1 and RuBP (500 μM), CbbR binding was stabilized and remained at a level similar to that seen with RuBP alone yet consistently higher than that seen with the combination of CbbR plus CbbRR1 or with CbbR alone (Table 2 and Fig. 4A).

It was previously shown via SPR studies that CbbR in the presence of ATP (500 μM) bound the DNA with 6-to-7-fold-higher affinity \( k_a = 23.4 \pm 2.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) and a 6-fold-lower dis-
TABLE 2 Kinetic constants and affinities calculated for the interaction of CbbR with the *R. palustris* cbbLS promoter in the presence of coinducers and CbbRR1

<table>
<thead>
<tr>
<th>Component(s)</th>
<th>$k_a (\times 10^9 M^{-1} s^{-1})$</th>
<th>$k_d (s^{-1})$</th>
<th>$K_d (k_d/k_a) (nM)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without coinducer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CbbR</td>
<td>3.6 ± 0.6</td>
<td>0.12 ± 0.01</td>
<td>32 ± 3.7</td>
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<tr>
<td>CbbR + CbbRR1</td>
<td>13.2 ± 1.03</td>
<td>0.13 ± 0.01</td>
<td>10.5 ± 0.32</td>
</tr>
<tr>
<td>With coinducer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CbbR + RuBP (500 M)$^a$</td>
<td>14 ± 2.7</td>
<td>0.08 ± 0.01</td>
<td>5.9 ± 0.1</td>
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<tr>
<td>CbbR + CbbRR1 + RuBP (500 M)$^a$</td>
<td>13.5 ± 0.5</td>
<td>0.11 ± 0.01</td>
<td>8.0 ± 0.99</td>
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<tr>
<td>CbbR + ATP (500 M)$^a$</td>
<td>23.4 ± 2.3</td>
<td>0.02 ± 0.004</td>
<td>0.9 ± 0.07</td>
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<td>CbbR + CbbRR1 + ATP (500 M)$^a$</td>
<td>283</td>
<td>0.023</td>
<td>0.085</td>
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<tr>
<td>CbbR + FBP (500 M)$^a$</td>
<td>11.8 ± 0.15</td>
<td>0.07 ± 0.01</td>
<td>5.7 ± 0.09</td>
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<tr>
<td>CbbR + CbbRR1 + FBP (500 M)$^a$</td>
<td>18.7</td>
<td>0.042</td>
<td>2.3</td>
</tr>
</tbody>
</table>

$^a$ Data were previously published (20).

$^b$ Values represent the averages of the results determined with two separate binding reactions.

The results seen with response regulator CbbRR2 in combination with CbbR and the coinducers RuBP, ATP, and FBP, as reflected by the significantly higher apparent association rate (0.02 s$^{-1}$) than CbbR alone (20). Interestingly, the combination of ATP (500 M) plus CbbRR1 had a synergistic effect ($k_a = 283 \times 10^9 M^{-1} s^{-1}$) (Table 2), with increased and stable CbbR binding that reached equilibrium ($K_d = 0.085$ nM) (Fig. 4B and Table 2). This was in total agreement with the gel mobility shift data and indicated a potential significant regulatory role for CbbRR1 under those conditions. CbbR in the presence of FBP (500 M) bound to the promoter DNA with a 3-fold-higher association rate ($k_a = 11.8 \times 10^9 M^{-1} s^{-1}$) than CbbR alone (20). However, the combination of ATP (500 M) plus CbbRR1 was not synergistic and resulted in only a slight increase in the $k_a$ over that observed for either FBP or CbbR alone (Fig. 4C and Table 2). Though 500 M NADPH enhanced the DNA binding ability of CbbR in gel mobility shift assays, it did not have the same effect on CbbR binding in SPR experiments (9); neither did CbbRR1 have any effect on CbbR binding in the presence of NADPH (data not shown).

Thus, to summarize, the CbbR binding affinity increased considerably in the presence of CbbRR1 and the coinducers RuBP, ATP, and FBP, as reflected by the significantly higher apparent association rates.

The results seen with response regulator CbbRR2 in combination with CbbR and coinducers (500 M) were strikingly different. In all cases, in the presence of the coinducer and CbbRR2, the CbbR binding response (RU) ultimately reached levels similar to those seen with CbbR plus CbbRR2 alone (Fig. 4D). This was also reflected in the association rates for each binding reaction; however, the dissociation rate differed significantly from that for CbbR plus CbbRR2 in the absence of the coinducer (Table 3).

**DISCUSSION**

This work is part of an ongoing investigation to understand the molecular regulation of *cbbLS* (form I RubisCO) expression in *Rhodopseudomonas palustris*. Accumulated experimental evidence presented in this study and from past investigations indicates that there is a complex mechanism of *cbbLS* gene regulation in *R. palustris*. It involves a multitude of metabolic and protein regulatory factors, all functioning to influence the interaction of the master regulator CbbR at the *cbbLS* promoter. We have probed the CbbR-cbbLS promoter interactions in great detail in order to eventually gain insight into the mechanism of transcriptional activation. Previously, the CbbR binding site was mapped by deletion analysis of the *cbbI* control region in gel mobility shift assays. CbbR interacted at two specific binding sites resembling canonical LTTR motifs (20). It is also known that form I RubisCO production in *R. palustris* absolutely requires a functional CbbR gene (1, 3) and the CbbRRS two-component system is required for efficient transcription of the *cbbLS* genes (1).

The response regulators of the CbbRRS system regulate *cbbLS* expression via protein-protein interactions with CbbR; any direct interaction of the response regulators with the DNA has been ruled out experimentally (14). In this scenario, it is apparent that understanding the influence of these regulatory proteins on CbbR-DNA binding becomes relevant. Therefore, we tested the ability of both the CbbRR1 and CbbRR2 response regulators to influence the CbbR footprint. The combination of CbbR with CbbRR1 or CbbRR2 did not change the length of the footprint but did result in some additional hypersensitive and protected sites exclusively of the addition of either response regulator alone. These results indicated a conformational change in the DNA, likely due to binding of the protein complex.

In addition to the above-described protein factors, the nature of the metabolic signals that *R. palustris* CbbR perceives for the regulation of transcription was partially addressed in a previous study (20). Four different coinducers, namely, RuBP, ATP, FBP, and NADPH (500 M each), were identified that altered (enhanced) CbbR-cbbLS promoter binding. These coinducer metabolites had different effects on the CbbR footprint, with the presence of ATP and FBP resulting in extensive hypersensitivity and DNA structural changes. RuBP and NADPH improved the footprint. Promoter activity may be strongly influenced by DNA bending, either by structural changes to the DNA helix or by facilitating interactions with RNA polymerase (RNAP) (15). Thus, it was speculated that under these conditions, there was a likely additional requirement for one or both response regulators for stabilizing CbbR-DNA interactions.

The presence of CbbRR1 in the binding reaction with CbbR and ATP/FPB (500 M) enhanced DNA binding of CbbR, resulting in the formation of a stable CbbR-DNA complex. This was evident as a single protein-DNA complex of increased intensity in gel mobility shift assays (Fig. 2). Indeed, the presence of CbbRR1 in a reaction with CbbR and ATP or FBP (500 M) during SPR binding analysis significantly increased the rate of CbbR binding, resulting in the formation of a stable CbbR-DNA complex (Fig. 4 and 5 and association rate $k_a$ in Table 2). A stable promoter-bound CbbR-CbbRR1 complex likely recruits RNAP to the promoter for transcription initiation. Thus, at the highest concentration tested (500 M), the coinducers ATP (an intracellular metabolite required for biosynthesis and an indicator of the energy status of the cell) and FBP (a CBB cycle intermediate) might signal the presence of conditions conducive for the CBB cycle to proceed. At lower concentrations, due to decreased carbon and energy pools in the cell, it is conceivable that these coinducers behave as negative effectors to prevent *cbbLS* transcription and diminished CBB cycle activity.
NADPH was identified as a coinducer of CbbR in *R. palustris*, as it increased the affinity of CbbR for its promoter in gel mobility shift assays. The CbbR footprint in the presence of NADPH resembled the RuBP footprint very closely, and no changes in the extent of protection were observed (20). It was somewhat surprising that CbbRR1 did not seem to influence the binding of the CbbR-NADPH complex in either the gel shift (Fig. 2B) or the SPR binding analyses. The CbbRRS system is a redox-regulated system that is functional under photoautotrophic (CO2-dependent) growth conditions, a growth regimen associated with increased intracellular levels of NADPH as well as induced levels of CBB cycle proteins required for the production of cellular carbon.

The results seen with response regulator 2 (CbbRR2) were clearly distinct from those seen with CbbRR1. Inclusion of CbbRR2 in gel shift assays with CbbR and a coinducer (RuBP, ATP, FBP, or NADPH) not only inhibited CbbR-CbbRR2 inter-

### TABLE 3

Kinetic constants and affinities calculated for the interaction of CbbR with the *R. palustris cbbLS* promoter in the presence of coinducers and CbbRR2

<table>
<thead>
<tr>
<th>Component(s)</th>
<th>$k_a$ ($\times 10^6$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_d (k_d/k_a)$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without coinducer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CbbR</td>
<td>3.6 ± 0.6</td>
<td>0.12 ± 0.01</td>
<td>32 ± 3.7</td>
</tr>
<tr>
<td>CbbR + CbbRR2</td>
<td>17.4 ± 6.2</td>
<td>0.13 ± 0.04</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td>With coinducer$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CbbR + CbbRR2 + RuBP (500 μM)</td>
<td>21</td>
<td>0.06</td>
<td>3</td>
</tr>
<tr>
<td>CbbR + CbbRR2 + ATP (500 μM)</td>
<td>12.2</td>
<td>0.02</td>
<td>1.7</td>
</tr>
<tr>
<td>CbbR + CbbRR2 + FBP (500 μM)</td>
<td>18.9</td>
<td>0.03</td>
<td>1.6</td>
</tr>
</tbody>
</table>

$^a$ Values represent the averages of the results determined with three separate binding reactions.

NADPH was identified as a coinducer of CbbR in *R. palustris*, as it increased the affinity of CbbR for its promoter in gel mobility shift assays. The CbbR footprint in the presence of NADPH resembled the RuBP footprint very closely, and no changes in the extent of protection were observed (20). It was somewhat surprising that CbbRR1 did not seem to influence the binding of the CbbR-NADPH complex in either the gel shift (Fig. 2B) or the SPR binding analyses. The CbbRRS system is a redox-regulated system that is functional under photoautotrophic (CO2-dependent) growth conditions, a growth regimen associated with increased intracellular levels of NADPH as well as induced levels of CBB cycle proteins required for the production of cellular carbon.

The results seen with response regulator 2 (CbbRR2) were clearly distinct from those seen with CbbRR1. Inclusion of CbbRR2 in gel shift assays with CbbR and a coinducer (RuBP, ATP, FBP, or NADPH) not only inhibited CbbR-CbbRR2 inter-

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**FIG 4** Sensorgram depicting the real-time interaction of CbbR with the *cbbLS* promoter and the effect of CbbRR1 and coinducer RuBP (500 μM) (A), ATP (500 μM) (B), FBP (500 μM) (C), and CbbRR2 and coinducer (ATP or RuBP) (D) on binding. Target DNA, probe SP90, was immobilized onto a SA sensor chip. Binding analysis was carried out by injection of the controls, CbbR (0.065 nM) alone and CbbR plus CbbRR1 (5 nM), for comparison of the results to those of assays run with a combination of CbbR plus coinducer, with or without CbbRR1 or CbbRR2. All samples were injected for 400 s at a flow rate of 5 μl/min. The results were expressed as response units (RU).
actions on the DNA but also reduced CbbR-DNA binding despite the presence of the coinducer. This raises the possibility that (i) a portion of the DNA binding domain on CbbR may be involved in interaction with CbbRR2, thus accounting for the reduced CbbR-DNA binding (OFF conformation), or (ii) the coinducer and CbbRR2 may share a binding region on CbbR. The occupancy of this region by either coinducer or CbbRR2 and the subsequent effect on CbbR may be governed by CbbRR1, presumably binding a distinct site on the protein. Thus, the presence of CbbRR1 likely caused a conformational change in CbbR that allowed stable DNA binding but also resulted in an interaction with CbbRR2 (14) (unlocked or ON conformation).

Thus, to summarize, the data presented here suggest that CbbR responds to both metabolic and protein effectors via distinct binding sites on the protein. CbbR undergoes significant conformational changes when in complex with the regulator proteins or coinducers. The CbbRRS system (especially CbbRR1) seems to play a coinducer-like concentration-dependent regulatory role to stabilize CbbR binding. CbbR-CbbRR1 interactions enhanced the binding affinity of CbbR, thus favoring formation of a CbbR-DNA complex (14). The presence or absence of coinducers and CbbRR1 governs the interaction of CbbR with CbbRR2 as well as with the DNA, strongly suggesting altered CbbR conformations (ON or OFF) induced by these regulatory factors.

Proposed model depicting CbbR-CbbRR1/CbbRR2 interactions and the regulation of cbbLS transcription. Fundamental to this model are the distinct DNA conformational changes that occur as the CbbR-protein complex assembles on the DNA, as well as its rearrangement in the presence of coinducers. CbbRR1 functions to increase the binding affinity of CbbR, and CbbR and CbbRR1 interact prior to CbbR binding the DNA (Fig. 5A). The interaction of CbbRR2 with CbbR occurs to allow or prevent CbbR binding, depending on the presence or absence of CbbRR1 and coinducers. In this scenario, the presence of CbbRR1 causes a conformational change in CbbR that not only allows CbbR DNA binding but also allows the formation of a stable three-protein (CbbR-CbbRR1-CbbRR2) complex (Fig. 5B).

In addition to CbbR interactions, the transition from chemoheterotrophic to photoautotrophic (PA) growth is sensed by the CbbRRS system, presumably resulting in phosphorylation of the response regulators by the sensor kinase, although this has not been unequivocally established in vivo to this point. Under PA growth conditions, when the intracellular concentrations of RuBP and NADPH are high, CbbR would be capable of responding to changes in the concentrations of these compounds, thus initiating and/or enhancing transcription of cbbLS. The energy status and biosynthetic readiness of the cell, as indicated by intracellular ATP and NADPH concentrations, would ensure productive operation of the CBB cycle. Both CbbRR1 and CbbRR2 sense the fluctuating coinducer concentrations. The data suggest that CbbRR1 functions in synergy with the coinducers to enhance CbbR binding to maximize cbbLS transcription. We propose that CbbRR2 functions as an activator or antiactivator, depending on the presence or absence of all the coinducers and CbbRR1. In their absence, CbbRR2 functions as an antiactivator and locks CbbR in a conformation unable to bind DNA. In the presence of one or all four coinducers and CbbRR2, CbbR binding is restored or completely abolished, respectively. This suggests a requirement of CbbRR1, in whose presence CbbR binding is enhanced (unlocked or ON conformation). In its capacity as an activator under these conditions, CbbRR2 dissociates and no longer interacts with CbbR either off or on the DNA. The effect of the coinducers is manifest by conformational changes to the protein as well as structural changes to the DNA helix, bending it into a conformation that likely favors contact with RNAP and transcriptional activation of the cbbLS operon (Fig. 5C). Thus, the cellular signals perceived by the CbbRRS system, as well as the fluctuating metabolite concentrations in the cell, are all transduced to the cbbLS promoter via the master regulator CbbR. In this context, CbbR likely functions as a global regulator that integrates distinct signals perceived by CbbRR1 and CbbRR2. Thus, the response regulators and the binding of the coinducer(s) to CbbR act in concert for efficient cbbLS transcription.

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