The LysR-Type Transcriptional Regulator CbbR Controlling Autotrophic CO₂ Fixation by Xanthobacter flavus Is an NADPH Sensor

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Autotrophic growth of Xanthobacter flavus is dependent on the fixation of carbon dioxide via the Calvin cycle and on the oxidation of simple organic and inorganic compounds to provide the cell with energy. Maximal induction of the cbb and gap-pgk operons encoding enzymes of the Calvin cycle occurs in the absence of multicarbon substrates and the presence of methanol, formate, hydrogen, or thiosulfate. The LysR-type transcriptional regulator CbbR regulates the expression of the cbb and gap-pgk operons, but it is unknown to what cellular signal CbbR responds. In order to study the effects of low-molecular-weight compounds on the DNA-binding characteristics of CbbR, the protein was expressed in Escherichia coli and subsequently purified to homogeneity. CbbR of X. flavus is a dimer of 36-kDa subunits. DNA-binding assays suggested that two CbbR molecules bind to a 51-bp DNA fragment on which two inverted repeats containing the LysR motif are located. The addition of 200 μM NADPH, but not NADH, resulted in a threefold increase in DNA binding. The apparent Kₘ,app of CbbR was determined to be 75 μM. By using circular permuted DNA fragments, it was shown that CbbR introduces a 64° bend in the DNA. The presence of NADPH in the DNA-bending assay resulted in a relaxation of the DNA bend by 9°. From the results of these in vitro experiments, we conclude that CbbR responds to NADPH. The in vivo regulation of the cbb and gap-pgk operons may therefore be regulated by the intracellular concentration of NADPH.

During autotrophic growth of Xanthobacter flavus, CO₂ is assimilated via the Calvin cycle (16, 17). The energy required to operate the Calvin cycle is provided by the oxidation of methanol, formate, thiosulfate, or hydrogen (20). To date, three unlinked transcriptional units encoding Calvin cycle enzymes have been identified: the cbb operon, the gap-pgk operon, and the tpi gene (18, 19, 21, 24). The key enzymes of the Calvin cycle, ribulose-1,5-bisphosphate carboxylase-oxygenase (cbbLS) and phosphoribulokinase, are encoded within the cbb operon (19, 23).

The LysR-type transcriptional regulator CbbR has been identified in several chemo- and photoautotrophic bacteria (5, 6, 13, 19, 25, 32, 37, 38, 42). This protein controls the expression of the cbb operon and, in X. flavus, also the gap-pgk operon (24). LysR-type proteins recognize inverted repeats containing the LysR motif (7). Two LysR motif-containing inverted repeats are present in the intergenic region between cbbR and cbbL, in which the promoter of the cbbLXSFPIAE operon is located (37). Promoter-distal repeat IR₁ is a perfect repeat, whereas promoter-proximal repeat IR₂ is imperfect (Fig. 1).

The expression of the cbb and gap-pgk operons is maximally induced during growth in the absence of multicarbon substrates and in the presence of suitable autotrophic substrates, e.g., methanol (4, 20, 24). Although it is firmly established that CbbR plays an important role in transducing cellular signals to the transcription apparatus, the nature of these signals is still unknown. The results from studies with mutants of X. flavus,Ralstonia eutropha, and Pseudomonas oxalaticus blocked in glycolysis and isocitrate lyase indicated that the intracellular concentration of a glycolytic intermediate, e.g., phosphoenolpyruvate or acetyl coenzyme A, is an important factor in the regulation of the cbb operon (10, 18, 21, 22, 28). A correlation between the generation of reducing equivalents and the induction of the Calvin cycle has been demonstrated in both chemo- and photoautotrophic bacteria, suggesting that the intracellular concentration of NAD(P)H could be important in the regulation of the cbb operon (4, 9, 15, 29, 40). A low intracellular phosphoenolpyruvate concentration signals that insufficient carbon is available, which would necessitate CO₂ fixation; a high level of NADH signals that sufficient reducing power is available for the Calvin cycle to proceed. Interestingly, the activity of bacterial phosphoribulokinase is inhibited by phosphoenolpyruvate and stimulated by NADPH (34).

A number of LysR-type proteins have been shown to respond to the presence of low-molecular-weight ligands by an altered affinity for their DNA-binding sites and a decrease in the DNA-bending angle introduced upon binding of the protein (31). To obtain further insight in the molecular mechanism by which CbbR regulates the transcription of the cbb operon, the effects of low-molecular-weight compounds on the interaction of CbbR with its cognate binding sites were investigated. This paper describes the purification of CbbR of X. flavus and its interaction with NADPH.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Escherichia coli strains were grown on Luria-Bertani medium at 37°C (30). When appropriate, the following supplements were added: ampicillin, 100 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 20 μg/ml; chloramphenicol, 100 μg/ml; isopropyl-β-D-thiogalactopyranoside (IPTG), 0.1 mM. Agar was added for solid medium (1.5% [wt/vol]).

DNA manipulations. Plasmid DNA was isolated via the alkaline lysis method of Birnboim and Doly (1). DNA-modifying enzymes were obtained from Boehr-
grown in 3 liters of Luria-Bertani medium (with 50 mM dithiothreitol, 10% [vol/vol] glycerol). and resuspended in ice-cold buffer A (25 mM Tris-HCl [pH 7.8], 1 mM EDTA, allowed to proceed for an additional 3 h. Cells were harvested via centrifugation reached. IPTG was added to a final concentration of 1 mM, and growth was of chloramphenicol per ml) at 30°C until an optical density at 663 nm of 0.5 was

PIG. 1. (A) Nucleotide sequences of the 277- and 56-bp fragments used in the band shift assays. The five nucleotides of the 56-bp fragment derived from the vector are not shown. The positions of the putative binding sites of CbbR (IR1 and IR2) are indicated by arrows. The translations of cbbL and cbbR are shown below the nucleotide sequence. The translation of cbbL is from the reverse complement (lowercase letters). Putative ribosome-binding sites are underlined. The transcriptional start site of the cbb operon (19) is indicated by an arrow. The nucleotides protected by CbbR from DNase I digestion are boxed, and the position of the DNase I-hypersensitive nucleotide is indicated by the asterisk. (B) Alignment of IR1 and IR2, the putative binding sites of CbbR. Identical nucleotides are indicated by asterisks.

The nucleotides making up the LysR motif (T-N11-A) are boxed. The nucleotides protected by CbbR from DNase I digestion are boxed, and the position of the DNase I-hypersensitive nucleotide is indicated by the asterisk. (B) Alignment of IR1 and IR2, the putative binding sites of CbbR. Identical nucleotides are indicated by asterisks.

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Preparation of DNA fragments used in binding studies. The intergenic region between cbbR and cbbL was amplified by PCR from pSR1 by using oligonucleotides Preind (5'-CGCGAATTCGTGTTCGGTGCTAG-3') and CR2 (5'-CATGGATACGCAGCCGCGCCGAGCCGACG-3'). The resulting 285-bp DNA fragment was ligated into pTZ19U digested with Smal, yielding pTZ0. A DNA fragment containing the CbbR-binding sites without flanking DNA sequences was obtained by a PCR with pSR1 as the template and oligonucleotides Pr2 (5'-CGGGGATCCATCTGAGGTCGGAAG-3') and Pr6 (5'-CTTGGGCGGG CGCGCGGCAAGGACG-3'). The resulting fragment was digested with BamHI and EcoRI and subsequently cloned into pBluescript KS digested with BamHI and EcoRI, which yielded pSR168. The nucleotide sequences of the inserts of pSR168 and pTZ0 were determined to verify that mutations were not introduced during the PCR.

Labeling of DNA fragments. To obtain DNA fragments for use in band shift assays, pTZ0 and pSR168 were digested with BamHI and EcoRI and labeled with [γ-32P]ATP in a mixture (30 μl) containing 50 ng of DNA, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 1 μCi of [γ-32P]ATP, 1 U of Klenow enzyme, 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM NaCl, and 1 mM β-mercaptoethanol. Following incubation at room temperature for 30 min, 50 μM deoxyribonucleoside triphosphates were added and the reaction was allowed to proceed for an additional 15 min. The reaction was stopped by adding 15 mM EDTA to the mixture, and the DNA fragment was subsequently purified by using the Qiaquick PCR purification kit (Qiagen). DNA fragments with blunt ends and oligonucleotides were labeled with [γ-32P]ATP (30 μCi) by using T4 polynucleotide kinase (30).

Band shift assay. Band shift assays were performed as described previously, by using [32P]CTP-labeled DNA fragments, except that 20 μg of BSA was included in the binding assay (37). Metabolites were included in the incubation mixture to a final concentration of 200 μM. The samples were subjected to nondenaturing gel electrophoresis using 6% acrylamide gels in Tris-borate buffer (30) and run at 4°C and 10 V/cm. Following drying, the gel was analyzed by autoradiography.

RESULTS

Preparation of CbbR. To facilitate purification of CbbR, an efficient expression system was constructed by replacing the GTG initiation codon of cbbR with ATG and by placing cbbR downstream from the T7 promoter present on pET3a. Following induction of T7 RNA polymerase in E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500, CbbR was present as the most abundant protein in the cell extract. CbbR was purified from E. coli BL21(DE3)/pLysE/pER500. MW

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FIG. 2. Coomassie brilliant blue-stained denaturing polyacrylamide gel showing CbbR (10 μg) purified from IPTG-induced E. coli BL21(DE3)pLysE/pER500. The MW standards used are shown.
absence of carbon sources and in the presence of methanol, formate, or hydrogen. We therefore tested whether metabolites associated with glycolysis (phosphoenolpyruvate, 2-phosphoglycerate, and 3-phosphoglycerate) or energy metabolism (ATP, ADP, NADH, NAD, NADPH, and NADP) influence the in vitro binding of purified CbbR to its cognate binding sites. The addition of 200 \( \mu M \) NADPH to the binding assay resulted in an increase in DNA binding, whereas the other metabolites tested did not affect DNA binding by CbbR (Fig. 4).

The addition of NADPH to the DNA-binding assay had two effects on DNA binding by CbbR (Fig. 5). The total amount of \( ^{32}P \)-labeled DNA bound to CbbR increased threefold when the NADPH concentration was increased from 0 to 500 \( \mu M \). Saturation occurred at approximately 200 \( \mu M \) NADPH; the apparent \( K_d \) of CbbR was estimated to be 75 \( \mu M \). In addition, the ratio of complex 1 to complex 2 changed dramatically. In the absence of NADPH, 65% of the \( ^{32}P \)-labeled DNA bound to CbbR was present in complex 1, representing the binding of CbbR to both cognate binding sites. In the presence of 500 \( \mu M \) NADPH, virtually all (97%) of the bound \(^{32}\)P-labeled DNA was present in complex 1.

**CbbR induces DNA bending.** A number of LysR-type proteins induce a bend in the DNA following binding (31). To investigate whether CbbR bends DNA, the 68-bp BamHI-HindIII DNA fragment of pSR168 containing the CbbR-binding sites was cloned into pBEND4, yielding pLG168. A series of circular permuted DNA fragments of the same length and differing only in the position of the CbbR-binding sites were isolated following digestion of pLG168 with various enzymes and used in band shift experiments (Fig. 1 and 6A). The results show that the electrophoretic mobility of the DNA-CbbR complex is dependent on the distance between the CbbR-binding sites and the ends of the DNA fragment (Fig. 6C). From the electrophoretic mobilities of the protein-DNA complexes, it was calculated that CbbR introduces a bend \( \alpha \) of 64 \( \pm \) 3° following binding to its cognate binding sites.

**NADPH relaxes CbbR-induced DNA bending.** It has been reported for some LysR-type proteins that the DNA-bending angle is reduced when the ligand is added to the binding assay (31). To determine whether DNA bending by CbbR is influenced by NADPH, 200 \( \mu M \) NADPH was included in the assay mixture (Fig. 6B and C). Following analysis on nondenaturing gels, a bending angle of 55 \( \pm \) 3° was calculated, 9° less than that calculated in the absence of NADPH.

**DISCUSSION**

Physiological studies have shown that the expression of the Calvin cycle in facultatively autotrophic bacteria depends on the availability of suitable carbon and energy sources. The discovery that CbbR is a transcriptional regulator of the \( cbb \) operons in chemo- and photoautotrophic bacteria (6, 13, 37, 38, 42) suggested that this protein transduces these physiological signals to the transcription apparatus. This paper describes the effects of metabolic intermediates on the in vitro DNA-binding characteristics of purified CbbR.

CbbR of *X. flavus* protects nucleotides −75 and −29 relative to the transcriptional start site of the \( cbb \) operon. A similar region is protected by CbbR of *Thiobacillus ferrooxidans* and *R. eutropha*, which overlaps the −35 region of the promoter of the \( cbb \) operon. The close proximity of CbbR-binding sites to the promoter of the \( cbb \) operon facilitates contact between CbbR and the \( \alpha \) subunit of RNA polymerase, which was shown to be important for transcriptional activation by LysR-type regula-
It has been shown that CbbR of *R. eutropha* acts as a repressor of its own synthesis by binding to the *cbbR* promoter. The DNase I footprint obtained by using CbbR from *X. flavus* shows that this protein binds to the same region as the protein of *R. eutropha*, which overlaps the initiation codon of *cbbR* and upstream sequences (Fig. 1). This strongly indicates that in *X. flavus*, transcription of the *cbbR* gene is also repressed by CbbR.

DNase I footprinting and band shift assays done by using CbbR of *R. eutropha* showed the presence of two binding sites in the region protected by CbbR from DNase I. The results presented here and in a previous study show that this is also
true for CbbR of X. flavus (37). DNA binding by CbbR of both species is therefore typical for LysR-type transcriptional regulators, which, in general, bind to two binding sites upstream from the promoter. Since the region protected by CbbR contains two inverted repeats with a LysR motif, it is likely that these inverted repeats represent CbbR-binding sites.

LysR-type transcriptional regulators use low-molecular-weight compounds as ligands which, upon binding, frequently cause a modest increase or decrease in the DNA-binding affinity of the transcriptional regulator and a decrease in the DNA-bending angle (31). Of all of the metabolites tested, only the addition of NADPH to the binding assay had an effect on the DNA-binding characteristics of CbbR. A classical pyridine dinucleotide binding motif (41) is not present in the primary structure of CbbR. This is not altogether surprising, since binding of NADPH to allosteric sites may be quite different from binding to catalytic sites, which usually display the pyridine dinucleotide binding motif. The addition of NADPH caused a threefold increase in total DNA binding by CbbR. Following the addition of NADPH, 97% of the bound CbbR is present in complex I, which is formed following the binding of two CbbR dimers to their cognate binding sites. In contrast, only 65% of the total bound CbbR interacts with both sites in the absence of NADPH. CbbR therefore resembles TrpI; binding of TrpI to the promoter-proximal binding site is dependent on the presence of the ligand indoleglycerol phosphate (3). Interestingly, CbbR from R. eutropha resembles NodD in that binding to both binding sites is independent of the protein concentration and the presence of a ligand. The CbbR proteins of R. eutropha and X. flavus therefore display different DNA-binding characteristics, although both bind to two binding sites.

CbbR from X. flavus induces DNA bending, which is relaxed in the presence of NADPH. The presence of DNAase I-hypersensitive sites between the two CbbR-binding sites in a footprint of the cbb promoter of R. eutropha suggests that CbbR of this bacterium also bends its target DNA (14). It has been shown that DNA bending strongly influences the activity of some promoters (27). This may be due to a conformational change in the DNA helix or, alternatively, may facilitate the formation of productive contacts with RNA polymerase. DNA bending and ligand-induced relaxation of the DNA bend were observed in studies on other LysR-type proteins (31). In OxyR and OccR, relaxation of the DNA bend is associated with repositioning of the LysR-type regulator in the promoter-proximal DNA-binding site (36, 39).

The results of the in vitro experiments described here strongly suggest, but do not prove, that in vivo transcriptional regulation of the cbb and gap-pgk operons by CbbR is regulated by the intracellular concentration of NADPH. A number of experiments show that autotrophic growth is associated with elevated levels of NADPH. The transition of P. oxalatus from heterotrophic to autotrophic growth is accompanied by an increase in the intracellular NADPH-to-NADP ratio (12). Furthermore, NADP is completely reduced during incubation of Rhodospirillum rubrum under anaerobic conditions in the light. NADPH was rapidly oxidized following exposure to oxygen or in the dark. Interestingly, R. rubrum induces the Calvin cycle under the former growth conditions but not under the latter two (11).

Although bacteria use NADH to drive assimilation of CO2 by the Calvin cycle, NADPH may be a better signal in the regulation of Calvin cycle gene expression. NADPH, produced from NADH by transhydrogenase, is used in biosynthesis. A high intracellular NADPH concentration may therefore signal that although sufficient reducing power is available, biosynthetic reactions do not proceed due to the lack of a source of carbon. This is alleviated by the addition of suitable carbon sources to the medium or by induction of the Calvin cycle, followed by the fixation of CO2. The regulation of the cbb operon by the intracellular concentration of NADPH therefore explains why the oxidation of unrelated compounds such as thiosulfate, molecular hydrogen, and methanol induces expression of the Calvin cycle, whereas virtually all carbon sources which are readily assimilated have a repressive effect. The present study strongly suggests that NADPH plays an important role in the transcriptional regulation of the Calvin cycle genes. Future research will aim to characterize the in vivo and in vitro transcriptional regulation of the cbb and gap-pgk operons by the intracellular concentration of NADPH and the interaction between CbbR and NADPH.

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