Constitutive Mutations of the OccR Regulatory Protein Affect DNA Bending in Response to Metabolites Released from Plant Tumors

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OccR is a LysR-type transcriptional regulator of Agrobacterium tumefaciens that positively regulates the octopine catabolism operon of the Ti plasmid and is also an autorepressor. Positive control of the occ genes occurs in response to octopine, a nutrient released from crown gall tumors. OccR binds to a site upstream of the occQ promoter in the presence and absence of octopine. Octopine causes prebound OccR to undergo a conformational change at the DNA binding site that causes changes in footprint length and DNA bending. To determine the roles of these conformational changes in transcriptional activation, we isolated 22 OccR mutants that were able to activate the occQ promoter in the absence of octopine. Thirteen of these mutants contained single amino acid substitutions, and nine contained two base pair changes resulting in two amino acid substitutions, which in most cases acted synergistically. These mutations spanned the entire length of the protein. Most of these mutant proteins in the absence of octopine displayed DNA binding and bending properties characteristic of transcriptionally active OccR-octopine complexes.

The LysR family of transcriptional regulators is the largest known family of DNA-binding regulatory proteins in Proteobacteria (1). The Escherichia coli K-12 genome encodes 45 LysR-type proteins, almost 15% of all predicted regulatory proteins of this organism (2). LysR proteins regulate diverse genes and functions, yet many are involved in regulating metabolic functions such as amino acid biosynthesis. These proteins have an N-terminal DNA-binding motif of ~75 amino acids, and a C-terminal inducer binding domain of ~225 amino acids. They generally bind to rather long DNA binding sites upstream of target promoters. Activation of these promoters usually requires binding of a low molecular weight ligand, although specific DNA binding generally does not require such a ligand (3). LysR proteins often act as both repressors of their own transcription and activators of regulated promoters.

The region of greatest amino acid conservation among LysR-type proteins lies in the DNA binding domain, which contains a helix-turn-helix DNA-binding motif. The less conserved ligand binding domains of two LysR proteins, OxyR and CysB, have been analyzed by X-ray crystallography (4, 5), and the OxyR fragment has been crystallized in its active and inactive forms. These structures closely resemble each other, and both have a pronounced structural similarity to certain periplasmic binding proteins, especially the histidine-binding protein and the lysine-arginine-ornithine-binding proteins of enteric bacteria (6). The proposed sensory domains of OxyR and CysB are composed of two subdomains, designated domains I and II. Domain I is composed of two amino acid sequences, corresponding to OccR residues 90–160 and 265–298, whereas domain II is composed of one contiguous sequence corresponding to residues 161–264 (Fig. 2).

It has been suggested from past mutational studies of several LysR proteins that two sequences corresponding to residues 95–175 and residues 196–206 play a role in inducer response (3, 7–13). Mutations isolated in these regions result in a range of phenotypes that alter ligand-responsive transcriptional activation. More recently, an additional region, comprising residues 227–255, has been proposed to be important in CysB (13) and OxyR (9). The crystal structures of both CysB and OxyR suggest that the cavity formed between domains I and II is the likely ligand binding site for LysR proteins, as observed in the family of periplasmic binding proteins. Residues that line this cavity, and hence are likely to participate directly in ligand binding, fall within these proposed sequences as short patches. Other residues in these proposed regions lie outside of this cavity, suggesting that the response to inducer extends beyond that of the binding pocket. The functionally active form of LysR proteins is either dimeric or tetrameric, and oligomerization is thought to be mediated by the C-terminal region of the protein (13–15). Studies of CatR (16), TrpI (17), and OxyR (18) suggest that LysR proteins contact the C-terminal domain of the α-subunit of RNA polymerase and act to increase polymerase recruitment. Positive control mutants have been isolated in several LysR-type proteins, and these mutations generally cluster within the N-terminal DNA binding domain, suggesting that this domain may contact RNA polymerase (13, 19).

Most LysR-type proteins bind their promoters under noninducing as well as inducing conditions, and inducing ligands often cause a conformational change in these complexes. For several of these proteins, including NahR (20), TrpI (21), MetR (22), and CatR (23), inducing ligands appear to increase the number of bound protein protomers. In such cases, these proteins remain bound at all times to a particular site, whereas inducing ligands cause additional monomers to bind to an adjacent site. A different phenomenon is observed in other LysR proteins such as OxyR bound at the oxyR-oxyS intergenic region (24), where the inducer does not alter the number of bound protomers but instead causes a translocation of bound protein. In the absence of inducer, one dimer remains bound at all times to a site centered at −62, whereas inducing stimuli appear to cause a second dimer to shift from a site centered at −32 to a site centered at −42.

It has been reported that a number of LysR-type regulatory
proteins induce DNA bends when bound to their respective promoter regions (24–29). For example, the TrpI protein of *Pseudomonas putida* induces a DNA bend at the trpBA promoter, and this bend angle is increased when indoleglycerol phosphate stimulates binding of additional monomers to the promoter (29). CysB of *Salmonella typhimurium* also induces a bend at cysR and cysP promoters that is partially relaxed by the inducer, N-acetyl-l-serine (27). CatR of *P. putida* bends the catBC and pheBA promoters to regions of different degrees in the absence of cis, cis-muconate, but addition of the inducer results in a similar degree of DNA bending at the two promoters (28). The DNA bending induced by CatR at the pheBA promoter is actually enhanced in the presence of inducer, whereas bending at the catBC promoter is relaxed in the presence of inducer. OxyR also causes a high angle DNA bend in the oxyR-oxyS intergenic region under non-inducing conditions and relaxes this bend under inducing conditions (24). DNA bending has developed as a common theme in transcriptional regulation of prokaryotic promoters (30). For example, the *E. coli* CAP (catabolite gene activator) protein induces sharp bends at target binding sites, and replacing the CAP binding site with appropriately phased DNA bending sequences or by another protein-induced DNA bend can increase the rate of transcription (31, 32). However, it is important to keep in mind that for a number of LysR proteins, the highly bent protein-DNA complex is the inactive conformation, and the less bent complex is the active conformation.

This report examines a LysR-type protein found in the plant pathogen *Agrobacterium tumefaciens* that genetically transforms host plants by conjugally transferring oncogenic fragments of DNA to host cell nuclei. After infection and transfer of the T-DNA to the plant host, the plant cells release a unique set of compounds called opines (33), which provide the bacteria with sources of carbon, nitrogen, and energy. These plant-released compounds must be detected by tumor-colonizing agrobacteria. One such opine, octopine, is produced by the *A. tumefaciens* pathogen. One such opine, octopine, is produced by *A. tumefaciens* pathogen. Octopine does not, however, affect the binding affinity for the operator or alter the oligomeric state of DNA-bound OccR. In the absence of octopine, OccR protects a region from −80 to −28 overlapping the intergenic region between its own gene and the positively regulated *occQ* gene, whereas in the presence of octopine, this region shrinks to an interval from −80 to −38. The upstream 20 nucleotides of the binding site contain a substrate essential and sufficient for full binding affinity (the “high affinity subsite”), whereas the downstream 30 nucleotides of the operator do not contribute greatly to binding affinity but are required for ligand-responsive DNA bending (37).

### Experimental Procedures

#### Chemical Mutagenesis of OccR

Constitutive OccR Mutants

Only vectors, host strains, and basic plasmids are shown. The construction of plasmid derivatives containing mutant *occR* genes is described in detail under "Experimental Procedures.”

| Strain or plasmid | Relevant genotype | Ref. |
|-------------------|------------------|-----|
| JM109             | *E. coli* K12 F′traD38 proA’ B′ lacIΔlacZΔM15 Dlac-proAB glnV44 e14 gyrA96 recA1 relA2 endA1 thi hsdR17 | (42) |
| BL21/DE3         | *E. coli* B Plac-gene 1 of bacteriophage T7 | (40) |
| RA101            | A. *tumefaciens* KYC1203 (pMM1018/pKY148) | This study |
| KYC1203          | A. *tumefaciens* R10 (arcB–TrpS-gusA774ocR–pKY135) KmR, SmR | (44) |
| pSW213           | InsP cloning vector, lacZ, lacI, TrcR | (41) |
| pALTER-1         | Phagemid for site-directed mutagenesis, TrcR | Promega |
| pBEND3           | Derivative of pBlueScript for assays of DNA binding, ApR | (45) |
| pCGN1559         | Broad host range cloning vector, GmR | (46) |
| pRSETA           | TT promoter cloning vector, ApR | Invitrogen |
| pKCY148          | PoccQ-locZ cloned into broad host range plasmid pUCD2, SpR | (39) |
| pRA201           | occQ-occR intergenic region cloned into pALTER-1 | This study |
| pRJM101          | occR cloned into pCGN1559 | This study |
| pRA260           | occR cloned into pALTER-1 | This study |
| pRA304           | occR cloned into pRSETA | This study |
| pLW132           | occQ-occR intergenic region cloned into pBEND3 | (37) |

#### Fig. 1. Catabolism of octopine by *A. tumefaciens* R10. The catabolism of octopine requires genes on the linear chromosome and Ti plasmid. The chromosomal arcB and Ti plasmid ocd genes are redundant, and both encode ornithine cyclodeaminase. In an *arcB*occR null strain, a constitutive mutation in *occR* provided on a plasmid can result in growth on arginine as a sole carbon source due to up-regulation of the *ocd* gene.
constitutive mutations. The N-terminal DNA binding and C-terminal inducer recognition/response regions are indicated. Residues constituting domains I and II of the C-terminal region based on OxyR are outlined according to Choi et al. (4). The proposed dimerization interfaces are indicated by gray boxes. Single amino acid changes (A) and double point mutations (B) conferring a constitutive phenotype in OccR as defined by this study are indicated. To represent double point mutations, lines connect linked amino acid changes.

**TABLE II**

Expression of the occQ promoter by constitutively active occR mutant strains under different octopine concentrations

| Amino acid mutation | Codon mutation | β-Galactosidase activity at octopine concentration (Miller units) |
|--------------------|---------------|---------------------------------------------------------------|
|                    |               | 0 nm  | 10 nm | 100 nm | 1,000 nm | 10,000 nm | 100,000 nm |
| None               | None          | 1.8   | 2.5   | 4.0    | 7.0      | 24         | 72         |
| L3F                | CTC → TTC     | 8     | 10    | 12     | 20       | 44         | 152        |
| E23G               | GAA → GGA     | 325   | 365   | 370    | 372      | 386        | 406        |
| G74R               | GGG → AGG     | 154   | 155   | 147    | 150      | 134        | 137        |
| A89T               | GCC → ACA     | 20    | 26    | 40     | 52       | 24         | 72         |
| A89V               | GCA → GTA     | 10    | 12    | 12     | 45       | 102        | 224        |
| F113L              | TTC → TTC     | 72    | 72    | 70     | 93       | 135        | 341        |
| L120F              | CTC → TTC     | 41    | 42    | 48     | 51       | 66         | 211        |
| S123F              | GCC → GCC     | 550   | 430   | 442    | 476      | 489        | 497        |
| 314S               | GGC → GCC     | 30    | 28    | 31     | 32       | 70         | 209        |
| R198H              | CGT → CAT     | 6     | 8     | 12     | 20       | 57         | 197        |
| R302P              | CCG → CCG     | 120   | 120   | 111    | 123      | 160        | 279        |
| P214S              | CCG → TCG     | 37    | 32    | 35     | 40       | 68         | 221        |
| S215L              | TCA → TTA     | 18    | 19    | 21     | 32       | 90         | 216        |

Double point mutations

| A3F/A111V          | CTC → TTC, GCT → GGT | 112  | 133  | 106    | 147      | 206        | 226        |
| A97F/189H          | GCA → ACA, CAT → CAT | 139  | 138  | 154    | 236      | 291        | 298        |
| L93F/A224V         | CTC → TTC, GCG → GTC | 47   | 46   | 53     | 62       | 77         | 215        |
| L120F/A71V         | TTC → TTC, GCG → GTC | 413  | 332  | 357    | 339      | 331        | 308        |
| L120F/R222W         | TTC, GCG → TCG      | 303  | 337  | 328    | 323      | 319        | 341        |
| L120F/A232V         | TTC, GCG → GTC      | 145  | 136  | 139    | 149      | 191        | 244        |
| S123F/P214S         | TCC → TTC, CCG → TCG | 692  | 631  | 555    | 568      | 591        | 598        |
| P149S/P214S         | CCA → TCA, CCG → TCG | 452  | 480  | 420    | 425      | 430        | 423        |
| A232G/G233W         | GCC → GCC, GGG → TGG | 189  | 207  | 235    | 274      | 307        | 349        |
To separate out the two bp changes in the double mutants, occR genes containing only one of the bp changes were created by site-directed mutagenesis and cloned into pCGN1559.

\[ \frac{\text{mutation}}{\text{bp}} \times \frac{\text{occR}}{\text{genes}} \times \frac{\text{mutagenesis}}{\text{and cloned}} \times \frac{\text{into}}{\text{pCGN1559}.} \]

\[ \frac{\text{containing}}{\text{only}} \times \frac{\text{one}}{\text{of}} \times \frac{\text{the}}{\text{bp}} \times \frac{\text{changes}}{\text{were}} \times \frac{\text{created}}{\text{by}} \times \frac{\text{site-directed}}{\text{mutagenesis}} \times \frac{\text{and}}{\text{cloned}} \times \frac{\text{into}}{\text{pCGN1559}.} \]

\[ \frac{\text{With}}{\text{saturated}} \times \frac{\text{cultures}}{\text{were}} \times \frac{\text{diluted}}{\text{100-fold}} \times \frac{\text{into}}{\text{fresh}} \times \frac{\text{AB}}{\text{minimal}} \times \frac{\text{glucose}}{\text{medium}} \times \frac{\text{without}}{\text{antibiotics}} \times \frac{\text{and}}{\text{containing}} \times \frac{\text{occR}}{\text{mutant}} \times \frac{\text{genes}}{\text{in}} \times \frac{\text{the}}{\text{genome}} \times \frac{\text{and}}{\text{occR}} \times \frac{\text{active}}{\text{mutant}.} \]

\[ \frac{\text{By}}{\text{guest}} \times \frac{\text{on}}{\text{July}} \times \frac{29,2018}{\text{http://www.jbc.org/}} \text{Downloaded from} \]

\[ \frac{\text{TABLE III}}{\text{Synergistic effects of individual amino acid changes in occR double mutants}} \]

| Amino acid substitution | \( \beta \)-Galactosidase activity* |
|-------------------------|----------------------------------|
|                         | \(-\text{Octopine}\) | \(+\text{Octopine} (100 \mu M)\) |
| None                    | 1.8                  | 152                     |
| L3F/A111V               | 112                  | 226                     |
| ACF                    | 136                  |                         |
| A111V                  | 3                    | 177                     |
| A89T/R189H             | 139                  | 298                     |
| A89T                  | 20                   | 220                     |
| R189H                 | 6                    | 197                     |
| L503/F224V             | 47                   | 215                     |
| L953F                 | 1.8                  | 180                     |
| A224V                 | 3                    | 211                     |
| L120F/A71V             | 413                  | 308                     |
| A71V                | 6                    | 202                     |
| L120F                | 41                   | 211                     |
| L120F/R202W          | 303                  | 341                     |
| R202W            | 3                    | 136                     |
| L120F                | 41                   | 211                     |
| L120F/A232V          | 145                  | 244                     |
| A232V           | 1.8                  | 178                     |
| L120F                | 41                   | 211                     |
| S123F/P214S          | 692                  | 598                     |
| S123F              | 550                  | 550                     |
| P214S            | 37                   | 211                     |
| P149S/P214S        | 452                  | 423                     |
| P149S             | 10                   | 181                     |
| P149S             | 37                   | 221                     |
| A232G/G233W         | 189                  | 349                     |
| A232G         | 1.8                  | 141                     |
| G233W          | 32                   | 174                     |

* \( \beta \)-Galactosidase assays—To quantitate the activity of the mutant occR alleles, derivatives of strain KYC1203(pKY148) (44) containing the mutant occR derivatives of pRJM101 were cultured overnight at 28 °C in 2 ml of AB minimal glucose medium supplemented with 100 \( \mu \)g/ml each spectinomycin and gentamycin. Saturated cultures were diluted 100-fold into fresh AB minimal glucose medium without antibiotics and containing octopine at the indicated concentrations. \( \beta \)-Galactosidase-specific activities were measured after overnight incubation at 28 °C.

**RESULTS**

**Isolation of Constitutive occR Mutants**—We have previously demonstrated that using mutant operators to force OccR into a conformation that causes a low angle DNA bend and a short footprint is not sufficient to cause constitutive activity. Here we asked the converse question: do OccR constitutive mutants have a low angle DNA bend and a short footprint in the absence of octopine? To address this question, we devised a scheme to select and simultaneously screen for OccR constitutive mutants. The selection was for the constitutive expression of the occ gene of the Ti plasmid (Fig. 1), which functions in the breakdown of octopine (44) (an isofunctional gene elsewhere on the genome, arcB, was inactivated by a null mutation). This gene is induced by octopine but not by arginine, and yet its function is needed to catabolize both compounds. Growth on arginine as the sole carbon source therefore creates a selection for unregulated \( \text{occ} \) expression, which could be achieved by an OccR constitutive mutation. Because other mutations could also lead to derepressed \( \text{occ} \) expression, the strain also contained a plasmid-borne \( \text{occQ-lacZ} \) fusion, and the selection was done in the presence of X-gal. Colonies that turned blue on this medium were expected to arise only by constitutive OccR activity. To facilitate recovery of occR mutant genes, we inactivated the Ti plasmid copy of this gene and complemented this mutation with a plasmid-borne copy of \( \text{occR} \). This strain, RA101, is a derivative of KYC1203 (an \( \text{arcB/occR} \) double mutant) (44) containing pRJM101 and pKY148, which contain \( \text{occR} \) and an \( \text{occQ-lacZ} \) fusion, respectively. After mutagenesis with MNNG and plating on selective medium, blue colonies appeared after 10–12 days of incubation at a frequency of \( \sim 10^{-7} \) mutants/viable cell.

To ensure that the mutation allowing for growth on arginine originated from the plasmid encoding \( \text{occR} \), this plasmid was isolated from each of the candidates and introduced into KYC1203(pKY148) by electroporation. All the resulting strains showed constitutive \( \text{occQ} \) activity, indicating that the original constitutive phenotype was caused by a mutation in \( \text{occR} \). Two candidates from each of the 70 mutagenized pools were analyzed.

**Mutations Span the Length of the Protein**—To determine the location and nature of the mutations, the plasmid containing the putative \( \text{occR} \) mutation was isolated, and the \( \text{occR} \) gene was sequenced. Each of the 140 mutant \( \text{occR} \) genes had a single or double mutation. Of these, 22 different mutations were isolated, 13 containing single mutations and nine containing double mutations resulting in two amino acid substitutions. Identical mutations were isolated from independent mutant pools, indicating that we had isolated most or virtually all possible \( \text{occR} \) constitutive mutations.

The nucleotide and amino acid substitutions of each mutant are shown in Table II and Fig. 2. Surprisingly, these mutations spanned virtually the entire length of the OccR protein. Two mutants contained 1-nucleotide substitutions in the N-terminal region, L3F and E23G, the latter being part of the helix-turn-helix DNA binding motif. A number of mutations were found in regions of \( \text{occR} \) in which no mutations in studies of other LysR

R. Akakura and S. C. Winans, submitted for publication.
family proteins have been identified (7–13). However, no mutations were isolated in the C-terminal 64 amino acids, a region thought to be involved in multimerization (13, 14, 15). Although studies on other LysR family regulators have identified amino acid changes leading to constitutive activity (7–13), only single nucleotide substitution mutants have been identified to date. The fact that we were able to identify nine constitutive mutants with double base pair substitutions demonstrates the strength and effectiveness of this selection procedure for the isolation of very rare mutations.

Quantitation of Constitutive Activity—Strains containing each occR mutant and an occQ-lacZ fusion were tested for β-galactosidase expression in the presence and absence of octopine. All mutants showed a 3–200-fold elevated basal level of expression (Table II). The level of induction of these mutants at the highest concentrations of octopine was comparable with wild-type levels of induction.

The second category of mutants was those that had intermediate levels of activity in the absence of octopine. They expressed 10–30-fold higher activity compared with the wild type and could still respond to octopine. The last group of mutants was those that did not appear to respond to octopine and were therefore fully constitutive. This activation was as strong or slightly stronger than that of the wild-type OccR in the presence of the highest levels of octopine.

Because these mutants were selected on plates containing arginine (which resembles octopine) as the sole carbon source, it seemed possible that some might require arginine for full activity. We therefore tested the ability of arginine to act as an inducer of occQ activity. Arginine could not function as an inducer for the wild-type or any of the constitutive mutants (data not shown). This indicated that the mutations conferring the constitutive phenotype did not simply affect the inducer specificity of the proteins.

Double Point Mutations Act Synergistically to Create Strong Constitutive Alleles—All of the double mutants showed very high levels of activity in the absence of octopine. These mutants were fully constitutive and showed little if any induction of β-galactosidase activity with the addition of octopine. Only one mutant, L93F/A224V, showed an intermediate phenotype.

To identify the contribution of each amino acid substitution in these double mutants, it was necessary to create occR alleles containing just one of these mutations. Some of these muta-
tions had already been isolated as single mutations from the original selection. For the remaining nine mutants, site-directed mutagenesis was used to create the individual point mutations. These mutated occR genes were introduced into KYC1203(pKY148) and quantitatively assayed for octopine-induced activation of /H9252-galactosidase activity (Table III). Many of the mutations created by site-directed mutagenesis behaved like the wild type and did not show more than a 2-fold increase in activity in the absence of octopine. These low basal activities of many of these mutants may explain why they were not isolated in the original selection. OccR with an A71V, L93F, or P149S mutation led to a minimal increase in basal /H9252-galactosidase activity, whereas a G233W mutation led to a 16-fold higher activity than the wild type under noninducing conditions. However, when the two separate amino acid changes occur in the same gene, a very strong constitutive allele results. This suggested that the two individual base pair substitutions act synergistically to create this strong phenotype. With one exception, all double mutations occur in amino acid residues that are distinctly separated from each other. Mapping of these residues onto the OxyR structure indicates that each pair of mutations are in almost all cases separated from each other in tertiary structure as well as primary sequence. Leu120, located at the dimerization interface, may play an important role in inducer recognition and response, because a mutation to phenylalanine (L120F) leads to constitutive activity, and the same mutation can interact with at least three other amino acid residues to confer even higher activity. Similarly, P214S alone and along with two other amino acid changes leads to high constitutive activity.

**DNA Bending by OccR Constitutive Mutants**—Each occR mutant was placed under control of the strong T7 promoter, and crude extracts from the resulting strains were used in DNA bending assays as described previously (25). The OccR binding site was placed into plasmid pBend3 (45), which is designed to measure DNA bending, and the mobilities of the resulting mutant OccR-DNA complexes were observed on 5% polyacrylamide gels. OccR-DNA complexes with high-angle DNA bends migrate slowly in these gels, whereas complexes with low angle DNA bends migrate more quickly. We hypothesized that constitutive OccR mutants would have low angle DNA bends in the absence of octopine or that they might acquire this conformation in the presence of lower octopine concentrations than wild-type OccR.

The single point mutations (Fig. 3) responded to octopine in varied ways. The majority of these mutants had the same gel mobility as the wild type in the absence of octopine and yet switched to the faster mobility conformation in response to low octopine concentrations. Several mutants responded to octopine concentrations as low as 100 nM, and at 300 nM almost all of the mutants in this group attained their maximal migration. This concentration of octopine does not induce a conformational change in the wild-type protein (Fig. 3). The second group were those that migrated slightly faster than the wild type in the absence of octopine but also retained the ability to respond to octopine. These included L3F, E23G, G74R, A89V, and L120F. These mutants respond to octopine concentrations as low as 100 nM. The third group of mutants has a single member, R202P, which has a rapid migration rate in the absence of octopine and at all octopine concentrations tested.

The double mutants were subjected to the same assays, and in general had properties quite unlike most of the single point mutations (Fig. 4). The majority of these proteins formed fast migrating DNA complexes in the absence of octopine and were not much affected by octopine. The two exceptions were L93F/A224V and P149S/P214S (Fig. 4, lanes 5 and 10, respectively). Even these mutants shifted to the faster migration rate with very low octopine concentrations.

In all of the assays described above, we detected a range of
Constitutive OccR Mutants

Fig. 5. DNase I footprinting of the OccR binding site with a constitutive mutant. A fragment containing the occQ-occR intergenic region was end-labeled on the top strand and incubated with no OccR (lane 2), with cell extracts overexpressing the wild type OccR protein (lanes 3 and 4), and with extracts overexpressing the L120F/R202W constitutive OccR protein (lanes 5 and 6) in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of 300 μM octopine. The G + A ladder is shown in lane 1.

gel mobilities under different conditions, and in all cases we detected only single bands. This could be interpreted to mean that complexes can take many different static conformations, each with a different bend angle. However, we strongly prefer the alternative hypothesis that OccR has only a small number of possible conformations (probably two) and that intermediate migration rates are due to a dynamic equilibrium between these conformations during electrophoresis.

We performed two tests to ensure that the mobility shifts that we had detected were due to changes in DNA bending rather than to differences in the number of bound protein monomers. First, gel retardation assays were repeated with operator DNA containing the bend center near the end of the fragment rather than at the middle of the fragment. All the mutant OccR-DNA complexes migrated at the same rate in the presence or absence of octopine (data not shown). Therefore, the mutations altered the conformation of bound protein rather than the number of bound OccR monomers.

Our second test was to use DNase I footprinting to ensure that the fast migrating OccR-DNA complexes had the characteristic short footprint. We selected a representative double mutant, chosen because of its strong phenotype both in vivo and in bending assays. As previously seen, slow migrating OccR-DNA complexes have a DNase I footprint of ~60 nucleotides, whereas fast migrating complexes have a footprint of about 50 nucleotides (Fig. 5, lanes 2 and 3). The longer footprint contains several sensitive or hypersensitive bases among the protected bases, whereas the shorter footprint displays fewer of these sensitive sites. In contrast, the constitutive mutant showed the characteristic short footprint conformation in both the presence and absence of octopine (Fig. 5, lanes 4 and 5).

These results correlate precisely with those obtained with the DNA bending assays, which also showed no effect of octopine on this mutant. We conclude from these tests that the rapid gel mobility of OccR-DNA complexes indicates a low angle DNA bend and a short DNase I footprint.

We detected a rough quantitative correlation between the in vivo β-galactosidase activity of these mutants and their in vitro DNA bonding properties. To illustrate this relationship, the constitutive activities of the mutants in vivo (i.e. their activity in the absence of octopine) were plotted against the minimum concentration of octopine required to show a detectable shift in gel mobility (Fig. 6). First, all mutants required less octopine than the wild type to shift to a low angle DNA bend. Second, mutants requiring little or no octopine for a relaxation of the DNA bend in vitro tended to show high constitutive expression in vivo, whereas mutants requiring higher amounts of octopine to form low angle complexes in vitro tended to have lower expression levels in vivo. However, a few mutants did not fit this trend in that they had very strong constitutive activities in vivo but still required moderate amounts of octopine for relaxation of the DNA bend. For example, the double mutant P149S/ P214S and the single mutant S123F showed very high activities in vivo and yet required 1000 nM octopine to assume the low angle DNA bend.

**DISCUSSION**

The 13 constitutive single mutations and nine double mutations of OccR described in this study may represent the most complete set of constitutive mutants isolated for any member of this gene family. The fact that identical mutations were frequently isolated from independent mutant pools indicates that our selection may have identified nearly all possible constitutive OccR mutations. This is also the first report of any constitutive LysR-type mutant protein resulting from two synergistic mutations. It was somewhat surprising that mutations were isolated along the entire length of the protein. It was not possible to correlate the variations in activity with the location of the amino acid substitutions on the protein. However, OccR variants with double point mutations generally were considerably more active in vivo than the majority of those with single point mutations.
Constitutive OccR Mutants

The central hypothesis of this study is that the biochemical properties of constitutive OccR mutants should, in the presence or absence of inducer, resemble those attained by the wild-type protein only in the presence of inducer. Specifically, we hypothesized that constitutive OccR mutants would adopt low angle DNA bends in the absence of octopine, which represents the structure required for transcriptional activation of the occQ promoter. We identified a variety of phenotypes with regard to DNA bending. The majority of OccR variants with single point mutations displayed wild-type mobility in the absence of octopine but switched to the fast migrating complex at lower octopine concentrations than that required for the wild-type protein. The OccR proteins containing double point mutations had more dramatic effects on DNA bending. Most gave rise to fast migrating complexes in the absence of octopine, and the addition of inducer had little effect on this mobility. The majority of the mutations lie on a single line, indicating a correlation between the strength of constitutive activity and the propensity for a low angle DNA bend (Fig. 6). These data largely confirm our original hypothesis. However, a few mutants do not conform to this trend. Specifically, the single mutant S123F and the double mutants S123F/P214S and P149S/P214S have the highest in vitro activity of all the isolated mutants, and yet all three mutant proteins required considerable octopine concentrations to switch from a high angle DNA bend to a low angle bend. These mutants must therefore act by some mechanism other than shifting the conformation of OccR-DNA complexes.

Although there is a clear tendency for OccR constitutive mutants to favor the low angle DNA bend conformation at lower concentrations of octopine in vitro, the mutants do not appear to be sensitized to octopine levels in vivo. The reason for this apparent discrepancy could be that in vivo activation assays require the efficient uptake of octopine by Agrobacterium cells via the octopine permease. The lower concentrations of octopine used in these assays could well be below the $K_m$ value of this permease, thereby making the cells less sensitive to very low concentrations of octopine provided in the assay medium.

Inducer-responsive decreases in DNA bends have been observed for several other LysR-type proteins. In some cases, constitutive mutants exhibit a low angle DNA bend, as reported here. For example, OxyR(G253K), CysB(T149M), and CysB(Y164N) bind to their respective promoters with a short footprint, low angle DNA bend conformation that resembles the active conformation of the wild-type protein (9, 10, 13). How- ever, several other OxyR constitutive mutants bound DNA with extended footprints, similar to those seen with many of the single point OccR mutants. Kullik et al. (9) showed that these mutants were able to stimulate RNA polymerase binding in vitro even under reducing conditions. They suggest that the constitutive phenotype of the OxyR mutants may be due to the exposure of a domain that allows OxyR to recruit RNA polymerase in both oxidizing and reducing conditions. It is plausible that the OccR mutants that have high in vitro activity and yet have high angle DNA bends in vitro may activate transcription in a similar manner by recruiting RNA polymerase in both the presence and absence of octopine.

Our results suggest that DNA bending plays an important role in OccR-mediated transcription of the occQ operon. Elsewhere, we have shown that DNA bending alone is not sufficient for activation, because using mutant OccR binding sites that cause a conformation resembling the active conformation did not lead to octopine-independent promoter activity. Octopine must therefore cause OccR to undergo other conformational changes at the promoter that allow it to form optimal protein-protein contacts with RNA polymerase. Yet it is clear from the present study that a low angle DNA bend is strongly associated with transcription activation.

The position of the ligand binding site remains elusive for all LysR-type proteins. However, the CysB crystal structure included a molecule of sulfate buried in the cleft between domains I and II. This sulfate was thought to occupy the site normally occupied by the inducing ligand. In the case of OxyR, one of the redox-sensitive Cys residues, Cys199, lies in a similar position. As mentioned above, the ligand-binding domains of these proteins have a strong structural resemblance to the periplasmic histidine-binding protein and the lysine-arginine-ornithine-binding proteins, in which the ligands are also buried in the cleft between the two domains of these proteins. These proteins undergo large conformational changes when binding ligand such that the cleft closes. It is therefore plausible that octopine might bind OccR at a similar site and that the cleft between domains I and II may close upon octopine binding. If so, it is interesting that only two of the constitutive mutants (G148D and R202P) have mutations within this cleft.

The dimerization interface of OxyR consists of residues Gly91–Glu127 (which form a $\beta$-a-$\beta$ structure) of each protomer that contact residues His121–Gly124 (which form a $\beta$-a-structure) of the opposite protomer. Three mutations that alter residues within this dimerization interface of OxyR (1110D, H114Y, and A233V) have been isolated and shown to cause constitutive expression of the oxyS target gene, indicating the importance of these dimeric interactions in OxyR function (4).

The corresponding regions of OccR are Gly91–Gly126 and Ser114–Gly121 (gray boxes in Fig. 2). Interestingly, many of the constitutive OccR mutations lie in one of these regions, including A89T, F113L, L120F, S123F, P214S, and S215L, and it seems probable that these mutations alter this dimer interface and possibly weaken it. Most of the double mutants have at least one and sometimes both amino acid substitutions in the same region. It therefore seems plausible that octopine might act in the wild-type protein by weakening the interactions between protomers.

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