GcvR interacts with GcvA to inhibit activation of the _Escherichia coli_ glycine cleavage operon

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The _Escherichia coli_ glycine cleavage enzyme system, encoded by the _gcvTHP_ operon, catalyses the oxidative cleavage of glycine to CO$_2$, NH$_3$ and a one-carbon methylene group. Transcription of the _gcv_ operon is positively regulated by GcvA and negatively regulated by GcvA and GcvR. Using a LexA-based system for analysing protein heterodimerization, it is shown that GcvR interacts directly with GcvA in _vivo_ to repress _gcvTHP_ expression. Several mutations in either _gcvA_ or _gcvR_ that result in a loss of _gcv_ repression also result in a loss of GcvA-GcvR heterodimerization. Finally, it is shown that the C-terminal half of GcvA is involved in its interaction with GcvR, whilst the entire GcvR protein appears to be necessary for heterodimerization.

Keywords: _gcvTHP_, repressor, activator, repression, antiactivation

INTRODUCTION

The _Escherichia coli_ glycine cleavage enzyme system, encoded by the _gcvTHP_ ( _gcv_ ) operon, catalyses the oxidative cleavage of glycine to CO$_2$, NH$_3$ and a one-carbon methylene group. Transcription of the _gcv_ operon is positively regulated by GcvA and negatively regulated by GcvA and GcvR. Using a LexA-based system for analysing protein heterodimerization, it is shown that GcvR interacts directly with GcvA in _vivo_ to repress _gcvTHP_ expression. Several mutations in either _gcvA_ or _gcvR_ that result in a loss of _gcv_ repression also result in a loss of GcvA-GcvR heterodimerization. Finally, it is shown that the C-terminal half of GcvA is involved in its interaction with GcvR, whilst the entire GcvR protein appears to be necessary for heterodimerization.

The _Escherichia coli_ glycine cleavage enzyme system, encoded by the _gcvTHP_ ( _gcv_ ) operon, catalyses the oxidative cleavage of glycine to CO$_2$, NH$_3$ and a one-carbon methylene group. Transcription of the _gcv_ operon is positively regulated by GcvA and negatively regulated by GcvA and GcvR. Using a LexA-based system for analysing protein heterodimerization, it is shown that GcvR interacts directly with GcvA in _vivo_ to repress _gcvTHP_ expression. Several mutations in either _gcvA_ or _gcvR_ that result in a loss of _gcv_ repression also result in a loss of GcvA-GcvR heterodimerization. Finally, it is shown that the C-terminal half of GcvA is involved in its interaction with GcvR, whilst the entire GcvR protein appears to be necessary for heterodimerization.

METHODS

Bacterial strains and plasmids. All strains used in this study were constructed in our laboratory and are derivatives of the _E. coli_ K-12 strain GS162 [pheA905 tbi Δ(argF–lac)U169 araD129 rpsL150 relA1 deoC1 βlB5301 ptsF25 rbsR]. Ad-
ditional strain designations and mutations are as follows: GS1111, ΔgcvR bcp2neo; GS1118, ΔgcvA orf2ΣaadA; GS1131, ΔgcvR bcp2neo, ΔgcvA orf2ΣaadA. All lysogens denoted with igcvT::lacZ carry a single chromosomal copy of a gcvT::lacZ reporter fusion in phage λgt2 (Stauffer et al., 1993). The lysogen GS1131suλA::lacZ carries a single chromosomal copy of the sulA::lacZ reporter fusion from strain SU202 (Dmitrova et al., 1998).

The multi-copy plasmid used in the gcvR mutant screen was pBR322 (Bolivar et al., 1977). The low-copy-number plasmid used in this study was pSC101 (Cohen & Chang, 1977) and the single-copy plasmid used was pGS311 (Ghrist & Stauffer, 1998).

**Media and growth conditions.** The rich medium used was Luria–Bertani broth (LB) (Miller, 1992). The minimal media used were minimal salts (Vogel & Bonner, 1956) supplemented with phenylalanine (50 µg ml−1), thiamin (1 µg ml−1), and either 0.4% glucose (GM) or 0.4% lactose (LM). Agar was added at 1.5% to make solid media. Additional supplements were added, where indicated, at the following concentrations: inosine, 50 µg ml−1; glycine, 300 µg ml−1; phenylethyl-β-d-thiogalactoside (TPEG), 2 mM; ampicillin (AP), 150 µg ml−1 for multi-copy plasmids and 30 µg ml−1 for single-copy plasmids; tetracycline (TC), 10 µg ml−1. igcvT::lacZ lysogens carry the c1857 mutation resulting in a temperature-sensitive repressor and were grown at 30°C. All other strains were grown at 37°C.

**Construction of LexA fusion plasmids.** Wild-type and mutant alleles of gcvA were cloned into pMS604 (Dmitrova et al., 1998) on PCR-generated BstEII–XhoI fragments, creating in-frame fusions with the LexA DNA-binding domain (DBD). Wild-type and mutant gcvR alleles were cloned into pDP804 (Dimitrova et al., 1998) on PCR-generated BssHII–BglII fragments, creating in-frame fusions with the LexA408 DBD variant. The nucleotide sequence of each PCR-generated fragment was verified by DNA sequence analysis.

**β-Galactosidase assays.** β-Galactosidase assays were performed as described by Miller (1992). Each experiment was repeated at least twice, with each sample assayed in triplicate.

**RESULTS**

**GcvR interacts with GcvA in vivo**

To determine if GcvR interacts with GcvA in vivo, we used a LexA-based system for analysing protein heterodimerization developed for use in *E. coli* (Dmitrova et al., 1998). In this system, the reporter strain carries a isulA::lacZ fusion under the control of a hybrid LexA operator containing a wild-type half-site and a mutant half-site (Fig. 2). The wild-type half-site is recognized by the wild-type LexA DBD and the mutant half-site is recognized by the LexA408 DBD variant. Interaction between protein domains fused to these LexA DBDs will create LexA/LexA408 heterodimers capable of repressing the isulA::lacZ reporter (Fig. 2). For this experiment, gcvA was fused downstream of the wild-type LexA DBD sequence of pMS604 and gcvR was fused downstream of the LexA408 DBD sequence of pDP804. The expression of both fusion proteins is under the control of a lacUV5 promoter with a down mutation in the ribosome-binding site. Since the reporter strain GS1131suλA::lacZ carries a lacI deletion, both fusion proteins are constitutively expressed in this strain. The reporter strain transformed with one or both of these fusion plasmids was grown in LB (plus appropriate antibiotics) and β-galactosidase activity was determined. Expression of either fusion protein alone resulted in a small decrease in β-galactosidase activity (less than 30%) (Table 1). However, co-expression of both fusion proteins resulted in a significant decrease in β-galactosidase activity (Table 1, compare rows 1 and 10). When co-expressed with the GcvR fusion protein, there was still a significant reduction in β-galactosidase levels (Table 1, compare rows 1 and 11). These results indicate that GcvA and GcvR interact to form heterodimers in vivo.

**Isolation of gcvR mutants unable to properly repress the gcv operon**

To determine if gcvR mutants unable to properly repress gcv expression might also be defective in their ability to interact with GcvA, the following strategy was used to isolate gcvR mutants impaired in their ability to negatively regulate the gcv operon. The wild-type lysogen GS162suλA::lacZ, which carries a chromosomal gcvT::lacZ reporter fusion, was transformed with a multi-copy plasmid pool carrying PCR-induced random base pair changes in gcvR. Transformants were selected on LB+AP plates and then transferred to LM+AP plates supplemented with the purine nucleoside inosine and TPEG (an inhibitor of β-galactosidase); this medium results in maximal repression of
The gcvT::lacZ fusion and the inability to grow with lactose as the carbon source. Assuming that GcvR acts as a dimer (G. Heil, unpublished results), we reasoned that transformants expressing mutant GcvR proteins that could not interact with GcvA but that could still dimerize with wild-type GcvR and interfere with its ability to repress gcv expression would be able to grow on the LM plates due to increased β-galactosidase activity. Five transformants with this phenotype were isolated. Plasmid DNA was purified from each of these transformants and used to transform the ΔgcvR strain GS1111gcvT::lacZ. The resulting transformants were grown in GM supplemented with inosine and assayed for β-galactosidase activity. All five were found to have increased β-galactosidase activity as compared to GS1111gcvT::lacZ transformed with a multi-copy plasmid carrying the wild-type gcvR allele, indicating that each encoded a GcvR protein defective in negative regulation of the gcv operon (data not shown).

Each of the mutant gcvR alleles was sequenced and the following mutations were identified: gcvRA99–189 and gcvRA131–189 contain stop codons at positions 99 and 189, respectively; gcvRA106 contains a single nucleotide deletion (C) at codon 106, resulting in a reading frameshift and a stop codon at position 108, producing a protein that is 107 amino acids in size with two missense amino acids at its C-terminus; gcvRA442 contains a frameshift and a stop codon at position 144.

Table 1. Heterodimerization of GcvA and GcvR and their mutants

<table>
<thead>
<tr>
<th>gcvA allele</th>
<th>gcvR allele</th>
<th>β-Galactosidase activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>1133 ± 145</td>
</tr>
<tr>
<td>None</td>
<td>gcvR</td>
<td>992 ± 33</td>
</tr>
<tr>
<td>gcvA</td>
<td>None</td>
<td>930 ± 5</td>
</tr>
<tr>
<td>gcvA</td>
<td>gcvR</td>
<td>109 ± 14</td>
</tr>
<tr>
<td>gcvA</td>
<td>gcvR129H</td>
<td>548 ± 3</td>
</tr>
<tr>
<td>gcvA</td>
<td>gcvRQ144P</td>
<td>484 ± 20</td>
</tr>
<tr>
<td>gcvAC169R</td>
<td>gcvR</td>
<td>286 ± 46</td>
</tr>
<tr>
<td>gcvAR197G</td>
<td>gcvR</td>
<td>493 ± 23</td>
</tr>
<tr>
<td>gcvAA292–305</td>
<td>gcvR</td>
<td>687 ± 16</td>
</tr>
<tr>
<td>gcvAR§</td>
<td>None</td>
<td>1157 ± 71</td>
</tr>
<tr>
<td>gcvR§</td>
<td>gcvR</td>
<td>176 ± 40</td>
</tr>
</tbody>
</table>

*gcva and its mutant alleles were fused in-frame to the lexA region of pMS604 (or pSC101§).†gcva and its mutant alleles were fused in-frame to the lexA region of pDP804.‡GS1131lexA::lacZ transformants were grown in LB plus appropriate antibiotics and assayed for β-galactosidase activity. Activity is expressed in Miller units (Miller, 1992).

Table 2. gcvA and gcvR mutations and their effects on gcvT::lacZ expression

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Plasmid-encoded gcvA or gcvR allele†</th>
<th>β-Galactosidase activity‡ in cells grown in§</th>
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</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>None</td>
<td>233 1395 22</td>
</tr>
<tr>
<td>ΔgcvR</td>
<td>None</td>
<td>1756 2122 1209</td>
</tr>
<tr>
<td>ΔgcvR gcvRA131–189</td>
<td></td>
<td>2041 2448 1340</td>
</tr>
<tr>
<td>ΔgcvR gcvRA106</td>
<td></td>
<td>2070 2561 1435</td>
</tr>
<tr>
<td>ΔgcvR gcvRA99–189</td>
<td></td>
<td>1863 2060 1111</td>
</tr>
<tr>
<td>ΔgcvR gcvRR129H</td>
<td></td>
<td>1156 1891 715</td>
</tr>
<tr>
<td>ΔgcvR gcvRQ144P</td>
<td></td>
<td>2011 1767 1362</td>
</tr>
<tr>
<td>ΔgcvR gcvAC169R</td>
<td></td>
<td>529 1006 261</td>
</tr>
<tr>
<td>ΔgcvR gcvAR197G</td>
<td></td>
<td>1522 2373 1066</td>
</tr>
<tr>
<td>ΔgcvR gcvAA292–305</td>
<td></td>
<td>1413 2026 1070</td>
</tr>
</tbody>
</table>

*Transformants of GS1625gcvT::lacZ (wild-type), GS1111- gcvT::lacZ (ΔgcvR) or GS1118gcvT::lacZ (ΔgcvA).†All mutant alleles were expressed from the single-copy plasmid pGS311.‡Activity is expressed in Miller units (Miller, 1992). All assays were repeated at least twice. Data from a representative experiment are shown.§Transformants were grown in GM + AP with supplements as indicated and assayed for β-galactosidase activity.
also led to a loss of repression, but the phenotype was less severe. It should be noted that the 1:5–2-fold repression observed in GM + inosine medium is due to the PurR repressor, which has been shown to repress transcription of the gcv operon independently of GcvA/GcvR-mediated repression (Wilson et al., 1993a).

### GevR mutants unable to repress gcv expression are impaired in their ability to interact with GcvA

If GcvA interacts directly with GcvR to regulate the gcv operon, one possible explanation for the loss of repression displayed by the gcvR mutants described above is that they are unable to interact with GcvA. Therefore, the gcvRQ144P and gcvRR129H alleles were cloned into pDP804 and tested for their ability to interact with GcvA using the protein heterodimerization system. Both mutant alleles were impaired in their ability to interact with GcvA, displaying an approximately 4.5–5.5-fold increase in expression of the isulA::lacZ reporter fusion as compared to the wild-type gcvR allele (Table 1, compare row 4 with rows 5 and 6).

### Deletion analysis of gcvR

To define the region(s) of GcvR involved in its interaction with GcvA, a 3′ deletion (gcvRA143–189) and a 5′ deletion (gcvRA1–35) of gcvR (Fig. 3a) were cloned into pDP804 and tested for their ability to interact with GcvA using the LexA heterodimerization assay. Since all of the gcvR mutations described above were located in the 3′ half of gcvR, it was not surprising that deletion of the C-terminal 47 residues of GcvR, which includes the glutamine at position 144, resulted in a loss of interaction with GcvA (Table 3, row 4). It was somewhat surprising, however, that deletion of the N-terminal 35 residues also resulted in a loss of interaction with GcvA (Table 3, row 5).

### Isolation of GcvA mutants refractory to inhibition by GcvR

In a previous study, two gcvA mutants that are able to activate but not repress gcv expression were isolated but not characterized (Jourdan & Stauffer, 1998). In that same study, a deletion of the sequence encoding the C-terminal 14 amino acids of GcvA (ΔgcvA944; renamed gcvAΔ292–305 in this study) (Fig. 3b) was constructed and shown to have the same phenotype. During this study, the uncharacterized gcvA mutant alleles were sequenced and the mutations were identified as a cysteine to arginine change at position 169 (GcvAR169R) and an arginine to glycine change at position 197 (gcvAR197G) (Fig. 3b). To measure the effects of these mutations on gcv expression, they were cloned into the single-copy plasmid pGS311 and the resulting constructs were used to transform the ΔgcvA reporter strain GS1118ΔsulA::lacZ. Transformants were grown in GM, GM + glycine and GM + inosine media and assayed for β-galactosidase activity. As expected, the ΔgcvA control lysogen showed no significant change in β-galactosidase activity under any growth condition, except for the twofold PurR effect (Table 2). The gcvAR197G and gcvAΔ292–305 mutations resulted in a complete loss of gcv repression, producing levels of β-
galactosidase activity similar to that observed in the ΔgcvR control (Table 2, rows 10 and 11). The gcvAC169R mutation also led to a loss of gcv repression, although the phenotype was not as severe (Table 2, row 9).

GcvA mutants unable to repress gcv expression are impaired in their ability to interact with GcvR

To test whether the gcvA mutants described above are defective in their ability to interact with GcvR, each mutant allele was cloned into pMS604 and its interaction with GcvR was measured using the LexA heterodimerization system. The gcvAC169R mutation resulted in a small loss of interaction with GcvR, whilst the gcvAR197G and gcvAA292–305 mutations resulted in more severe losses of interaction with GcvR (Table 1, compare row 4 with rows 7, 8 and 9). These results are consistent with the effects of the mutations on repression of the gcv operon.

The C-terminal half of GcvA is able to interact with GcvR

To define the region(s) of GcvA that is involved in its interaction with GcvR, several 5’ and 3’ deletions of gcvA (gcvAA1–151, gcvAA1–230 and gcvAA1–151·∆231–305) (Fig. 3b) were cloned into pMS604 and tested for their ability to interact with GcvR using the protein heterodimerization system. Deletion of the N-terminal 151 amino acids of GcvA had no effect on its ability to interact with GcvR (Table 3, row 6). However, deletion of the N-terminal 230 amino acids, which deletes the cysteine at position 169 and the arginine at position 197, or deletion of amino acids 1–151 and 231–305, resulted in significant loss of interaction with GcvR (Table 3, rows 7 and 8). These results suggest that the GcvA/GcvR interaction requires the C-terminal half of GcvA.

The C-terminal half of GcvA interferes with inhibition of wild-type GcvA by GcvR

Sequence and mutational analysis indicate that the N-terminal half of GcvA is involved in DNA binding and activation (Wilson & Stauffer, 1994; Jourdan & Stauffer, 1998) and results from this study suggest that the C-terminal half of GcvA is involved in an interaction with GcvR. To determine if overexpression of the C-terminal half of GcvA would lead to activation of the gcv operon by interfering with the ability of GcvR to inhibit wild-type GcvA, the wild-type strain GS162::gcvT::lacZ and the ΔgcvA strain GS1118::gcvT::lacZ were transformed with plasmids carrying the lexA–gcvAA1–151 and lexA–gcvAA1–230 alleles used in the heterodimerization experiment. Transformants were grown in GM, GM+glycine and GM+inosine media and assayed for β-galactosidase activity. Transformation of the ΔgcvA strain GS1118::gcvT::lacZ with either plasmid resulted in low non-inducible gcv expression, identical to that observed in the untransformed control (data not shown). Thus, neither LexA–ΔGcvA protein fusion is able to activate the gcvT::lacZ fusion. Transformation of wild-type strain GS162::gcvT::lacZ resulted in normal regulation of gcv expression in the case of the lexA–gcvAA1–230 allele, whereas transformation with the lexA–gcvAA1–151 allele led to constitutive activation in all three media (Table 4).

**DISCUSSION**

In this report, we present evidence to support a model in which GcvR interacts with GcvA to prevent activation of the gcv operon. Using a LexA-based system for measuring protein heterodimerization, we were able to show that GcvA and GcvR interact in vivo. In addition, mutations in both gcvA and gcvR which result in a loss of gcv repression also result in a loss of GcvA/GcvR interaction. Thus, there is a strong correlation between the ability of these proteins to form heterodimers and their ability to repress expression of the gcv operon.

Three lines of evidence suggest that the C-terminal half of GcvA is involved in heterodimerization with GcvR. First, substitution mutants of GcvA which result in a loss of interaction with GcvR lie in the C-terminal half of GcvA. Second, deletion of the N-terminal half of GcvA has no effect on its ability to interact with GcvR, whereas deletion of the C-terminal 14 amino acids results in a complete loss of heterodimerization. Finally, although unable to activate gcv transcription, the C-terminal half of GcvA is able to interfere with GcvR-mediated repression of wild-type GcvA, probably by titrating the available antiantiactor, leading to constitutive activation of the operon (Table 4). Together, these results suggest that the C-terminal half of GcvA is involved in an interaction with GcvR.

All of the gcvR mutations identified in this study that result in a decrease in repression of the gcv operon as

<table>
<thead>
<tr>
<th>gcvA allele*</th>
<th>β-Galactosidase activity† in cells grown in‡</th>
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<tbody>
<tr>
<td></td>
<td>GM</td>
</tr>
<tr>
<td>None</td>
<td>135</td>
</tr>
<tr>
<td>gcvAA1–151</td>
<td>847</td>
</tr>
<tr>
<td>gcvAA1–230</td>
<td>165</td>
</tr>
</tbody>
</table>

* gcvA deletion alleles were fused to the lexA408 region of plasmid pMS604.
† Activity is expressed in Miller units (Miller, 1992). All assays were repeated at least twice. Data from a representative experiment are shown.
‡ GS162::gcvT::lacZ transformants were grown in GM+TC medium with supplements as indicated and assayed for β-galactosidase activity.
well as a decrease in heterodimerization lie in the C-terminal half of the protein, suggesting that this region is involved in an interaction with GcvA. Consistent with these results, a deletion of the C-terminal 47 amino acids resulted in a loss of interaction with GcvA (Table 3). However, deletion of the N-terminal 35 amino acids also resulted in a loss of heterodimerization (Table 3). Since our screen relied on the ability of mutant GcvR to interact with wild-type GcvR, it is possible that mutations in the N-terminal region that would have resulted in a loss of GcvR interaction with GcvA were excluded from the screen as the mutations might have also resulted in a loss of GcvR homodimerization. It is also possible that deletion of this region of gcvR results in a decrease in expression or protein stability.

While it is clear that GcvR interacts with GcvA to repress the gcv operon, it is not clear how this interaction is modified by glycine and purines and, in turn, how this modification leads to the correct regulation of gcv expression. In previous studies, we have shown that neither glycine nor purines have an effect on the expression of gcvA or gcvR (Wilson & Stauffer, 1994; Ghrist & Stauffer, 1998). These results led us to propose a model in which GcvA homocomplexes function as activators, whilst GcvA/GcvR heterocomplexes function as repressors. In this model, the coeffectors influence the type of complex formed; glycine promotes the formation of activation complexes, whilst purines promote the formation of repression complexes. Increasing the expression of GcvA or GcvR would force the formation of activation or repression complexes, respectively. We used the LexA-based heterodimerization system to measure the effects of glycine and inosine on the ability of GcvA and GcvR to interact by growing transformants in GM, GM + glycine and GM + inosine media prior to assaying for β-galactosidase activity. However, glycine and inosine had no significant effect on the GcvA/GcvR interaction (data not shown). This may be due to the high level of expression of both fusion proteins in this assay and in vitro studies with purified GcvA and GcvR will be needed to provide further data to prove or disprove this model.

Regulation of the gcv operon by GcvA and GcvR is part of a growing number of prokaryotic regulatory systems involving an activator/antiactivator complex, including TraR/TraM regulation of conjugal transfer of the Ti plasmid in Agrobacterium tumefaciens (Hwang et al., 1999), NifA/NifL regulation of nitrogen fixation in Klebsiella pneumoniae (Narberhaus et al., 1995), ComK/MecA/ClpC regulation of competence in Bacillus subtilis (Turgay et al., 1998) and CRP/CytR regulation of operons encoding the nucleoside transport and biosynthesis enzymes (Valentin-Hansen et al., 1996). In the case of TraR/TraM, NifA/NifL and ComK/MecA/ClpC, the activator is sequestered by the antiactivator(s), thus preventing binding and activation. In the case of CRP/CytR, the antiactivator binds DNA between activator-binding sites, forming a nucleo-protein complex which prevents activation (Valentin-Hansen et al., 1996). Evidence suggests that GcvR could act in both ways to negatively regulate gcv expression. As discussed above, the C-terminal half of GcvA, deleted for the DNA-binding region (Jourdan & Stauffer, 1998), prevents GcvR-mediated repression. This suggests that GcvR may function by sequestering GcvA in an inactive form. On the other hand, overexpressing GcvR in a gcvA+ strain results in a lower level of gcv expression than that observed in a gcvA mutant (Ghrist & Stauffer, 1995). In addition, GcvA/GcvR, in the presence of inosine, repress a gcvT::lacZ fusion four- to fivefold in a GcvA-binding site 1-dependent manner (Wonderling et al., 2000). These results suggest that GcvA and GcvR act together at the gcv control region to directly repress gcv expression.

ACKNOWLEDGEMENTS
We thank Michele Granger for generously providing pMS604, pDP804 and SU202.

REFERENCES


Received 29 December 2000; revised 23 March 2001; accepted 23 April 2001.