Glycine binds the transcriptional accessory protein GcvR to disrupt a GcvA/GcvR interaction and allow GcvA-mediated activation of the *Escherichia coli* gcvTHP operon

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**INTRODUCTION**

In *Escherichia coli*, the primary source of glycine and one-carbon (C1) units is through the serine hydroxymethyltransferase reaction (*glyA* gene product) (Mudd & Cantoni, 1964). A secondary pathway for the production of C1 units is the glycine cleavage (GCV) enzyme system, which catalyses the oxidative cleavage of glycine into CO$_2$, NH$_3$ and a C1 unit (Kikuchi, 1973). The glycine and C1 units generated by these reactions are not end products, but serve as substrates in other metabolic pathways. Glycine, in addition to its role in protein synthesis, is used as a substrate in the biosynthesis of purines and cell walls (Stauffer, 1996; Blakely, 1969; Jordan & Sheman, 1972; Pizer & Potochny, 1964), and C1 units are used in the biosynthesis of purines, thymidine, histidine, coenzyme A and methionine (Matthews, 1996). Methionine, in turn, is converted to S-adenosylmethionine, which serves as a methyl donor for the methylation of proteins and nucleic acids (Mudd & Cantoni, 1964; Marinus, 1996). The current hypothesis for the physiological role of the GCV enzyme system in *E. coli* is to balance the requirements of the cell for glycine and C1 units used in these various biosynthetic and methylation reactions.

The GCV enzyme system consists of four proteins designated T-protein, H-protein, P-protein and L-protein (Kikuchi, 1973). The *gcvT*, *gcvH* and *gcvP* genes, encoding the T-, H- and P-proteins, respectively, form an operon at 65–7 min on the *E. coli* chromosome (Plamann et al., 1983; Stauffer et al., 1993, 1994). The *lpd*-encoded lipoamide dehydrogenase, common to the pyruvate and 2-oxoglutarate dehydrogenase enzyme complexes, functions as the L-protein (Steiert et al., 1990). The *lpd* gene maps at 2–8 min (Berlyn et al., 1996). Transcription of the *gcv* operon is under the control of three global regulatory proteins [Lrp, PurR and cAMP-receptor protein (CRP)] and two *gcv*-specific transcriptional regulatory proteins (GcvA and GcvR) (Ghrist & Stauffer, 1995; Lin et al., 1992; Stauffer & Stauffer, 1994; Wilson et al., 1993a; Wilson & Stauffer, 1994; Wonderling & Stauffer, 1999). Together these
proteins modulate transcription of the operon in response to the levels of glycine and purines within the cell.

The leucine-responsive protein (Lrp), a global regulator involved in the control of transcription of several genes involved in amino acid metabolism (Calvo & Matthews, 1994), binds to multiple sites in the gcv control region from bp −229 to −92 relative to the transcriptional start site (Fig. 1) (Stauffer & Stauffer, 1994). Lrp’s role in gcv regulation appears to be structural, binding and bending the DNA to facilitate the formation of the appropriate regulatory complexes for activation or repression of the operon (Stauffer & Stauffer, 1999), although the possibility that Lrp also activates transcription through interactions with RNA polymerase (RNAP) or a second regulatory protein has not been completely ruled out.

PurR, a repressor for numerous genes involved in nucleotide metabolism (Kilstrup et al., 1989; Rolfes & Zalkin, 1988), mediates a twofold decrease in gcv transcription in the presence of exogenous purines (Stauffer et al., 1994; Wilson et al., 1993a). PurR binds to the gcv control region from bp −3 to +17 relative to the transcription start site (Fig. 1) and likely interferes with RNAP binding to the gcv promoter (Wilson et al., 1993a).

CRP binds to a site from approximately bp −303 to −324 relative to the transcription start site (Fig. 1) and is required for a fourfold activation of the gcv operon in glucose minimal (GM) medium (Wonderling & Stauffer, 1999). The specific mechanism for the observed effect of CRP is unknown. However, the dependence of the CRP effect on the repressor function of GcvA suggests that its role is to antagonize GcvA-mediated repression.

GcvA, a member of the LysR family of transcriptional regulatory proteins, functions as an activator of the gcv operon in the presence of exogenous glycine and as a repressor of the operon in the absence of glycine (Wilson et al., 1993a, b). GcvA-mediated repression is enhanced by the presence of exogenous purines. GcvA binds to three sites in the gcv control region: site 3 from bp −271 to −242, site 2 from bp −242 to −214 and site 1 from bp −69 to −34 (Fig. 1). All three of these sites are required for repression of the operon, while only sites 2 and 3 appear to be necessary for activation (Wilson et al., 1995; Wonderling et al., 2000). In addition, DNase I footprint studies with GcvA showed hypersensitive cleavage sites in the region between the upstream sites 3+2 and the downstream site 1 when GcvA was bound to these sites. These results suggest that GcvA might also be involved in bending DNA to form appropriate nucleoprotein complexes at the gcv promoter.

GcvR is necessary for repression of the gcv operon (Ghrist & Stauffer, 1995). In the absence of GcvR, there is constitutive expression of a gcvT::lacZ fusion, and overproduction of GcvR results in super-repression of the fusion. Recently, we showed that GcvA and GcvR interact in vivo using a LexA-based two-hybrid system (Ghrist et al., 2001). Since GcvR has no repressor capabilities in the absence of GcvA (Ghrist & Stauffer, 1995), the results suggest that a GcvR/GcvA interaction might be required for repression. Furthermore, since mutations in any of the three GcvA binding sites result in reduced repression (Wilson et al., 1995; Wonderling et al., 2000), at least part of the repression response of the gcv operon likely requires that a GcvR/GcvA repressor complex be bound to gcv DNA.

Most LysR-type transcriptional regulators (LTTRs) are believed to directly bind their specific co-inducer molecules, although in most cases this binding has only been indirectly demonstrated through the isolation of mutants that either no longer respond to their respective co-inducer or have altered co-inducer specificity (Colyer & Kredich, 1996; Jørgensen & Dandanell, 1999; Schell, 1993). Co-inducer binding by LTTRs is manifested in several ways including altered DNA binding and DNA bending, which facilitate LTTR activation or repression of transcription (Gao & Gussin, 1991; Wek & Hatfield, 1988; van Keulen et al., 1998; Ogawa et al., 1999; Hryniwicz & Kredich, 1994; Wang et al., 1992). How glycine and purines affect GcvA and GcvR to allow an appropriate response at the gcv promoter is unknown. In the present study, we determined the likely role glycine plays as a co-inducer in transcriptional control of the gcv operon. The results suggest that GcvA/GcvR-mediated regulation of gcv utilizes a mechanism that may be unique among LTTR regulated operons.

**METHODS**

**Strains, plasmids and media.** Genotypes of strains and plasmids used are listed in Table 1. The defined medium used was Vogel & Bonner minimal salts (Vogel & Bonner, 1956) supplemented with 0.4% glucose (GM). GM medium was supplemented with phenylalanine and thiamin since most strains used carry the pheA905 and thi mutations. Luria-Bertani broth (LB) and Terrific Broth used for the culturing and overexpression of fusion proteins for purification were
### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
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<td>Stratagene</td>
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<td>Wilson et al. (1995)</td>
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<td>pGS498</td>
<td>pET-3d; T7 promoter-controlled gcvA&lt;sub&gt;Δhis&lt;/sub&gt; allele</td>
<td>This study</td>
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<td>*These strains also carry thi, pheA905, lacU169, araD129 and rpsL150 mutations.† KN, kanamycin.‡ SP, spectinomycin.</td>
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Prepared as described by Sambrook et al. (1989). Supplements were added where indicated at the following concentrations: ampicillin (AP), 200 μg ml<sup>-1</sup> for the maintenance of multi-copy expression vectors and 50 μg ml<sup>-1</sup> for the maintenance of single-copy plasmids; tetracycline, 10 μg ml<sup>-1</sup>; inosine, 50 μg ml<sup>-1</sup>; X-gal, 40 μg ml<sup>-1</sup>; phenylethyl-β-D-thiogalactoside (TPEG), 2 mM; IPTG, 600 μM.

**Enzyme assays.** β-Galactosidase assays were performed on mid-exponential phase cells (OD<sub>600</sub> ~ 0.5) as described by Miller (1992) using the chloroform/SDS lysis procedure. All results are the means of two or more assays, with each sample done in triplicate. Standard deviations for each data point were within 15% of the mean.

**Protein purification.** Plasmid pGS473 carries gcvA on an EcoRI–HindIII fragment (Jourdan & Stauffer, 1999). In this construct gcvA is under transcriptional control of the inducible lac promoter, contains an artificial Shine–Dalgarno sequence and has six histidine codons at the 3’ end of the gcvA ORF. pGS473, however, did not express the hexa-histidine-tagged GcvA (GcvA<sub>Δhis</sub>) to levels sufficient for use in this work. To increase levels of GcvA<sub>Δhis</sub> we constructed pGS498 as follows. pGS473 was digested with EcoRI, the ends filled using the large fragment of DNA polymerase I and XhoI linkers added. The resulting fragment was then digested with XhoI/HindIII, the fragment gel-purified and ligated into the corresponding sites in the pET-3d vector (New England Biolabs), placing the gcvA<sub>Δhis</sub> allele under transcriptional control of the T7 promoter. Since GcvA and GcvR interact in vivo (Ghrist et al., 2001), pGS498 was used to transform the ΔgcvR::KN<sup>+</sup> strain GS1128 on LB agar + AP, and an AP<sup>+</sup> transformant isolated. Expression of GcvA<sub>Δhis</sub> in strain GS1128 prevents the possible co-purification of GcvA<sub>Δhis</sub> and WT GcvR. The AP<sup>+</sup> transformant was used to inoculate 3 ml Terrific Broth + AP and the culture was incubated at 37 °C for 3 h. To prevent the accumulation of β-lactamase in the growth medium that could result in the loss of the expression plasmid from cells and lower protein expression, cells from the 3 ml culture were harvested by centrifugation and used to inoculate 1 l Terrific Broth + AP. The culture was grown at 30 °C to OD<sub>600</sub> between 1.2 and 1.4. Expression of the T7 RNAP was induced by the addition of IPTG, resulting in transcription of gcvA<sub>Δhis</sub>. Induced cultures were then incubated at 24 °C for 7 h, the cells harvested by centrifugation, resuspended in loading buffer A (50 mM HEPES/NaOH pH 7.4, 500 mM NaCl, 1 mM EDTA, 0-1% Triton X-100) and sonicated. The lysate was clarified by...
centrifuging at 12000 r.p.m. in a Sorvall SS-34 rotor for 30 min at 4°C, followed by centrifugation at 40000 r.p.m. in a Beckman 60Ti rotor for 1 h at 4°C. Solid ammonium sulfate was added to 33% saturation and incubated further at 4°C for 2 h. The precipitate was recovered by centrifugation, dissolved in 5 ml loading buffer A and loaded onto a 5 ml Hi Trap metal chelating column (Pharmacia Biotech) pre-equilibrated with loading buffer A. The column was washed with 20 vols loading buffer A followed by 10 column vols wash buffer A (50 mM HEPES, 20 vols loading buffer A followed by 10 column vols wash buffer A (50 mM HEPES, pH 7.4, 500 mM NaCl, 0.1 mM EDTA, 10% glycerol, 20 mM imidazole). GcvA

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agar

GcvR and WT GcvA, and transformants selected on LB

plates containing Xba

fragments. M, MuI; X, Xhol; E, EcoRV; S, StuI; B, BamHI; XbaI, XbaI. The centre of each fragment is indicated with an arrow. Only relevant restriction enzyme sites are shown.

Impact I protein purification system (New England Biolabs) as follows. Plasmid pGS318 carries an in-frame gcrT::lacZ fusion (Stauffer & Stauffer, 1998a). Plasmids pGS420 and pGS428 were derived from pGS318 and carry a 15 bp and a 25 bp insert between GcvA binding site 3+2 and site 1, respectively (Stauffer & Stauffer, 1998b). Plasmid pGS357 has a 4 bp change in site 1 that reduces GcvA binding to site 1 about twofold (Wilson et al., 1995; Wonderling et al., 2000). These plasmids were used as the starting templates in PCR reactions with primers containing XbaI sites and sequences complementary to gcr upstream and downstream the GcvA binding sites. The resulting 244, 259 and 264 bp DNA fragments generated from pGS318, pGS420 and pGS428, respectively, carry GcvA binding sites 3+2+1, and were cloned into the XbaI site of plasmid pBend2. Five different restriction enzymes were then used to generate DNA fragments of identical length (365 bp), but with different locations of the GcvA binding sites (Fig. 2). The fragments were 32P-labelled at their 5' termini with T4 polynucleotide kinase (Sambrook et al., 1989). Target DNA was added to 20 μl reaction mixtures containing DNA binding buffer (10 mM Tris/HCl pH 7.5, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM DTT) plus 125 μgBSA ml−1. Reaction mixtures were pre-incubated for 5 min at 37°C, 2 μl purified GcvA

was added at the concentrations indicated in Figs 3 and 5, and incubation continued for 15 min. One microlitre of loading dye (0.1% xylene cyanol and 50% glycerol in water) was added to each reaction mixture and the samples were loaded onto a 5% polyacrylamide gel and run at 12 V cm−1. Gels were transferred to 3MM Whatman paper, dried and autoradiographed. DNA bending by GcvA

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Co-precipitation (co-ppt) experiments. The following buffers were used in the GcvA/GcvR affinity co-ppt experiments: loading buffer C (20 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol), wash buffer B (loading

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Equilibrium dialysis. Equilibrium dialysis was conducted using a micro-chamber equilibrium dialysis apparatus with pairs of 100 µl chambers separated by a 12000–14000 molecular mass cut off Spectrapor dialysis membrane (Spectrum Medical Industries). Each protein tested was diluted to 25 µM in dialysis buffer (20 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol) and placed in chambers (inside) opposite chambers containing [14C]glycine (~16 500 c.p.m.) in dialysis buffer (outside). Following overnight mixing of the dialysis cells at 20 °C, 50 µl samples from each chamber on opposite sides of the membrane were collected. The amount of [14C]glycine in each of the samples was determined by counting the samples in 10 ml Budget-Solve scintillation cocktail (Research Products International). The amount of [14C]glycine bound was determined by subtracting the counts ‘outside’ from the counts ‘inside’ each pair of chambers (Miller, 1992).

Random mutagenesis. Mutagenesis of gcvR was performed using a PCR mutagenesis protocol (Zhou et al., 1991). PCR products were generated using primers complementary to the DNA template outside of the target region and overclamping on the EcoRI restriction site at the 5′ end and a HindIII site at the 3′ end. The PCR products were digested with EcoRI/HindIII, cloned into plasmid pBR322 and used to transform the gcvA mutant lysogen GS998 using a PCR mutagenesis protocol (Zhou et al., 1991). The amount of [14C]glycine bound was determined by subtracting the counts ‘outside’ from the counts ‘inside’ each pair of chambers (Miller, 1992).

RESULTS

Effects of glycine on GcvA binding to the gcv control region and bending DNA

Most LTTRs have been shown to bind to their promoters in the absence of their respective co-inducers (Schell, 1993). The presence of the co-inducer molecule usually results in either an altered DNA binding affinity of the LTTR, observed as an increase in the number of protein monomers bound to a specific site, or altered DNA bending of the promoter region by the LTTR (Gao & Gussin, 1991; Wek & Hatfield, 1988; Ogawa et al., 1999; Rhee et al., 1998; van Keulen et al., 1998). These effects result in appropriate transcriptional regulation of the respective operons. Previous DNase I protection studies using crude extracts containing over-expressed GcvA did not show alterations in the degree of protection or in the pattern of protection of the gcv control region upon the addition of glycine (Wilson et al., 1995), and previous GMS experiments were con-

buffer C + 20 mM imidazole, pH adjusted to 7.4 after addition of imidazole) and elution buffer (loading buffer C + 500 mM imidazole, pH adjusted to 7.4 after addition of imidazole).

Glycine was added to the loading and wash buffers where indicated at 1 mM.

Ni²⁺-NTA agarose affinity matrix (Qiagen) was dispensed into 30 µl aliquots for each co-ppt and washed twice with 400 µl loading buffer C to equilibrate, each time briefly centrifuging and decanting the supernatant with a Pasteur pipette. GcvA<sub>A<sub>4</sub></sub> (‘bait’) was diluted to 50 µg ml⁻¹ in 200 µl loading buffer C, added to 30 µl affinity matrix and incubated with gentle mixing at 20 °C for 15 min. The affinity matrix was collected by centrifugation as above and the supernatant saved as sample 1. The Ni²⁺-NTA agarose was washed twice with 200 µl loading buffer C to remove unbound protein. Following each of the washing of the Ni²⁺-NTA agarose was centrifuged, and the supernatants decanted and saved as samples 2 and 3. GcvR (‘target’) was diluted to 50 µg ml⁻¹ in 200 µl loading buffer C and added to the Ni²⁺-NTA agarose–GcvA<sub>A<sub>4</sub></sub> complex and incubation continued at 20 °C for 15 min. The affinity matrix was collected by centrifugation and the supernatant saved as sample 4. The matrix was then washed twice with 200 µl loading buffer C to remove unbound target protein. Following each of the washes the Ni²⁺-NTA agarose was centrifuged, the supernatants decanted and saved as samples 5 and 6. The Ni²⁺-NTA agarose–protein complex was washed twice with 200 µl wash buffer B to remove target protein non-specifically bound to the matrix. Each wash was incubated for 5 min at 20 °C with gentle mixing, the mixtures centrifuged, and the supernatants collected as samples 7 and 8. The Ni²⁺-NTA agarose–protein complex was then washed twice with 200 µl elution buffer B. Each wash was incubated for 5 min at 20 °C with gentle mixing, the Ni²⁺-NTA agarose was collected by centrifugation, and the supernatants collected and combined as sample 9. Four volumes of acetone were added to each sample, the proteins precipitated on ice for 30 min, and pelleted by centrifugation at 14000 r.p.m. [16000 g in a refrigerated (4 °C) Eppendorf microfuge]. The supernatants were decanted and the proteins dried under vacuum. The protein samples were resuspended in SDS loading buffer, heated at 95 °C for 5 min and loaded onto a 10% polyacrylamide/SDS gel.
GcvA is shown as a circle, although biophysical experiments show it as a tetramer in solution (G. Heil, G. V. Stauffer and A. Robertson, unpublished). A 25 bp insertion is indicated as a thick bar. 1. Positions of the three GcvA binding sites. 2. Binding of GcvA to sites 3→2 results in about a 100° bend in the DNA. 3. Binding of GcvA to sites 3→2+1 results in an apparent 12° bend, possibly due to a GcvA-DNA looped complex whose mobility is relatively independent of the location of the GcvA sites. 4. Binding of GcvA to sites 3→2+1 with a 4 bp change (AGTT) in site 1 away from consensus results in about an 80° bend, possibly due to an inability of GcvA to bind DNA at site 1 to allow formation of a looped complex. 5. Binding of GcvA to sites 3→2+1 with an insertion of 25 turns of DNA in the intervening region between sites 3→2 and site 1 results in a 25° bend, possibly due to phasing the bend at sites 3→2 and the bend at site 1 in opposite orientations such that the complex migrates as an apparent linear DNA–protein complex.

GcvA is shown as a circle, although biophysical experiments show it as a tetramer in solution (Jourdan & Stauffer, 1999; Wonderling et al., 2000; Wilson et al., 1995). Since glycine acts as the co-inducer with this LTTR controlled system, effects of the small molecule on the ability of GcvA to bind its specific sites in either the gcv or gcvA control region, or to mediate bending of the DNA when bound to these sites could not be examined under the assay conditions used. To determine whether glycine affects either of these properties of GcvA, GMS assays were done with purified GcvA in a non-glycine-based buffer.

Initially we tested whether GcvA binding alone induces a bend in DNA. DNA fragments carrying GcvA binding sites 3→2+1 were cloned into the pBend2 vector (see Methods), and five different restriction enzymes were used to generate DNA fragments of the same length, but with the GcvA binding sites at different positions relative to the centre of the fragments (Fig. 2). Gel electrophoresis of the unbound fragments showed no significant intrinsic DNA bending (Fig. 3). The addition of GcvA resulted in three shifted bands. Although we did not perform DNase I footprinting on the shifted complexes, previous studies showed that the three GcvA binding sites have affinities for GcvA in the order 2→3→1 (Wilson et al., 1995). The different positions of the GcvA binding sites within the equal length fragments resulted in different relative mobilities of each of the three GcvA/DNA complexes (Fig. 3). The apparent bend angle was calculated for the gcv control region using the formula μM/μE = cosα/2, where μM is the mobility of the DNA with the protein bound near the centre of the fragment and μE is the mobility of the DNA fragment with the binding site located near the end of the DNA (Kim et al., 1989). When GcvA was bound to site 2 (band A in Fig. 3), a bend angle of about 30° was calculated, when bound to sites 2→3 (band B in Fig. 3) a bend angle of about 100° was calculated, and when bound to all three sites (band C in Fig. 3), a bend angle of about 12° was calculated. One explanation for the loss of bending when all three sites are occupied is that a GcvA-mediated loop forms, the mobility being relatively independent of the position of the binding sites within the loop (Fig. 4).

Although other explanations are possible, we favour this interpretation based on results discussed below. When the GMS assay was performed on a DNA fragment carrying only GcvA site 1, a bend angle of about 75° was calculated (not shown). If phased with the bend induced at sites 3→2, a looped structure is...
Novel co-inducer mediated regulation of gcvTHP

Table 2. GcvA-mediated activation of gcv is independent of glycine

<table>
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<tr>
<td>GS162 gcvT::lacZ</td>
<td>WT</td>
<td>GM + glycine: 963, GM + Gly-Ser-Phe: 295</td>
</tr>
<tr>
<td>GS1089 gcvT::lacZ</td>
<td>glyA gcvR</td>
<td>GM + glycine: 1061, GM + Gly-Ser-Phe: 1049</td>
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</table>

Cells were grown in GM medium with the indicated supplements and assayed for β-galactosidase activity (Miller, 1992). Each data point is the mean of two separate assays, each performed in triplicate. Standard deviations from the mean were less than 15% for all data presented.

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possible. We also used a DNA fragment carrying the three GcvA binding sites but with a 4 bp change in site 1 that reduced the affinity of GcvA for site 1 about twofold and that resulted in a loss of GcvA-mediated repression (Wilson et al., 1995; Wonderling et al., 2000). The ability of GcvA to bind sites 2 + 3 and bend the DNA were unchanged (Fig. 5a). However, when GcvA was bound to all three sites a bend angle of about 80° was calculated. Thus, although the bp changes only resulted in a small decrease in the ability of GcvA to bind to site 1, they significantly altered GcvA-induced bending of the DNA. We also inserted 1-5 and 2°5 turns of DNA between site 1 and sites 2 + 3. For both the +15 bp insert (not shown) and the +25 bp insert (Fig. 5b), GcvA binding to sites 2 + 3 and its ability to bend DNA were essentially unchanged. However, when bound to all three sites, a bend angle of about 25° was calculated. If the 75° bend induced by GcvA bound to site 1 is phased opposite to the 100° bend induced by GcvA bound to sites 3 + 2, the data would be consistent with the bending pattern observed. The results are summarized in Fig. 4.

We then tested whether glycine effects the GcvA/DNA interaction. The addition of glycine (3 mM) to the GMS assays did not show any alteration in either GcvA’s binding affinity to the three sites in the control region, nor were there any changes in the calculated bend angles induced by GcvA binding to the DNA (data not shown).

Does glycine directly affect GcvA-mediated expression of gcv?

Since glycine does not significantly affect GcvA’s ability to bind or bend DNA, we wanted to determine how glycine is involved in the activation mechanism. It was reported previously that mutations in gcvR result in constitutive expression of the gcv operon, but a reduced growth rate (Ghrist & Stauffer, 1995). It is possible that the reduced growth rate is a result of glycine limitation due to its rapid catabolism by the GCV enzyme system. If true, this would suggest that activation of the gcv operon could occur in the absence of glycine and would be consistent with the GMS assay results. Therefore, we tested whether the reduced growth rate in the gcvR mutant strain GS1053 was due to altered glycine levels in the cell. The generation times of the WT strain GS162 grown in GM medium vs GM + glycine at 30 °C are 85 and 84 min, respectively, and for the gcvR strain GS1053 are 130 and 84 min, respectively. Thus, the gcvR mutation results in glycine auxotrophy, but constitutive expression of gcv. Growth of the gcvR strain in GM + inosine also reduced the generation time to 84 min. Since a glycine molecule is incorporated into the purine ring structure, the reduced generation time is possibly due to a sparing effect of glycine by purine supplementation.

We also constructed a glyA gcvR double mutant strain GS1089. This strain requires an exogenous source of glycine for growth due to the glyA mutation (Pizer, 1965). We used the Gly-Ser-Phe tripeptide to limit glycine (glycine concentration 50 µg ml⁻¹). The generation time of the WT strain in GM + tripeptide at 30 °C was 82 min and the double mutant was more than 174 min. The addition of glycine to the medium reduced the generation time of the mutant to that of the WT, suggesting that the double mutant is starved for glycine. In the WT lysogen, gcvT::lacZ expression was threefold lower in the presence of the tripeptide compared to glycine, suggesting that the concentration of glycine as tripeptide is limiting for activation (Table 2). In the glyA gcvR mutant lysogen, gcvT::lacZ expression was constitutive, although the cells were starved for glycine. These results and the results of the GMS assays suggest that glycine probably does not interact directly with GcvA to activate the gcv operon.

Glycine binds to GcvR

If glycine does not affect GcvA’s ability to bind or bend DNA, and the gcv operon can be activated under glycine starvation conditions if gcvR is inactivated, what is the role of glycine? We used micro-chamber equilibrium dialysis to determine where glycine fits into the regulatory mechanism. Purified GcvA exploits at 25 µM and purified native GcvR at 25 µM were each dialysed against 250 µM [¹⁴C]glycine overnight at 20 °C (see Methods). 50 µl aliquots of ‘outside’ and ‘inside’ buffer were collected from each sample and the radioactivity measured as c.p.m. GcvA did not show a significant level of radioactivity as c.p.m. bound compared to a negative control (no protein ‘inside’), indicating that glycine does not bind GcvA at the protein and ligand concentrations.
examined (Fig. 6). GcvR, in contrast, showed a high level of radioactivity as c.p.m. bound compared to the negative control and to GcvA (Fig. 6). Although this assay was conducted using a single protein and ligand concentration, the difference in ligand bound between GcvA and GcvR as compared to the ‘no protein’ control at the ligand and protein concentrations used suggests that glycine is likely bound by GcvR.

Co-ppt of GcvA and GcvR

Previous results suggested that a GcvA/GcvR complex is likely to form in vivo (Ghrist et al., 2001). Since the co-inducer glycine binds GcvR rather than the activator GcvA, it is possible that glycine’s role is to prevent a GcvA/GcvR interaction required for repression, permitting GcvA to function as an activator. We used a qualitative co-ppt assay to demonstrate in vitro that an interaction between GcvA and GcvR occurs and to determine whether glycine affects this interaction. Purified GcvA<sub>6-his</sub> was bound to a Ni<sup>2+</sup>-NTA agarose affinity matrix, followed by the addition of purified native GcvR. The matrix was washed with loading buffer to remove any unbound GcvR, and subsequently washed with loading buffer containing a low concentration of imidazole (20 mM) to remove any nonspecific binding of GcvR to the matrix. GcvA was then eluted from the matrix with buffer containing a high concentration of imidazole (500 mM). The final elution of GcvA<sub>6-his</sub> from the Ni<sup>2+</sup>-NTA agarose affinity matrix also resulted in elution of a significant amount of GcvR (Fig. 7a, lane 9). When GcvR alone was incubated with the Ni<sup>2+</sup>-NTA affinity matrix and subsequently washed with low imidazole (20 mM), all of the GcvR was eluted from the affinity matrix, as no protein was eluted by the subsequent high imidazole wash (Fig. 7c, compare lanes 4 and 5 with lane 6). Thus, the GcvR co-eluting with GcvA (Fig. 7a, lane 9) was likely due to an interaction of GcvR with GcvA that allowed it to remain in complex with GcvA and the Ni<sup>2+</sup>-NTA agarose affinity matrix until the high imidazole elution. When this experiment was performed in the presence of glycine (1 mM) in the loading and elution buffers, all GcvR eluted either in the loading buffer or in the low concentration imidazole wash, and no longer eluted with GcvA (Fig. 7, compare lanes 1 and 2 with lanes 9 and 10). These results suggest that glycine disrupts a GcvA/GcvR interaction.

GcvR proteins unable to bind glycine

Previously, a point mutation in gcvA was isolated that resulted in GcvA’s inability to mediate full repression of a <i>gcvT::lacZ</i> transcriptional fusion, but that did not alter GcvA’s ability to function as an activator (Jourdan

\[
\text{\begin{tabular}{cccccccc}
\text{Control} & GcvA & GcvR & GcvR<sub>F50S</sub> & GcvR<sub>T130M</sub> & GcvR<sub>R18H</sub> & GcvR<sub>L44P</sub> & GcvR<sub>L44P</sub>\\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0
\end{tabular}}
\]

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\text{\begin{tabular}{cccccccc}
\text{Control} & GcvA & GcvR & GcvR<sub>F50S</sub> & GcvR<sub>T130M</sub> & GcvR<sub>R18H</sub> & GcvR<sub>L44P</sub> & GcvR<sub>L44P</sub>\\
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0 & 0 & 0 & 0 & 0 & 0 & 0 & 0
\end{tabular}}
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& Stauffer, 1998). The mutation resulted in an arginine to glycine change at residue 197 (R197G) and disrupted the GcvA/GcvR interaction as measured in a LexA two-hybrid system (Ghrist et al., 2001). Our initial effort was to verify the GcvA/GcvR interaction. Convincing genetic evidence would be the isolation of allele-specific gcvR mutations that restore the GcvA/GcvR interaction. The gcvA strain GS998::gcvT::lacZ carrying the gcvR::AR197G mutant allele on a single-copy plasmid (pGS311) was transformed with a multi-copy plasmid pool carrying PCR-induced random bp changes in gcvR (see Methods). We assumed that transformants expressing mutant GcvR proteins that restore the interaction between GcvA-R197G and GcvR would produce either white or light blue colonies due to the ability of GcvR to repress expression of the gcvT::lacZ fusion. Four separate transformants with the correct phenotype were identified. Plasmid DNA was isolated from each transformant and the gcv sequence was determined. Three of the plasmids contained single bp mutations resulting in amino acid changes R18H, L44P and T130M. The fourth clone contained two bp changes, one silent and the other resulting in the amino acid change F50S. The WT and mutant gcvR alleles were cloned into the single copy plasmid pGS311 alone. The plasmids were then used to transform the gcvA gcvR strain GS1053::gcvT::lacZ to determine the effects of the mutations on regulation of the gcv operon when the WT and mutant GcvR proteins were at more physiological concentrations. Unexpectedly, when both gcv::AR197G and the individual mutant gcvR alleles were expressed from the single-copy plasmid, none of the gcvR mutants had any suppressor effect on gcvT::lacZ expression when compared to the WT gcvR allele (data not shown). Since the four gcvR mutations result in amino acid changes at different positions in GcvR, we hypothesized that the mutations might have altered GcvR to make it a more efficient repressor. Thus, when expressed from a multicopy plasmid, the mutant GcvR proteins are able to interact with GcvA-R197G to cause repression, but not when expressed from the single-copy plasmid. If this hypothesis is correct, then the mutations might have an effect on WT GcvA expressed from a single chromosomal gcvA gene even though they did not have an effect when GcvA-R197G was expressed from the single-copy plasmid. We tested this hypothesis by measuring the effects of the gcvR mutant alleles in the presence of a single copy of the WT gcvA gene. As controls, we used the gcvA mutant GS998::gcvT::lacZ to show basal levels of expression in the absence of the activator protein and the gcvR mutant GS1053::gcvT::lacZ to show constitutive levels in the absence of GcvR. The gcvA mutant GS998::gcvT::lacZ is neither activated in the presence of glycine nor repressed in the presence of inosine, except for a twofold PurR-mediated repression (Wilson et al., 1993a), whereas the gcvR mutant GS1053::gcvT::lacZ shows constitutive expression, except for the twofold PurR-mediated repression (Table 3). The WT and mutant gcvR alleles were cloned into the single copy plasmid pGS311 alone. The plasmids were then used to transform the gcvR strain GS1053::gcvT::lacZ to determine the effects of the mutants in the presence of the single copy of the WT gcvA allele on the chromosome. In GM medium, the single-copy plasmid carrying either the gcv::AR18H, gcv::L44P or gcv::T130M allele resulted in a 17- to 22-fold reduction in β-galactosidase levels compared to a sevenfold reduction by the WT gcvR; in GM + glycine there was a 2.5-fold reduction in β-galactosidase levels compared to no significant reduction by the WT gcvR; and in GM + inosine, there was a 101- to 115-fold reduction in β-galactosidase levels compared to a 54-fold reduction for WT gcvR (Table 3). The effects of the gcv::F50S allele were more severe, resulting in a 31-fold reduction in GM, a 14-fold reduction in GM + glycine and a 101-fold reduction in GM + inosine (Table 3).

<table>
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<tr>
<th>Strain/plasmid*</th>
<th>Plasmid-encoded gcvR allele</th>
<th>β-Galactosidase activity</th>
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<tr>
<td></td>
<td></td>
<td>GM</td>
</tr>
<tr>
<td>GS998::gcvT::lacZ</td>
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<td>78</td>
</tr>
<tr>
<td>GS1053::gcvT::lacZ</td>
<td>None</td>
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<tr>
<td>GS1053::gcvT::lacZ[pGS338]</td>
<td>WT gcvR</td>
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<tr>
<td>GS1053::gcvT::lacZ[pGS558]</td>
<td>gcv::RR18H</td>
<td>71</td>
</tr>
<tr>
<td>GS1053::gcvT::lacZ[pGS559]</td>
<td>gcv::RL44P</td>
<td>82</td>
</tr>
<tr>
<td>GS1053::gcvT::lacZ[pGS589]</td>
<td>gcv::RT130M</td>
<td>45</td>
</tr>
<tr>
<td>GS1053::gcvT::lacZ[pGS589]</td>
<td>gcv::RT130M</td>
<td>64</td>
</tr>
</tbody>
</table>

* Lysogens GS998::gcvT::lacZ and GS1053::gcvT::lacZ carry gcvA and gcvR mutations, respectively. The gcvR lysogen GS1053::gcvT::lacZ was transformed with a single-copy plasmid carrying either WT or mutant gcvR alleles.
One possible cause for the phenotypes seen in the gcvR mutants could be that the mutated GcvR proteins are reduced in their ability to bind glycine. To test this hypothesis, the GcvR-R18H, GcvR-L44P, GcvR-F50S and GcvR-T130M proteins were purified and the mutant proteins (25 μM) dialysed against [14C]glycine (250 μM). No significant accumulation of label across the membrane was seen with any of the mutant proteins at the protein and ligand concentrations used as compared to the WT GcvR protein (Fig. 6).

**DISCUSSION**

Most characterized LTTR proteins require small co-effectors molecules to carry out their regulatory functions (Schell, 1993). These low molecular mass co-effectors are believed to directly bind their respective LTTR proteins, resulting in altered binding site affinity or relaxed DNA bending by the LTTR proteins at the regulated promoters (Schell, 1993). These changes are necessary for appropriate transcriptional regulation (Gao & Gussin, 1991; Wek & Hatfield, 1988; Ogawa et al., 1999; Rhee et al., 1998; van Keulen et al., 1998). The addition of glycine to GM medium results in GcvA-mediated activation of the gcv operon (Plamann et al., 1993). However, several lines of evidence suggest that the co-inducer effect of glycine might not occur directly through GcvA. First, although GcvA demonstrates a preferential binding to its three sites in the gcv control region (231), glycine did not affect the order in which the three sites were filled or the apparent affinity for the sites (Wilson et al., 1995). Second, GcvA induces constitutive expression of a gcvT::lacZ fusion in the absence of glycine if GcvR is inactivated, a growth condition that results in a partial glycine auxotrophy (Table 2; Ghrist & Stauffer, 1995). The GMS assays in this study are consistent with this hypothesis. GcvA bound to the gcv promoter region and induced a bend in the DNA in the absence of glycine (Fig. 3). The addition of 3 mM glycine, however, had no significant affect on either the affinity of GcvA binding for its target sites or the degree of DNA bending.

Since glycine did not alter GcvA’s ability to bind or bend gcv DNA, and since GcvR is required for GcvA to mediate negative regulation of the gcv operon, as well as for GcvA to show a positive response to glycine (Ghrist & Stauffer, 1995), it seemed possible that glycine might interact with GcvR rather than GcvA. We tested this possibility by equilibrium dialysis. The results showed that glycine binds GcvR rather than GcvA (Fig. 6), suggesting that the co-activator glycine functions by a different mechanism for gcv control compared to the co-activators for other LTTR-regulated operons. Results from in vivo studies using a LexA two-hybrid system suggested that a direct interaction occurs between GcvA and GcvR (Ghrist et al., 2001). The in vitro results from this study showed that when GcvA×his was bound to Ni2+-NTA agarose affinity matrix, followed by addition of WT GcvR, a significant amount of GcvR co-eluted with GcvA during a high imidazole wash (Fig. 7a).

Furthermore, the addition of glycine (1 mM) to the loading and wash buffers inhibited this interaction (Fig. 7b). These results are consistent with a direct GcvA/GcvR interaction and suggest that the role of the co-inducor glycine is either to block this association or to cause a dissociation of the complex once formed. The isolation of gcvR mutants that encode proteins with reduced abilities to bind glycine and that result in a reduced ability of glycine to activate gcvT::lacZ expression is consistent with this model. We used purified GcvR in a GMS assay with gcv DNA and in both the absence and presence of glycine. However, we could not show any binding of GcvR to gcv DNA (data not shown). These results suggest that GcvR interacts directly with GcvA to form a repression complex: a somewhat unconventional mechanism for prokaryotes (Fig. 8A).

The above model predicts that transcription of the gcv operon is inversely proportional to the level of GcvA/GcvR complex formed, which in turn, is inversely proportional to the level of glycine present in the cell. Thus, glycine does not act as a classical co-activator in this system, but rather through a mechanism of de-repression by binding to GcvR and preventing GcvR from interacting with GcvA to block GcvA’s activator function. In mid-exponential phase cells growing in GM medium it was determined that the intracellular con...
centration of glycine is approximately 41 μM (Raunio & Rosenqvist, 1970). This level is apparently insufficient to allow full de-repression of the gcv operon as seen in the gcvR mutant GS1053::gcvT::lacZ, but is sufficient to bind some GcvR and to allow de-repression above the basal level seen in the gcvA mutant GS998::gcvT::lacZ. As the intracellular glycine concentration increases due to transport of exogenous glycine, more GcvR is bound by glycine, resulting in a further loss of repression and increased activation of gcvT::lacZ. Of interest, the GcvR-R18H, GcvR-L44P, GcvR-F50S and GcvR-T130M proteins did not bind glycine in the equilibrium dialysis experiment, but did show some response to glycine in vivo (Fig. 6 and Table 3). If glycine binds to GcvR in a concentration-dependent manner to disrupt the GcvA/GcvR complex, then the GcvR mutant proteins likely still bind glycine, but with reduced affinity. Additional equilibrium dialysis experiments at different GcvR and ligand concentrations will be necessary to verify this hypothesis.

It is worth noting that full repression of the gcv operon requires the addition of purines to GM medium (Wilson et al., 1993a; Ghrist & Stauffer, 1995). Although the mechanism of purine involvement is unknown, purines could either enhance the interaction between GcvA and GcvR, forming a more stable repressor complex, or change the conformation of the repressor complex at the promoter to prevent transcriptional initiation by RNAP. Furthermore, the addition of both glycine and purines to the growth medium results in derepression of the gcv operon (Stauffer & Stauffer, 1994; Wilson et al., 1993a). The dominance of glycine to purines in the regulatory mechanism, and the ability of glycine to disrupt the GcvA/GcvR interaction are consistent with a GcvA/GcvR complex being necessary for purine-mediated repression. Since all three GcvA binding sites are required for normal repression of the gcv operon (Wilson et al., 1995; Wonderling et al., 2000), it is likely that GcvA and GcvR form a repression loop (Fig. 8A). DNA looping normally occurs between identical proteins (Gralla & Collado-Vides, 1996). Verification of this as a heterologous protein system for DNA looping will provide a novel perspective into the basic mechanism by which protein/protein interactions control gene expression.

Previously it was shown that specific amino acid residues in the α-subunit of RNAP (α-carboxy-terminal domain), when mutated, disrupt GcvA-mediated activation of the gcv operon, but not GcvA-mediated repression (Jourdan & Stauffer, 1999). In addition, positive control mutants were isolated in gcvA that decreased GcvA’s ability to activate gcv transcription, but that did not affect DNA binding or GcvA-mediated repression (Jourdan & Stauffer, 1998). Since GcvA-mediated activation requires GcvA bound to sites 2 and 3, located 214–271 bp upstream of the transcription start site, it is reasonable to assume that a looped nucleoprotein structure is required for an appropriate GcvA/RNAP contact for activation (Fig. 8B). Although the results of this study show that GcvA also induces a bend in the DNA, it is not known whether the GcvA-induced DNA bending plays a direct role in GcvA-mediated regulation of the gcv operon.

ACKNOWLEDGEMENTS

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