Activation of Gene Expression by a Ligand-induced Conformational Change of a Protein-DNA Complex*

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IlvY protein binds cooperatively to tandem operator sites in the divergent, overlapping, promoter-regulatory region of the ilvYC operon of Escherichia coli. IlvY positively regulates the expression of the ilvC gene in an inducer-dependent manner and negatively regulates the transcription of its own divergently transcribed structural gene in an inducer-independent manner. Although binding of IlvY protein to the tandem operators is sufficient to repress ilvY promoter-specific transcription, it is not sufficient to activate transcription from the ilvC promoter. Activation of ilvC promoter-specific transcription requires the additional binding of a small molecule inducer to the IlvY protein-DNA complex. The binding of inducer to IlvY protein does not affect the affinity of IlvY protein for the tandem operator sites. It does, however, cause a conformational change of the IlvY protein-DNA complex, which is correlated with the partial relief of an IlvY protein-induced bend of the DNA helix in the ilvC promoter region. This structural change in the IlvY protein-DNA complex results in a 100-fold increase in the affinity of RNA polymerase binding at the ilvC promoter site. The ability of a protein to regulate gene expression by ligand-responsive modulation of a protein-DNA structure is an emerging theme in gene regulation.

LysR-type proteins are the most common class of transcriptional regulatory proteins in prokaryotes (1). Regulation by LysR-type proteins is distinguished by several highly conserved unique properties. LysR-type proteins typically activate transcription of a target gene(s) and autoregulate their own synthesis from a single regulatory locus. In most cases, the target gene is divergently transcribed from the structural gene encoding the LysR-type protein. Activation of the target gene requires the binding of a metabolically important small molecule inducer, whereas autoregulation of the LysR gene is inducer-independent. Inducer binding, however, does not significantly alter the DNA binding affinity of these proteins (1). Thus, unlike other well characterized inducer-responsive activator proteins, LysR-type proteins are unusual in that the binding of inducer activates transcription by affecting an activator property of the protein distinct from its DNA binding activity. However, the molecular basis of this regulation has not been determined for any member of this important class of transcriptional regulatory proteins.

IlvY protein is a prototypic member of the LysR family of transcriptional regulatory proteins and is required for the regulation of ilvC gene expression in Escherichia coli (2–5). The ilvC gene encodes acetoacetyl acid isomeroreductase (EC 1.1.1.86), an enzyme involved in the biosynthesis of the branched chain amino acids, l-isoleucine, l-valine, and l-leucine (5). Together with IlvY protein, either substrate of this enzyme, α-acetolactate or α-acetoxybutyrate, induces the activation of ilvC gene expression. Like other LysR-type proteins, IlvY protein also represses transcription of its own divergently transcribed structural gene in an inducer-independent manner (3, 4, 6).

In this report, we describe the first molecular mechanism for inducer-mediated activation by a LysR-type protein. Specifically, we show that IlvY protein binds cooperatively to the tandem operator sites in the divergent-overlapping promoter region of the ilvYC operon (Fig. 1) in an inducer-independent manner and autoregulates its own gene expression by an RNA polymerase occlusion mechanism. We further show that the binding of IlvY protein to the tandem operators is necessary but not sufficient to activate transcription from the ilvC promoter. Activation of transcription from the divergent ilvC promoter requires the additional binding of an inducer molecule to an IlvY protein-operator DNA complex. Inducer binding effects the partial relief of an IlvY protein-induced DNA bend centered around the −35 hexanucleotide element of the ilvC promoter. This ligand-induced structural change in the DNA helix enhances the binding affinity of RNA polymerase at the ilvC promoter. Thus, IlvY protein activates transcription from the ilvC promoter by the binding of an inducer molecule that directs a conformational change in the structure of an IlvY protein-operator DNA complex and enhances the recruitment of RNA polymerase to the ilvC promoter without affecting the occupancy of IlvY protein at the tandem operator sites.

MATERIALS AND METHODS

Chemicals and Reagents—Polyethyleneimine was purchased from Miles Laboratories. Restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were purchased from New England Biolabs. E. coli RNA polymerase, pancreatic RNAsin, and DNAse I were purchased from Boehringer Mannheim. Shrimp alkaline phosphatase was purchased from United States Biochemicals. Radiolabeled nucleotides were purchased from NEN Life Science Products. DNA oligonucleotides were synthesized by Operon Technologies. Site-directed mutagenesis was performed using the oligonucleotide directed in vitro mutagenesis kit version 2.0 from Amersham Pharmacia Biotech. DNA sequencing was performed using the Sequenase™ kit from United States Biochemicals.

Plasmids—Plasmid DNA isolation and all recombinant DNA manipulations were carried out using standard methods (7). Plasmid pET3cY, used for the overexpression of the ilvY gene product, was created by site-directed mutagenesis of the ilvY gene, contained on a 1400-bp BamHI-PvuII DNA fragment (derived from plasmid pRWY) (3). An NdeI restriction site was created at the beginning of the ilvY protein
The specific DNA binding activity of the purified IlvY protein was estimated from a titration of its binding to a molar excess of DNA fragments containing the tandem operator sites. The concentration of O1O2-containing DNA fragments used yielded an operator site concentration of 4.4 × 10⁻⁷ M, which was titrated with substoichiometric concentrations of IlvY protein dimer between 1.9 × 10⁻⁸ M and 1.5 × 10⁻⁷ M.
Hydroxyl Radical Footprinting Experiments—Hydroxyl radical footprinting was performed as described by Tallius and Dombroski (18) using the same DNA fragments (<1×10^12 m) used in the DNaSe I footprinting reactions. Binding reactions were performed under equilibrium binding conditions as described above with the exception that the transcription reaction mixture (containing purified IlvY protein and/or inducer) was analyzed with hydroxyl radical for exactly 2 min at 25 °C and quenched with the addition of 20 μl of 0.2 M thiourea to ensure conditions of single-hit kinetics as described above. Reaction products were resolved by electrophoresis on a 10% denaturing polyacrylamide gel (9.5% acrylamide, 0.5% N,N'-methylenebisacrylamide) containing 8 μl urea in TBE buffer following the migration of the bands of a digital image of each lane.

In Vitro Transcription Reactions—In vitro transcription reactions were conducted using the closed circular supercoiled (>80%) plasmid pDDSY (described above), in the absence and presence of purified IlvY protein and/or inducer, according to the procedures of Hauser et al. (19). RNA polymerase-plasmid DNA complexes were formed by preincubating 1 unit (2.4 pmol) of RNA polymerase and 100 ng of plasmid DNA (0.2 pmol) in a 20-μl reaction mixture (0.04 μl Tris-HCl (pH 8.0), 0.1 μl KCl, 0.01 μl MgCl2, 1.0 μl dithiothreitol, 0.1 μl EDTA, 200 mg CTP, 200 mg UTP, 100 mg GTP, 10 mCi of [35S]UTP, 0.1 mg/ml bovine serum albumin and 40 units of RNasin for 10 min at 25 °C. Transcription reactions were initiated by the addition of 2 μl of 2 mM ATP, 2 mM GTP solution. Reactions were terminated after 4 min with the addition of 20 μl of stop solution (95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol). Transcription under these conditions was determined to be linear with respect to time for at least 6 min and was proportional to the amount of plasmid DNA template used. To correct for differences in the total amount of transcription between reactions, the amount of ilvE-specific transcription was determined by normalizing the relative signal of ilvE-specific transcription to the relative signal of transcription arising from the RNA-I promoter present on each plasmid DNA template in the transcription reaction. Reaction products were separated by electrophoresis on an 8% denaturing polyacrylamide gel (7.5% acrylamide, 0.4% N,N'-methylenebisacrylamide) containing 8 μl urea in TBE buffer (7) and visualized by autoradiography as described above.

Circular Permutation Assays—Binding reactions were performed as described above using isometric DNA fragments containing the tandem O2_O2 operators derived from the plasmid pBENDY. Products were resolved on a 6% polyacrylamide gel (9.5% acrylamide, 0.4% N,N'-methylenebisacrylamide) containing 8 μl urea in TBE buffer (7) and visualized by autoradiography as described above.

Abortive Transcription Assays—Abortive transcription initiation assays were performed in the presence or absence of 2 mM inducer (α-acetoxyhydroxybutyrate) according to the methods of Hawley et al. (23) as described by Parekh and Hatfield (24). Briefly, reactions were initiated by the addition of RNA polymerase at final concentrations of 20, 40, 60, 80, and 100 nM to a preformed IlvY protein-DNA complex. IlvY protein-DNA complexes were preformed by incubating 30 μl IlvY protein dimer with 1 mM supercoiled DNA plasmid template, pDDSY, for 20 min at 37 °C in reaction buffer A (0.04 μl Tris-HCl (pH = 8.0), 0.1 mM KCl, 0.01 mM MgCl2, 1.0 mM dithiothreitol, 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin), 40 μM UTP, 100 mg CTP-[α-32P]UTP, and 0.5 mM Apa. The inclusion of the dinucleotide ApA in the reaction mixture restricts transcription initiation to the ilvE promoter of the plasmid DNA template, pDDSY, yielding the abortive product ApAUpU. Abortive transcription products were isolated by nondenaturing electrophoresis in a 25% polyacrylamide gel (23.75% acrylamide, 1.25% N,N'-methylenebisacrylamide) in 1× TBE buffer (7), and quantitated by PhosphorImager analysis. τ is a measure of the lag time required for open complex formation observed in a product versus time plot. τ values were determined by fitting a least-squares linear regression to the experimental data using the program LAGPLOT described by Goodrich (25). The apparent binding affinity of RNA polymerase (Kb) and the first-order isomerization rate constant (kₙ) were determined by plotting τ₂ₙ as a function of I/([RNA polymerase]) on the basis of a least-squares fit to the equation τ₂ₙ = (1/kₙ) + (I/([RNA polymerase])) using the TAUPLOT program of Goodrich (25).

Hydroxyl radical footprinting was performed as described by Tallius and Dombroski (18) using the same DNA fragments (<1×10^12 m) used in the DNaSe I footprinting reactions. Binding reactions were performed under equilibrium binding conditions as described above with the exception that the transcription reaction mixture (containing purified IlvY protein and/or inducer) was analyzed with hydroxyl radical for exactly 2 min at 25 °C and quenched with the addition of 20 μl of 0.2 M thiourea to ensure conditions of single-hit kinetics as described above. Reaction products were resolved by electrophoresis on a 10% denaturing polyacrylamide gel (9.5% acrylamide, 0.5% N,N'-methylenebisacrylamide) containing 8 μl urea in TBE buffer (7) and visualized by autoradiography as described above. Analysis of bend mapping was performed as described by Wü and Coenders (21). Estimates of bend angles were obtained as described by Thompson and Landy (22) using the empirical relationship cos(θ) = −2τ/(R (slowest)/R (fastest)).
effective affinity of 2.2 nM, consistent with a previous estimate. The concentration required to half-saturate O1 alone is 8-fold greater than 15% of the DNA migrated as bound in gel shift assays (6). The half-saturation point for IlvY binding to the tandem operator sites in the divergent promoter region and that this binding is cooperative (6). To explore the quantitative nature of this cooperativity, binding titrations of purified IlvY protein to DNA fragments containing the tandem operators (O1O2) and to DNA fragments with either O1 or O2 deleted were performed (Fig. 2). These assays were conducted using concentrations of operator sites well below the effective binding dissociation constant for the tandem operator sites to evaluate the microscopic binding constants governing the assembly of IlvY protein-operator DNA complexes. The gel mobility shift of the tandem operator-containing DNA fragment showed only a single liganded complex, which we interpret to be O1O2 with both sites bound by IlvY protein dimer. The half-saturation point for IlvY binding to the DNA fragment containing O1 and O2 corresponds to an affinity for O1O2 together (6). The observation that the apparent binding affinity for either operator site is reduced when the other site is removed indicates cooperative binding to the adjacent sites. To determine the affinity of IlvY protein for site O1 and to more precisely evaluate the cooperativity, the IlvY protein treatments of O1O2 and O2 alone (Fig. 2) were analyzed according to a general cooperative binding model (17). The adjustable parameters are the Gibbs free energy changes corresponding to the microscopic equilibrium constants for IlvY protein binding to O1 ($\Delta G_1 = -RT \ln K_1$) and to O2 ($\Delta G_2 = -RT \ln K_2$), and for cooperativity ($\Delta G_{coop}$). When a complete titration of site O2 alone is absent, the analysis does not provide independent estimates of $\Delta G_2$ and $\Delta G_{coop}$. Instead, we found such a high numerical correlation that increasing values of one compensated for decreasing values of the other. This correlation did not affect $\Delta G_1$, for which a precise estimate, $\Delta G_1 = -10.4 \pm 0.1$ kcal/mol, was obtained. Thus, it was possible to define a lower limit to the cooperativity and an upper limit to the affinity for O2 alone by conducting a series of analyses of the O1 alone and O1O2 data in which different values of $\Delta G_{coop}$ were entered as fixed input parameters. For each fit, estimates of $\Delta G_1$, $\Delta G_2$, and the variance of the fit were obtained. A minimum in the variance was obtained for $\Delta G_2 = -3.0$ kcal/mol. For $\Delta G_2 < -2.6$ kcal/mol the variance increased substantially reflecting the fact that it is not possible to account for the offset between the curves for IlvY protein binding to O1 alone versus to O1O2 without significant cooperativity. By comparing ratios of variances obtained for fits with different values of $\Delta G_2$ against an F-statistic, we conclude that $\Delta G_2 < -2.6$ kcal/mol, corresponding to at least 120-fold cooperativity, is required to obtain an adequate fit. This also sets a maximum affinity for O2 of 35 nM ($\Delta G_2 > 10.0$ kcal/mol). Setting $\Delta G_2 = -8.5$ kcal/mol corresponding to our previous estimate of Kd (0.45 µM) yields $\Delta G_2 = -4.3$ kcal/mol or 1500-fold cooperativity. Whether 1500-fold or only 120-fold, the cooperativity is sufficient that the O1O2 operator exists as liganded at either O1 alone or at O2 alone less than 1% of the time at any IlvY dimer concentration, a conclusion that is consistent with the absence of any intermediate band in the gel shift assay. The effect is that IlvY protein fills the tandem operator sites in a single concerted step.

RESULTS

Operator Binding by Purified IlvY Protein—Previous analyses with IlvY protein-enriched cell-free extracts indicated that IlvY protein binds to tandem operator sites (O1O2) in the divergent-overlapping ilvYC promoter region and that this binding is cooperative (6). To explore the quantitative nature of this cooperativity, binding titrations of purified IlvY protein to DNA fragments containing the tandem operators (O1O2) and to DNA fragments with either O1 or O2 deleted were performed (Fig. 2). These assays were conducted using concentrations of operator sites well below the effective binding dissociation constant for the tandem operator sites to evaluate the microscopic binding constants governing the assembly of IlvY protein-operator DNA complexes. The gel mobility shift of the tandem operator-containing DNA fragment showed only a single liganded complex, which we interpret to be O1O2 with both sites bound by IlvY protein dimer. The half-saturation point for IlvY binding to the DNA fragment containing O1 and O2 corresponds to an effective affinity of 2.2 nM, consistent with a previous estimate (2 nM) obtained with IlvY protein enriched cell free extracts (6). The lack of an intermediate band corresponding to IlvY protein bound to either O1 or O2 alone in the gel mobility shift experiment performed to generate the data in Fig. 2 is indicative of highly cooperative binding (17). In fact, the IlvY protein concentration required to half-saturate O1 alone is 8-fold greater than the concentration required to half-saturate the tandem operator sites (Fig. 2). We found previously that it was not possible to saturate the O2 site alone; at $8 \times 10^{-13}$ M IlvY dimer, the highest concentration used in the binding experiments, less than 15% of the DNA migrated as bound in gel shift assays (6). Based on this measurement, the affinity of IlvY protein for the O2 site alone is calculated to be $K_2 < 0.45 \mu M$. When this experiment was repeated with the highly purified IlvY protein reported here, the same result was obtained. Therefore, the affinity for O2 alone is at least 200-fold weaker than for sites O1O2 together (6). The observation that the apparent binding affinity for either operator site is reduced when the other site is removed indicates cooperative binding to the adjacent sites. To determine the affinity of IlvY protein for site O1 and to more precisely evaluate the cooperativity, the IlvY protein treatments of O1O2 and O2 alone (Fig. 2) were analyzed according to a general cooperative binding model (17). The adjustable parameters are the Gibbs free energy changes corresponding to the microscopic equilibrium constants for IlvY protein binding to O1 ($\Delta G_1 = -RT \ln K_1$) and to O2 ($\Delta G_2 = -RT \ln K_2$), and for cooperativity ($\Delta G_{coop}$). When a complete titration of site O2 alone is absent, the analysis does not provide independent estimates of $\Delta G_2$ and $\Delta G_{coop}$. Instead, we found such a high numerical correlation that increasing values of one compensated for decreasing values of the other. This correlation did not affect $\Delta G_1$, for which a precise estimate, $\Delta G_1 = -10.4 \pm 0.1$ kcal/mol, was obtained. Thus, it was possible to define a lower limit to the cooperativity and an upper limit to the affinity for O2 alone by conducting a series of analyses of the O1 alone and O1O2 data in which different values of $\Delta G_{coop}$ were entered as fixed input parameters. For each fit, estimates of $\Delta G_1$, $\Delta G_2$, and the variance of the fit were obtained. A minimum in the variance was obtained for $\Delta G_2 = -3.0$ kcal/mol. For $\Delta G_2 > -2.6$ kcal/mol the variance increased substantially reflecting the fact that it is not possible to account for the offset between the curves for IlvY protein binding to O1 alone versus to O1O2 without significant cooperativity. By comparing ratios of variances obtained for fits with different values of $\Delta G_2$ against an F-statistic, we conclude that $\Delta G_2 < -2.6$ kcal/mol, corresponding to at least 120-fold cooperativity, is required to obtain an adequate fit. This also sets a maximum affinity for O2 of 35 nM ($\Delta G_2 > 10.0$ kcal/mol). Setting $\Delta G_2 = -8.5$ kcal/mol corresponding to our previous estimate of Kd (0.45 µM) yields $\Delta G_2 = -4.3$ kcal/mol or 1500-fold cooperativity. Whether 1500-fold or only 120-fold, the cooperativity is sufficient that the O1O2 operator exists as liganded at either O1 alone or at O2 alone less than 1% of the time at any IlvY dimer concentration, a conclusion that is consistent with the absence of any intermediate band in the gel shift assay. The effect is that IlvY protein fills the tandem operator sites in a single concerted step.

DNA Binding Site Specificity of Purified IlvY Protein—DNase I footprinting experiments were conducted to demonstrate that the purified IlvY protein exhibits site-specific binding to the tandem operator sites in the divergent promoter region of the ilvYC operon (Fig. 1). The results in Fig. 3A show that purified IlvY protein protects 2 adjacent 27-bp regions of DNA (ilvC bp -76 to -50 and -44 to -18) separated by 4 intervening DNase I-sensitive nucleotides on a 240-bp EcoRI-HindIII DNA fragment containing the tandem operator sites. These observed regions of protection on the nontranscribed strand of the ilvC gene correspond to those previously defined as O1 and O2 with partially purified IlvY protein (6). The results in Fig. 3B demonstrate that the purified protein protects three regions of DNA on the transcribed strand of the ilvC gene (ilvC bp -78 to -55, -51 to -43, and -41 to -19). These sequences are also contained in the DNA regions of dyad symmetry previously defined as O1 and O2.

Effect of IlvY Protein Binding to O1O2 on Transcription from the ilvY Promoter—To investigate how IlvY protein binding to the tandem operators, O1O2, affects transcription from the ilvY promoter, transcription from the ilvY promoter was assayed in vitro using a negatively supercoiled plasmid DNA template, pDD3Y. This plasmid contains the divergent ilvC and ilvY promoters flanked by Rho-independent ribosomal, rnnB T1 T2, terminators. Transcription from the ilvY and ilvC promoters generates 369 nucleotide and 154 nucleotide products, respectively. The results in Fig. 4 demonstrate that transcription from the ilvY promoter is repressed as a function of increasing concentration of IlvY protein. The fractional repression calculated from the data in Fig. 4 and the fractional saturation of O1O2 taken from Fig. 2 have similar dependences on IlvY.
protein concentration (Fig. 5). Both repression of transcription from the ilvY promoter and binding of the IlvY protein to O1O2 are unaffected by the addition of inducer (6).

The simplest model consistent with these repression data and with the observation that O1O2 and the ilvY promoter occupy the same region of DNA on the same face of the DNA helix (6), is that repression is mediated by competition between IlvY protein and RNA polymerase binding. To assess whether such a model can account for the repression curve in Fig. 5, these data were analyzed using a simple competitive binding model in which the rule is that RNA polymerase binding to the ilvY promoter and IlvY protein dimer binding to either operator are mutually exclusive. The ΔG values for IlvY protein binding to O1O2 were set as equal to the best estimates obtained from analysis of the data in Fig. 2. The fitted parameter was the free energy change corresponding to the equilibrium dissociation constant for RNA polymerase binding to the ilvY promoter. The results of this analysis, shown as the solid curves in Fig. 5, match the offset between the two curves with a predicted $K_d$ for RNA polymerase binding to the ilvY promoter in the absence of IlvY protein of $2 \pm 0.6 \times 10^{-9} \text{ M}$. We conclude, therefore, that IlvY protein represses transcription from the ilvY promoter by an RNA polymerase occlusion mechanism in an inducer-independent manner.

**Effects of Inducer on IlvY Protein-ilvC Promoter Interactions**—The binding of inducer to IlvY protein does not significantly increase the affinity of IlvY protein for the tandem operators. Yet, it results in the appearance of a DNase I hypersensitive band in the region of the O2 operator sequence that overlaps the −35 region of the ilvC promoter (6). These results suggested that the binding of inducer might direct a conformational change of the IlvY protein-operator DNA complex. In addition, the binding of RNA polymerase to the ilvC promoter is detectable by DNase I footprinting only when both IlvY protein and inducer are present (6). We proposed, there-
fore, that the role of inducer is to facilitate a conformational change in the IlvY protein-O1O2 complex, which recruits RNA polymerase to the ilvC promoter (6). To test this hypothesis, in vitro transcription and DNase I footprinting assays were conducted to quantitatively compare the effects of inducer on the DNase I hypersensitivity in the ilvC—35 hexanucleotide region and on transcription from the ilvC promoter.

The autoradiogram in Fig. 6 shows the effect of increasing inducer concentrations on the DNase I footprinting pattern produced by the binding of IlvY protein to O1O2O2. At all inducer concentrations, IlvY protein remains bound to both operator sites. However, the DNase I hypersensitive site at bp −37 on the nontranscribed strand (relative to the ilvC transcription start site; Fig. 6A) increases in intensity as inducer concentration is increased. Inducer binding also results in the appearance of several DNase I hypersensitive sites on the transcribed strand (Fig. 6B). The inducer concentration dependence of the hypersensitive site on the nontranscribed strand is plotted in Fig. 7 (open squares).

In vitro transcription assays were performed with a negatively supercoiled plasmid DNA template, pDB3Y. In the absence of IlvY protein, basal level transcription from both the ilvY and ilvC promoters is observed (Fig. 8, lane 1). Addition of 4 × 10^{-8} M IlvY protein dimer, which yields greater than 99% saturation of O1O2 (Fig. 2), represses production of the 369-nucleotide ilvY transcript but does not significantly affect production of the 154-nucleotide transcript originating from the ilvC promoter (Fig. 8, lane 2). The addition of inducer, however, activates transcription from the ilvC promoter 10–15-fold (Fig. 8, lanes 3–11). This inducer-mediated activation is plotted in Fig. 7 (open triangles).

The data derived from these ilvC transcription and DNase I hypersensitivity titrations (Fig. 7) were analyzed to estimate the affinity of the inducer, α-acetohydroxybutyrate, for the IlvY protein-O1O2 complex. Using a simple, noncooperative binding model, a $K_d$ value of 0.31 mM ($\Delta G = -4.4 \pm 0.2$ kcal/mol) was obtained from the transcription data and a $K_d$ value of 0.55 mM (4G = −4.4 ± 0.2 kcal/mol), was obtained from the DNase I data. Since these experimental values are indistinguishable from one another, both transcriptional induction and DNase I hypersensitivity appear to be the result of a single inducer binding event. Neither binding transition shows any indication of either positive or negative cooperativity.

These results demonstrate that the binding of IlvY protein to the tandem operators is necessary, but not sufficient, for activation of transcription from the ilvC promoter. Activation requires the additional binding of inducer to an IlvY protein-operator DNA complex.

Effects of Inducer on the Conformation of the IlvY Protein-DNA Complex—The appearance of DNase I hypersensitive sites in and around the protected regions of DNase I footprints (Fig. 6) are often indicative of protein-induced DNA bends. In fact, other LysR type activator-repressor proteins have been shown to induce DNA bends at their target DNA binding sites; and, in some cases, these bends are modulated by the binding of an effector ligand (26–28). To examine the possibility that IlvY protein might incite a bend in the DNA helix, circular permutation assays were conducted according to the methods of Wu and Crothers (21). A 240-bp EcoRI-HindIII DNA fragment containing the tandem operator sites of the ilvYC operator-promoter region, was ligated into the unique SalI site of pBEND2 (9), and isometric (circularly permuted) DNA fragments containing the tandem operators were generated by cleavage at six tandemly repeated restriction endonuclease sites flanking the SalI site. The relative base pair positions of the ilv specific DNA fragment within each of these circularly permuted fragments is identified in Fig. 9B. These DNA fragments were incubated with IlvY protein in the presence and absence of inducer, and the products of these binding reactions were resolved by non-denaturing polyacrylamide gel electrophoresis.

The binding of IlvY protein to these circularly permuted DNA fragments results in the formation of protein-DNA complexes with differing position-dependent electrophoretic mobil-
ities (Fig. 9A), indicating a protein-induced DNA bend. The center of the IlvY protein-induced DNA bend was estimated by plotting the relative mobility of each of the IlvY protein-DNA complexes against the relative bp position of the center of each DNA fragment and extrapolating this curve to the relative bp position of the IlvY protein-DNA fragment with the slowest mobility (21). This localized the center of the IlvY protein-induced DNA bend to within the O2 site near the DNase I-hypersensitive site at bp ∼37. In the presence of inducer, the center of the IlvY protein-induced bend of the DNA helix did not change but the difference between the mobilities of the fastest and slowest migrating complexes decreased (Fig. 9B). This result suggests that the binding of inducer to the IlvY protein-DNA complex relaxes the angle of the IlvY protein-induced bend of the DNA helix. In the absence of IlvY protein, the free DNA fragments also exhibited differing position-dependent electrophoretic mobilities. Using the empirical relationship cos a/2 = Rf (slowest)/Rf (fastest) (22), the angles of IlvY protein-induced DNA bending were estimated to be 60° in the absence of inducer and 50° in the presence of 1 mM inducer. In the absence of IlvY protein, a sequence-specific DNA bend of 36°, also centered in the O2 region, was observed (Fig. 9B).

Although the difference between these bending angles is small and may not be significant, additional evidence for this inducer-mediated change of IlvY protein-induced bending of the DNA helix was obtained from the results of DNase I footprinting experiments of the transcribed strand of the ilvC gene. In the absence of substrate inducer, IlvY protein protects three contiguous regions of DNA ilvC bp −78 to −55, −51 to −43, and −41 to −19 in the operator sequences defined as O1 and O2 (Fig. 3B). The addition of inducer to an IlvY protein-DNA complex, however, results in the appearance of several DNase I-hypersensitive sites (ilvC bp −76, −70, −61, −57, −49, −47, and −30) aligned along one face of the DNA helix (Fig. 6B). The addition of inducer also results in the protection of an additional DNase I reactive site at ilvC bp −55 on the opposite face of the DNA helix. A schematic summary of these DNase I footprinting protection patterns in the presence and absence of inducer on both the transcribed and nontranscribed strands of the ilvC gene is shown in Fig. 10. Given the pattern and periodicity of DNase I reactive sites induced and protected, these results and the DNA bending assays suggest that the binding of inducer facilitates the conversion of a sharp IlvY protein-induced DNA bend to a smoother bend of a smaller angle.

The results of hydroxyl radical footprinting experiments also support an inducer-mediated conformational change in an IlvY protein-operator DNA complex. The binding of IlvY protein to the tandem operators results in the protection of nucleotide

**FIG. 8.** In vitro transcription reactions of the divergent overlapping ilvYC promoter region in the presence of a minimally saturating concentration of purified IlvY protein and increasing concentrations of the co-inducer α-acetohydroxybutyrate (AHB). Autoradiogram of the RNA products of in vitro transcription reactions using the supercoiled DNA template, pDD3Y (see “Materials and Methods”). IlvY and ilvC identify the 369- and 154-nucleotide RNA transcripts that originate from their respective promoters. Lane 1, no IlvY protein; lanes 2–11, [IlvY protein] = 4.0 × 10−4 M dimer and [AHB] = 4.7 × 10−3 mM; 9.4 × 10−3 mM; 1.8 × 10−2 mM; 3.75 × 10−2 mM; 7.5 × 10−2 mM; 1.5 × 10−1 mM; 3.0 × 10−1 mM; 1.25 mM; 2.5 mM; 5 mM, respectively. The 108-nucleotide transcript designated ori originates from the RNA-I promoter.

**FIG. 9.** Circular permutation analysis of IlvY protein binding to the tandem operators. A, autoradiogram of a minimally saturating concentration of IlvY protein (4 × 10−4 M dimer) bound to isometric “circularly-permuted” 32P-end-labeled DNA fragments containing the tandem operators O1−O2. Bound denotes IlvY protein-bound DNA complexes and Free denotes unbound DNA fragments. Relative base pair position of an ilv-specific DNA fragment (bp positions −111 to +1 relative to the start of ilvC transcription) (3) contained within a 270-bp circularly permuted DNA fragment: lanes I and 11, 10 bp; lanes 2 and 10, 43 bp; lanes 3 and 9, 61 bp; lanes 4 and 8, 67 bp; lanes 5 and 7, 79 bp; lane 6, 110 bp. Lanes 11–7 are repeats of lanes 1–5, respectively. B, table of relative Rf value as a function of the relative base pair position of an ilv-specific DNA fragment (bp positions −111 to +1 relative to the start of ilvC transcription) (3) contained in a 270-bp DNA fragment and calculated angles of bending for Free, IlvY protein-bound, and IlvY protein–α-acetolactate-bound DNA fragments. The relative Rf value of each of these species was determined by normalizing the migration of the DNA fragment yielding the fastest migrating species as an Rf value of 1. Calculated angles of DNA bending were determined using the empirical relationship cos (a/2) = Rf (slowest)/Rf (fastest).
residues predominantly aligned along one face of the DNA helix, and alters the relative chemical reactivity of several residues within the protected regions. These results are shown in Fig. 11 and schematically summarized in Fig. 10. In addition, it is important to note that in both DNase I and hydroxyl radical footprinting experiments, IlvY protein remains bound to both operator sites even in the presence of inducer.

Effects of Substrate Inducer on the Kinetics of the Transcription Initiation Reaction at the ilvC Promoter—To determine the effects of inducer binding to a preformed IlvY protein-DNA complex on the kinetics of the transcription initiation reaction at the ilvC promoter, abortive transcription assays of the ilvC promoter region complexed with IlvY protein were performed in the presence and absence of inducer (23). An IlvY protein dimer concentration of \(4 \times 10^{-8}\) M, which yields greater than 99% saturation of O1O2 (Fig. 2) was used in each transcription reaction. The results of these assays showed that the addition of 1 mM inducer to an IlvY protein-operator DNA complex increases the binding affinity of the ilvC promoter for RNA polymerase (\(K_d\)) nearly 100-fold and decreases the isomerization rate for open complex formation (\(k_2\)) approximately 7-fold (Table I). Thus, the principal effect of substrate-inducer binding to an IlvY protein-DNA complex on the transcription initiation reaction at the ilvC promoter is to increase the affinity of ilvC promoter for RNA polymerase.

**DISCUSSION**

We previously demonstrated that, in vivo, IlvY protein autoregulates expression of its own structural gene in the divergent-overlapping ilvYC operon in an inducer-independent manner and that both inducer and IlvY protein are required for the activation of ilvC gene expression (6). We further demonstrated that both activation and repression appear to be mediated from the same regulatory locus. Thus, we suggested that the binding of inducer to IlvY protein targets an activator property distinct from its DNA binding activity and that the inducer-directed activation of transcription from the ilvC promoter might functionally be correlated with a conformational change of a preformed IlvY protein-operator DNA complex (6). Previous work also demonstrated that the binding of inducer to IlvY protein does not significantly affect the occupancy of IlvY protein at the tandem operators (6). The results of DNase I and hydroxyl radical footprinting experiments reported here demonstrate that, in the absence or presence of inducer, IlvY protein remains bound to both operator sites. Therefore, these results definitively establish that the role of inducer in the IlvY protein-mediated activation of transcription from the ilvC promoter is not to recruit IlvY protein to the ilvC promoter region.

The binding of inducer to an IlvY protein-DNA complex evokes the induction of a hypersensitive DNase I site in the −35 hexanucleotide region of the ilvC promoter (Fig. 6) and other changes in the DNase I and hydroxyl radical footprinting patterns in the tandem operator region (Figs. 6, 10, and 11). These results suggest a ligand-induced conformational change in the IlvY protein-operator DNA complex. Quantitative analyses of these ligand-induced DNA structural changes further establish that this conformational change is functionally cor-
related with transcriptional activation. For example, the inducer concentration that results in half-maximal induction of the DNase I hypersensitive site at bp −37 in the −35 hexanucleotide region of the ilvC promoter is the same concentration that half-maximally activates transcription from this promoter (Fig. 7).

The nature of the inducer-directed conformational change in the IlvY protein-operator DNA complex is suggested by the results of circular permutation assays (Fig. 9). These experiments indicate that the binding of IlvY protein to DNA fragments containing the tandem operator sites increases a sequenced-directed DNA bend of approximately 36° to a bend angle of approximately 60°. The mean geometric center of both the sequence-directed and IlvY protein-induced bends is positioned in the −35 hexanucleotide region of the ilvC promoter. The addition of inducer to the IlvY protein-operator DNA complex causes the partial relief of this bend to about 50°. This conclusion is supported by the distribution of hydroxyl radical protection patterns obtained in the presence of both IlvY protein (4 × 10⁻⁸ M dimer) and AHB (1.25 mM) and indicated with a thin gray line.

![Image of hydroxyl radical footprint](http://example.com/hydroxyl footprint)

**Fig. 11. Hydroxyl radical footprint of IlvY protein-operator DNA interactions in the ilvC promoter region.** Plots of the relative band intensities in digitized images of autoradiograms of hydroxyl radical protection patterns obtained in the absence (thick line) and presence (thin black line) of a minimally saturating amount of purified IlvY protein (4 × 10⁻⁸ M dimer) bound to a 240 bp EcoRI-HindIII DNA fragment containing ilv bp positions −111 to +1 (relative to ilvC transcription) (3). DNA fragments were uniquely ³²P-end-labeled on either the nontranscribed (A) or the transcribed (B) strand of the ilvC gene. Hydroxyl radical protection patterns obtained in the presence of both IlvY protein (4 × 10⁻⁸ M dimer) and AHB (1.25 mM) are indicated with a thin gray line.

| Condition | $K_b$ | $h_2$ |
|-----------|------|------|
| +Inducer  | 10⁶ M⁻¹ | 10⁶ sec⁻¹ |
| −Inducer  | 2 ± 0.7 | 7.7 ± 1.2 |

Protein-induced DNA bending is known to influence the rate of transcription initiation at many promoters. In some cases, protein-induced bending of the DNA helix enhances the binding of RNA polymerase to the promoter region (29). In other cases, it enhances the rate of open complex formation (23, 24). The results of abortive transcription assays (Table I) indicate that the principal effect of the inducer-mediated conformational change in the IlvY protein-operator DNA complex is to increase the binding affinity of RNA polymerase for the ilvC promoter nearly 100-fold. This suggests that the functional effect of the inducer-mediated conformational change in the IlvY protein-DNA complex and the associated alterations in DNA bending is to remodel the structure of the poor −35 hexanucleotide region of the ilvC promoter region to make it a better template for RNA polymerase recognition. The results of these abortive transcription assays are also in general agreement with the proposal that the −35 region of a promoter is involved in the initial recognition of a promoter by RNA polymerase (29). The nucleotide sequence of the −35 hexanucleotide region of the ilvC promoter (TTTCCG) exhibits only a 3/6 match to the consensus sequence (TTGACA).

The results reported here also demonstrate that IlvY protein represses the expression of its own structural gene by a simple RNA polymerase occlusion mechanism. The results of *in vitro* transcription reactions conducted in the presence of increasing concentrations of IlvY protein show that IlvY protein binding to the tandem operators represses transcription from the ilvC promoter and that the fractional repression of ilvY expression and the fractional saturation of the tandem operator sites exhibit the same dependences on IlvY protein concentration (Fig. 5). The simplest interpretation consistent with this set of data is that IlvY protein competitively inhibits the binding of RNA polymerase at the ilvC promoter site. This conclusion is consistent with the results of chemical and enzymatic structural probing experiments, which demonstrate that both IlvY protein and RNA polymerase bind to overlapping DNA se-
quences on the same face of the DNA helix (6).

It is interesting that the inducer titration curves of ilvC transcription shown in Fig. 7 correspond to a simple noncooperative binding model. This result shows that the activation of transcription from the ilvC promoter is not dependent on cooperative binding of inducer to multiple sites on the IlvY protein at the O₁O₂ sites. These experiments also show that the measured inducer concentration required to half-maximally activate in vitro transcription from the ilvC promoter is nearly the same as that required to half-maximally saturate the enzyme product of the ilvC gene (5). These concentrations are in good agreement with the inducer concentrations required for in vivo activation of ilvC expression (6, 30, 31).

It is interesting to consider the activation mechanism described here in relation to that of the MerR protein (32), a mechanistically similar ligand-responsive activator protein which is not a member of the LysR family of proteins. In this case, the binding of the inducer, mercuric ion, to a MerR-operator DNA complex in the divergent-overlapping merR-merTPCAD operator-promoter region also facilitates the partial relief of a MerR-induced DNA bend. However, in this case the primary effect of the binding of inducer, and relief of protein-induced DNA bending, is the partial unwinding of the spacer region between the −35 and −10 hexanucleotide regions of the merTPCAD promoter. This unwinding facilitates open complex formation and/or promoter clearance by the transcription apparatus rather than the recruitment of RNA polymerase to the promoter site as is the case for IlvY protein. Thus, whereas both of these cases illustrate an important role for ligand-induced conformational changes of preformed protein-DNA complexes in the regulation of gene expression, they also highlight the functionally distinct roles these conformational changes may play. However, both of these examples emphasize the importance of protein-induced conformational changes on the structure of the DNA helix for the regulation of gene expression.

The kinetic effect of the ligand-induced, IlvY protein-directed, structural change in the IlvY protein-ilvC promoter DNA complex reported here is to recruit RNA polymerase to the promoter site. This might be accomplished by remodeling the DNA helix to facilitate improved RNA polymerase-DNA interactions. Alternatively, the primary effect of the inducer-mediated IlvY protein conformational change might be to facilitate protein-protein interactions between IlvY and RNA polymerase. In this case, the relaxation of the bend angle in the ilvC promoter region might be the fortuitous consequence of the inducer-mediated IlvY protein conformational change, and the primary role of IlvY protein might be to enhance RNA polymerase recruitment via facilitated protein-protein interactions with RNA polymerase across opposite faces of the DNA helix. The relative contributions of each of these components awaits further analysis.

Finally, it is interesting to note that inducer-responsive alterations in the angle of protein-induced bending of the DNA helix have also been reported for other LysR-type proteins such as OccR (33), CysB (28), and CatR (27). Thus, ligand-mediated alterations in protein-induced bending of the DNA helix appears to be a general feature among members of the LysR family. However, the functional relationship between ligand-induced conformational changes and the activation mechanism in these cases is uncertain. Thus, the initial elucidation of the activation mechanism employed by the IlvY protein reported here should facilitate our understanding of the regulatory mechanisms of other LysR-type regulatory systems.

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