Allosteric Mechanism of Induction of CytR-regulated Gene Expression

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Transcription from cistrons of the Escherichia coli CytR regulon is activated by E. coli cAMP receptor protein (CRP) and repressed by a multiprotein complex composed of CRP and CytR. De-repression results when CytR binds cytidine. CytR is a homodimer and a LacI family member. A central question for all LacI family proteins concerns the allosteric mechanism that couples ligand binding to the protein-DNA and protein-protein interactions that regulate transcription. To explore this mechanism for CytR, we analyzed nucleoside binding in vitro and its coupling to cooperative CytR binding to operator DNA. Analysis of the thermodynamic linkage between sequential cytidine binding to dimeric CytR and cooperative binding of CytR to deoP2 indicates that de-repression results from just one of the two cytidine binding steps. To test this conclusion in vivo, CytR mutants that have wild-type repressor function but are cytidine induction-deficient (CID) were identified. Each has a substitution for Asp281 or neighboring residue. CID CytR281N was found to bind cytidine with three orders of magnitude lower affinity than wild-type CytR. Other CytR mutants that do not exhibit the CID phenotype were found to bind cytidine with affinity similar to wild-type CytR. The rate of transcription regulated by heterodimeric CytR composed of one CytR281N and one wild-type subunit was compared with that regulated by wild-type CytR under inducing conditions. The data support the conclusion that the first cytidine binding step alone is sufficient to induce.

The transport proteins and enzymes required for nucleoside utilization in Escherichia coli are encoded by genes belonging to the CytR regulon (1). This gene family consists of nine unlinked transcriptional units whose expression is coordinately controlled by the interplay of two gene regulatory proteins. Transcription is activated in response to intracellular cAMP levels by CRP1 and repressed by a three-protein, CRP-CRP-CRP, complex. Transcription is induced when CytR binds cytidine. A central feature of this coordinate regulation is that CytR and CRP bind cooperatively to their respective operators (2). This is so despite the role of CytR as a functional antagonist of CRP. The critical role that cooperativity plays is highlighted by the fact that expression is induced, because this cooperative interaction is lost when CytR binds cytidine. Cytidine binding has no effect on intrinsic CytR binding to DNA.

CytR is a member of the LacI family of bacterial repressors (3). The gene regulatory activity of each of these proteins is modulated by binding a peripheral ligand, which functions as either inducer or co-repressor. The basic DNA binding unit of each of these proteins is a homodimer in which helix-turn-helix domains from both subunits combine to form the DNA binding interface. Since both subunits harbor identical ligand binding sites, the allosteric mechanism that couples inducer or co-repressor binding to changes in the macromolecular interactions that regulate transcription is an important issue to this entire family of proteins.

For both PurR and LacI, conformational transitions that accompany ligand binding have been investigated by x-ray crystallography (4–6). In these two cases, binding of co-repressor or inducer, respectively, causes a change in tertiary structure that alters substantially the dimer interface. In the non-DNA binding conformation, hinge helices that connect the helix-turn-helix motif to the ligand binding globular core domain are destabilized, and the helix-turn-helix motifs from the two subunits are thought to be out of register with successive DNA major grooves. In this manner, cooperative ligand binding (7, 8) to the individual subunits controls a concerted quaternary conformational change of the dimer. These features are consistent with MWC allostery. While the structural mechanisms that couple ligand binding to tertiary conformation differ in the two proteins (4–6), the tertiary and quaternary structural perturbations are remarkably similar.

The structures of the LacI family proteins, including CytR, appear to be highly conserved (5, 6, 9, 10). Given the structural resemblance among family members plus the similarity of allosteric mechanism for LacI and PurR, a similar mechanism might be anticipated for CytR. Yet CytR differs from all LacI family members in that it is cooperative that is allosterically controlled and not intrinsic DNA binding. Allostery thus appears to have a different structural basis in CytR than in other LacI/PurR proteins.

Understanding the allosteric mechanism is central to understanding coordinate regulation of the CytR regulon genes. Recently, we showed that CytR binds to multiple operators at one CytR regulated promoter, deoP2 (12). CytR binding to the operator responsible for repression interacts cooperatively with CRP binding to flanking CRP sites, CRP1 and CRP2. However, by binding to additional specific sites, CytR competes with CRP.

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1 The abbreviations used are: CRP, E. coli cAMP receptor protein (CRP is also referred to as CAP, catabolite activator protein); CID, cAMP induction defective; MWC, Monod Wyman and Changeux; KNP, Koshland Nemethy and Filmer; CDA, cytidine deaminase; UDP, uridine diphosphorylase; Cm’, chloromphenicol resistant; Amp’, ampicillin-sensitive; MOPS, 4-morpholinepropanesulfonic acid; bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.
for binding to CRP1 and CRP2. The net result of cooperativity and competition is that while CRP recruits CytR to form the repression complex, there is no significant reciprocal recruitment of CRP by CytR. This effect has also been reported for the nupG promoter (13). These interactions presumably function to direct both a multistage activation of transcription, using both Class I and Class II CRP mechanisms (14) and also a similar multistage repression mediated by CytR. We have proposed that this might be a general feature of CytR-mediated gene regulation (12).

The unique mechanism of cytidine mediated induction also suggests a multistage process. The cooperativity to which cytidine binding is linked appears to be complementary pair wise in nature. This follows from the observation that the free energy change characterizing cooperativity in the three protein complex, CRP-CytR-CRP bound to DNA, is equal to the sum of free energy changes characterizing pairwise cooperativity between CytR and CRP bound either to CRP1 or to CRP2 (12). If cooperativity in the three-protein repression complex is pairwise, then it is easy to envision that the two subunits of the dimer might react independently to cytidine binding. This would result in sequential elimination of pairwise, CytR-CRP cooperativity, hence sequential relief from repression, in response to sequential cytidine binding to the subunits.

The most general possibilities for coupling between ligand binding and transcription initiation are presented in Fig. 1. We have combined biophysical chemical and molecular genetic approaches to investigate these possibilities. First, CytR binding to CRP-saturated deoP2 was analyzed to evaluate the total contribution from cooperativity. Subsequently, CytR binding titrations were conducted as a function of cytidine concentration. The shape of the transition characterizing loss of cooperativity as cytidine binds CytR indicates that induction is an all or nothing process that occurs concomitant with only one of the cytidine binding steps. Second, CytR mutants were isolated and characterized as fully functional repressors, but which do not induce. The only defect in these mutants is inability to bind cytidine. By co-expressing cytidine induction-defective subunits and wild-type subunits, we evaluated whether the resulting heterodimers would support induction with only one subunit capable of binding cytidine. The combined data from these studies indicate that induction results when cytidine binds to the first subunit of the CytR dimer.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—Table I lists the bacterial strains and plasmids used in this study. The CytR allele, cytRD281N, was transferred to the bacterial chromosome as described by Winans et al. (15). First, the cat gene was inserted into plasmid pCB071-161 at a position 44 bp of the cytR termination codon, resulting in plasmid pCB122. Second, E. coli strain JS803 was transformed with linearized pCB122, and a recombinant strain, SS6140, was selected as chloramphenicol-resistant (Cmr) and ampicillin-lin-sensitive (Amp'). The cytRD281N allele was subsequently transferred to other strains by P1 transduction. The presence of the cytRD281N allele was verified in each Cm' isolate by enzyme assays. The tsx-lac gene fusions carried by strains GP4, Tex-lac500, Tex-lac501, Tex-lac502 and Tex-lac503 (16) were transferred into strain SS6003 by P1 transduction. The cytR:TexIdTet insertion was then moved from SS6018 into each SS6003 derivative, yielding strains SS6117 through SS6121 (Table I).

To express CytR, the coding sequences of wild-type and mutant cytR alleles were subcloned as an NdeI/BamHI fragment downstream of the T7 promoter carried by plasmid pSS584. Strain BL21(DE3) (17, 18) was transformed with each construct. The control plasmid for these experiments is pCB135, a pSS584 derivative devoid of cytR coding sequence.

Bacteria were collected from exponentially growing cultures for enzyme assays. The medium contained Vogel and Bonner salts (19) supplemented with vitamin B1 at 5 μg/ml, 0.02% casamino acids, and 0.4% glycerol (20). BL21(DE3) derivatives harboring CytR plasmids were grown in a 1% Bacto-tryptone, 0.4% glycerol medium containing Vogel and Bonner salts (TV medium). Either L-broth or 2 × YT was used for transformations and plasmid preparations (21). The Lac- phenotypes of the various strains were determined on solid TTC-Lac medium as described previously (20). When used, the final cytidine concentration was 2 mM. Antibiotic concentrations used in the media were: ampicillin, 100 μg/ml; tetracycline, 15 μg/ml; chloramphenicol, 20 μg/ml; and kanamycin, 25 μg/ml in minimal medium or 50 μg/ml in rich medium.

Generation, Identification, and Characterization of CytR Mutants—A...
mixture of mutagenic oligonucleotides complementary to cytR codons 276 through 284 was synthesized using an Applied Biosystems model 381A DNA synthesizer. The spiking protocol of Hutchison et al. (22, 23) was used to create degeneracy in the oligonucleotide sequence. The mutagenic oligonucleotide mixture and a site-directed mutagenesis kit from Amersham Corp. was used to mutate the cytR gene on an M13mp18 cytR10 template. Both single and multiple mutations were obtained, the frequency of single mutations being about 30%. Phage pooled from about 5000 mutagenized M13mp19 cytR10 plaques was obtained, the frequency of single mutations being about 30%. Phage M13mp19 cytR10 template. Both single and multiple mutations were used to create degeneracy in the oligonucleotide sequence. The superscript 2 indicates that the designated endonuclease cleavage site has been removed by reaction with either the Klenow fragment of DNA polymerase I or with T4 DNA polymerase.

| E. coli strains                | Characteristics         | Source       |
|-------------------------------|-------------------------|--------------|
| SS6003                        | F′ thi leu rpsL Δ(argF-lac) U169 | S. Short     |
| SS6004                        | F′ Δcid281 D281N         | S. Short     |
| SS6005                        | F′ Δcid281 D281N         | S. Short     |
| SS6018                        | F′ Δcid281 D281N         | S. Short     |

| Plasmids                      | Characteristics         | Source       |
|-------------------------------|-------------------------|--------------|
| pCB071–161                    | Amp′, an Nde I ′ , Cia I ′, pcBR322 derivative carrying cid allele cytRD281N | S. Short     |
| pCB093                        | A Kan′, pcB071 derivative carrying the wild-type cytR gene | S. Short     |
| pCB094                        | A Kan′, pcB093 derivative lacking the cytR gene and flanking sequence | S. Short     |
| pCB095                        | A Kan′, pcPL4 derivative carrying the wild-type cytR gene and flanking sequence | S. Short     |
| pCB096                        | A Kan′, pcB093 derivative deleted only for the cytR coding sequence | S. Short     |
| pCB122                        | An Amp′, pCB071–161 derivative. The cat gene from pACYC184 has been inserted into the AflII site in the tetracycline resistance gene | This study   |
| pCB123                        | A Kan′, pcSS584 derivative carrying the wild-type cytR gene | This study   |
| pCB124                        | A Kan′, pcSS584 derivative carrying cytRD281N | This study   |
| pCB127                        | A Kan′, pcSS584 derivative carrying cytRM151V | This study   |
| pCB128                        | A Kan′, pcSS584 derivative carrying cytRM151I | This study   |
| pCB131                        | A Kan′, pcSS584 derivative carrying cytRV15A | This study   |
| pGLP4                         | A Kan′, pcACYC184 derivative | S. Short     |
| pSS584                        | A Kan′, pcB093 derivative. Contains the galK coding sequence fused to a T7 promoter | S. Short     |
| pSS1332                       | An Amp′, pUC19 derivative with an 858-bp insert containing the deoP2 region of the deo operon | S. Short     |

| Plasmids                      | Characteristics         | Source       |
|-------------------------------|-------------------------|--------------|
| M13mp19-cytR10                | M13mp19 derivative containing the cytR coding sequence bounded by Smal and BamHI | This study   |

- The envelope of gene segment on a purified, double-stranded template (20).
- Bacteria used for enzyme assays were grown and cell extracts prepared as described previously (25). Purification of CytR—CytR was purified using a simpler protocol than that reported several years ago (2) but which yielded a higher yield of CytR with similar purity. All purification steps were carried out at 4 °C. Pellets from cells harvested 165 min postinduction were resuspended in 20 mM MOPS (pH 6.8), 2 mM MgSO4, 1 mM Na4EDTA, 10 mM NaCl, and treated with lysozyme, added to 10 mg/ml. The cells were frozen at −20 °C, thawed at 23 °C, and broken by sonication. The final cell extract was the clear supernatant remaining following centrifugation at 100,000 × g for 1 h at 4 °C.

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main stable for at least several years when stored in this manner.

Sedimentation equilibrium analysis shows this material to be homogenous dimer, with no evidence for either dissociation to monomer or association to higher order polymers over the concentration range, 0.1–10 μM.2 More recent analysis of gel mobility shift assays of CytR binding to DNA has been interpreted to indicate that CytR remains as stable dimer over the range of concentrations at which it binds DNA operators (11). Based on these data, the binding experiments were analyzed according to the simplest model in which CytR exists only as dimer.

Cytidine Binding Assays—Binding of [3H]cytidine to purified wild-type CytR and to both wild-type and mutant CytR containing cell-free extracts was measured using a filter binding assay. Binding reaction mixtures contained either 18–50 nM purified CytR dimer or 15–30 μg of cell extract protein in a 100-μl volume containing 0.04–110 μM [3H]cytidine (NEN Life Science Products). Two different buffers were used: 1) 20 mM MOPS (pH 6.8), 2 mM MgSO4, 1 mM NaEDTA, 200 mM NaCl and 20 mM bis-Tris (pH 7.0); 100 mM NaCl, 0.5 mM MgCl2, 0.5 mM CaCl2. Both buffers contained 100 μg/ml bovine serum albumin and 1 μg/ml calf thymus DNA. Following a 5-min incubation at 23 °C, the CytR-bound [3H]cytidine contained in 80 μl of assay mix was collected on a prewashed nitrocellulose filter (Millipore HAWP 02500; Millipore Corp., Bedford, MA). The filters were washed once with 500 μl of assay buffer, air-dried, and then dissolved in 3.5 ml of Packard Filter-Count LSC mixture (Packard Instrument Co.). Radioactivity was measured using a Packard model 1900TR scintillation counter.

For determination of nucleoside binding constants, binding assays were conducted as titrations by varying the nucleoside concentration at constant CytR concentration. The data were analyzed according to a simple Langmuir binding model as described below (see Equation 1) using a nonlinear least squares curve fitting program (32). The CytR concentration used in some titrations was not negligible. To analyze data under these conditions, the conservation polynomials for total cytidine and total CytR were solved for the concentration of free cytidine for each data point and at each iteration of the nonlinear least squares analysis. The program NONLN (33) was used for this purpose.

In experiments to compare [3H]cytidine binding by CytR mutants in cell-free extracts and wild-type CytR, the CytR content of the cell-free extracts was estimated using Western immunoblots as described above. For each extract, 25–200 ng of extract protein was electrophoresed on SDS-16.5% acrylamide gels. Proteins were electrotransferred to Immobilon-P membranes (Millipore Corp.) as described previously (20). CytR was complexed with anti-CytR antibody and [35S]protein A. [35S]Protein A in the complex was quantitated using a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Competition by other nucleosides for [3H]cytidine binding to wild-type CytR in extracts was measured in the MOPS assay buffer (20). CytR was complexed with anti-CytR antibody and [35S]protein A. [35S]Protein A in the complex was quantitated using a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

CRP Protein Used in DNA Binding Studies—CRP was the kind gift of James C. Lee (University of Texas Medical Branch, Galveston, TX). This protein preparation shows no evidence of any contaminating material by Coomassie staining of overloaded SDS-polyacrylamide gels, from which we conservatively estimate at least 98% purity.

Individual Site Binding Experiments—A series of DNase I footprint titrations of CytR binding to deoP2 was conducted to assess the effect of cytidine binding to CytR on heterologous cooperativity between CRP-cAMP and CytR (Fig. 3). An 879-bp NotI/HindIII restriction fragment containing the deoP2 regulatory sequence was isolated from a derivative of pSS1332 (Table I) in which an 8-bp NotI linker was inserted into the Smal site of the polylinker. The fragment contains the E. coli deo sequence from +151 to −801 from the P2 start site for transcription. The fragment was labeled with 32P at the NotI site by using the Klenow fill-in reaction and purified as described (34).

Each separate titration of CytR binding to this fragment (Fig. 4) was conducted at a different, fixed concentration of cytidine ranging from 1 nM to 10 μM and at a saturating concentration of CRP-cAMP. CytR was the only nucleic acid DNase I footprint reactions were conducted as described (12) in the bis-Tris (pH 7) cytidine binding buffer described above. CRP and cAMP were added to final concentrations of 0.10 μM (as dimer) and 100 μM, respectively. Each titration was analyzed using NONLN (33) according to Equation 1 to obtain the apparent free energy

| ctyR allele | Induction, 2 μl cell extract | Specific activity in cell extractsa |
|------------|-----------------------------|-----------------------------------|
|            | [3H] cytidine              | CDA units/mg protein               |
| Wild-type  | −                           | 2                                 |
| cytRG273C  | +                           | 119                               |
| cytRD281A  | +                           | 53                                |
| cytRD281E  | +                           | 3                                 |
| cytRN282I  | +                           | 2                                 |

3 Confidence limits to equilibrium constants are asymmetric. Asymmetric limits are reported as absolute values in parentheses following the maximum likelihood estimate.

RESULTS

Identification and Characterization of CID Mutants—We previously described two CytR mutants, D281N and D281Y, that are repression-competent but nonresponsive to induction by cytidine (20). We denote such cytidine induction-defective mutants as CID. Degenerate oligonucleotide-directed mutagenesis of cytidine codons 276 through 284 was used to identify other residues that are critical for cytidine induction. This mutagenesis yielded 21 mutant ctyR genes that produced stable CytR. Twelve of these mutant genes encoded inactive repressors. Eleven of these inactive repressors have amino acid substitutions for either His277 or Asp274 and are recessive. The other inactive repressor (C279R) has an inactive, dominant negative phenotype. The remaining nine of these mutant genes produced CID phenotypes. Three of these are previously identified mutants, and six are newly identified mutants. Four of the newly identified mutants have single amino acid substitutions, three for Asp281 (Table II) and one for Glu279. The others carried double substitutions (F280/D281A and F280/S/N282I).

The CID phenotype of the four new single substitutions was verified by measuring enzyme synthesis from two CytR-controlled genes, cdd and udp, in the presence and absence of cytidine. As was found with the original CID mutants, expression of these CytR-controlled genes is repressed by the mutant CytR proteins and is unaffected by cytidine (Table II). Only D281A was a significantly less active repressor than the wild-type protein. However, like all CID mutants, D281A did not
response to cytidine. All CID cytR alleles were expressed identically and all produced steady-state cellular CytR concentrations equal to that of the wild-type protein based on Western blot analysis of soluble extracts from the mutant strains. As found for other CytR mutants (20), the CytR controlled enzyme levels and the phenotype observed for bacteria expressing these CID mutants reflect directly the change in repressor function.

Functional repressor might show the CID phenotype if repression was no longer dependent on heterologous cooperative interactions with CRP-cAMP. To determine whether CID mutants require such interactions for repression, we compared the ability of each repressor to regulate transcription from wild-type and mutant tsx-lac reporter gene fusions. The tsx-lac gene fusions were constructed and characterized by Gerlach et al. (16). The tsx DNA of each mutant reporter gene fusion contains a single bp substitution in CRP2, which greatly reduces its affinity for CRP-cAMP binding. These point mutations have no direct effect on intrinsic CytR binding; CytR binding is affected only indirectly via loss of CytR-CRP cooperativity. This loss of cooperativity prevents repression of mutant tsxP2 promoters by wild-type CytR in vivo, even when expressed at high levels from a multicopy plasmid (37). Thus, expression from tsx-lac gene fusions provides a specific, direct assessment of CytR-CRP cooperativity. It avoids the use of bacteria having either cyar- or crp- mutations and their pleiotropic effects.

Both wild-type and CID CytR repress β-galactosidase synthesis from wild-type tsxP2. Regulation of mutant tsx-lac fusions by wild-type and CID repressors was also identical (Table II). Neither protein represses expression from the mutant promoter. Therefore, repression by mutant CID CytR is dependent on cooperative interactions with operator-bound CRP-cAMP, the same as repression by wild-type CytR. Thus, the only identifiable defect in function of the CID CytR proteins is their failure to respond to cytidine induction.

Allosteric Mechanism of Cytidine-mediated Induction

Cytidine Binding by Wild-type and CID CytR—The induction-defective phenotype of CID CytR could result either if the protein fails to bind inducer or if it binds but fails to respond to inducer. To assess these options, cytidine binding was studied directly. Titration of purified wild-type CytR yielded a single binding transition (Fig. 2). When analyzed according to a simple binding model (Equation 1), an apparent $K_d$ value of 0.17 (0.11,0.33) μM was obtained. The limiting ratio of cytidine retained to CytR dimer is 0.82. While this is similar to a stoichiometry of one per dimer, it doesn’t account for CytR protein that isn’t retained by the nitrocellulose filters and so only represents a lower limit to the stoichiometry. Cytidine binding is unaffected by addition of cell free extracts from an E. coli strain that is deleted of the cytR gene ($K_d = 0.16 (0.11,0.30)$ μM). Cytidine binding was also the same whether using purified CytR or cell free extracts from an E. coli strain containing the wild-type cytR gene (data not shown). Ligand binding is also specific. In competition assays conducted in the presence of a 1000-fold excess of nucleoside competitor (1 mM), only adenine competed with cytidine ($K_d = 22.5 ± 1.5$ μM). Uridine, 2′-deoxycytidine, 2′,3′-dideoxycytidine, cytidine arabinoside, and both 2′- and 3′-O-methylcytidine had no effect on cytidine binding in vitro, consistent with the inducer specificity in vivo (38–40).

Since addition of CytR-free E. coli extracts has no effect on cytidine binding to purified CytR, cytidine binding to wild-type and mutant CytRs in cell free extracts can be compared directly. Extracts were prepared from isogenic strains expressing either wild-type CytR, CytR from one of the different classes of mutants, or no CytR. There was no detectable cytidine binding in extracts lacking CytR. Of the mutant CytR containing extracts, only that containing the CytR repressors (CytR8281N) showed a significant decrease in cytidine binding (Table IV) as compared with the extract containing wild-type CytR. At a cytidine concentration (11 μM) that essentially saturates wild-type CytR (0.98 saturation), this CID CytR bound less than 1% as much cytidine as wild-type. This indicates a $K_d$ for the mutant CID CytR of 1 mM or greater, an affinity at least 2000-fold reduced from that of wild-type CytR. An effect of such magnitude can only be readily explained by differences between CytR in the different cell-free extracts. By contrast to this result, CytR with amino acid substitutions in domains proposed previously (20) to function in DNA binding (CytR815A) and in signal transduction (CytRM151I and CytR151M) bound roughly the same amount of cytidine as wild-type CytR, thus indicating no effect on cytidine binding affinity.

Effect of Cytidine Binding to CytR on Heterologous Cooperative Binding of CytR to deoP2—DNase I footprint titration was used to determine the apparent affinity of CytR for deoP2 at
The contribution from cooperativity is reduced when CytR binds to CytR-cAMP (Equation 1) and is accompanied by only a negligible effect on intrinsic CytR binding (Equation 2). The square root of the variance of the fit is: $s = 0.22$. Analysis according to complete Scheme I (broken curve) yields $\Delta G_{app} = -9.96 \pm (-11.87, -8.86)$ kcal/mol ($K_{1} = 2.7 \times 10^{9} \text{ M}^{-1}$), $\Delta G_{2} = -8.04 \pm 0.33$ kcal/mol ($K_{2} = 1.0 \times 10^{9} \text{ M}^{-1}$), $\Delta G_{3} = -13.15 \pm 0.18$ kcal/mol ($K_{3} = 6.5 \times 10^{9} \text{ M}^{-1}$), $\Delta G_{4} = -12.77 \pm (-13.07, -12.60)$ kcal/mol ($K_{4} = 3.4 \times 10^{9} \text{ M}^{-1}$), $\Delta G_{5} = -10.67 \pm 0.13$ kcal/mol ($K_{5} = 9.2 \times 10^{9} \text{ M}^{-1}$), and $s = 0.23$. Confidence limits (calculated at the 68% level) are listed in parentheses when asymmetric. The horizontal dotted line indicates the intrinsic (noncooperative) $\Delta G$ for CytR binding to deoP2 reflecting the data shown in Fig. 5.

### Scheme I. Equilibria between CytR, cytidine, and deoP2-(CytR-cAMP)$_{2}$

$$K_{app} = \frac{K_{1} + K_{2}[\text{cyt}] + K_{3}[\text{cyt}]^{2} + 1}{[\text{cyt}] + [\text{cyt}]^{2}}$$

Equilibrium constants, $K_{1}$-$K_{5}$, are independent parameters. Since $K_{1}$, $K_{2}$, and $K_{5}$ comprise a thermodynamic cycle, $K_{6}$ is defined by $K_{6} = K_{1}K_{2}/K_{5}$. Similarly, $K_{7} = K_{2}K_{3}K_{4}$. $K_{5}$ was set equal to zero.

Equation 2 was used to analyze the values in Fig. 3 ($\Delta G_{app} = -RT\ln K_{app}$) to estimate the Gibbs free energy changes corresponding to $K_{1}$-$K_{5}$. Results of this analysis are indicated by the broken curve in Fig. 3. The maximum likelihood parameter estimates indicate an affinity for binding of the first cytidine, $K_{1} = 2.7 \times 10^{9} \text{ M}^{-1}$. In this model, this binding step is accompanied by only a negligible effect on $\Delta G_{app}$ ($0.26$ kcal/mol). The second subunit binds cytidine with significantly lower affinity ($K_{2} = 1.0 \times 10^{9} \text{ M}^{-1}$) and is accompanied by a major reduction in $\Delta G_{app}$ (40-fold decrease). (Note: $K_{2}$ is a stepwise microscopic equilibrium constant; it corresponds to a microscopic constant for cytidine binding to the remaining cytidine binding site equal to $2.0 \times 10^{9} \text{ M}^{-1}$.) This result suggests that cytidine binding is negatively cooperative and that the major allosteric switch occurs at only one of the two cytidine binding steps.

An alternative possibility that is also consistent with this conclusion is that the allosteric switch occurs at the first cytidine binding step. If so, the transition in Fig. 3 represents cytidine binding to only the first of the two CytR subunits. To test this possibility, the data were analyzed using a simplified version of Scheme I, in which $K_{1}$ and $K_{2}$ were set equal to zero. Results of this analysis, indicated by the solid curve in Fig. 3, yielded $K_{1} = 2.0 \times 10^{9} \text{ M}^{-1}$. Thus, both models estimate the same cytidine affinity as being allosterically coupled to cooperative binding of CytR to the deoP2-(CytR-cAMP)$_{2}$ complex.
Goodness of fit criteria such as the variances and distributions of residuals provided no basis for discriminating between the two models. By contrast, all other models tested, such as the model in which the two subunits have independent (noninteracting) cytidine binding sites, which are either separately or together coupled to cooperative operator binding, failed to provide an adequate fit to the data.

Inducibility of Wild-type-CID Heterodimers in Vivo—The existence of well characterized CID alleles provides the means to test, in vivo, the conclusion that cooperativity is coupled primarily to only one cytidine binding step and also to determine whether the first or second step. To do so, steady-state expression from CytR-regulated promoters was examined in bacteria that co-express both wild-type, inducer-responsive CytR subunits and CID, nonresponsive CytR subunits. Wild-type and CID subunits were expressed by cistrons that had identical promoter and operator regions. Since both wild-type and CID CytR are fully functional repressors, their co-expression should result in heterodimeric CytR with one wild-type subunit and one CID subunit.

To promote heterodimer formation, CID allele cytRD281N was first recombined in single copy into the chromosome. As shown in Table V, CytRD281N that is expressed from the bacterial chromosome is indistinguishable from wild-type CytR in its ability to repress CDA and UDP synthesis, but retains its CID phenotype. Second, isogenic strains that differ only in their chromosomal cytR allele (either wild-type or CID) were transformed by a plasmid that expresses the wild-type cytR gene. A low copy number plasmid (41, 42) containing a P15A origin was used. These constructions yielded bacteria with the same cytoplasmic CytR level (see “Materials and Methods”) as one another. This is as expected, since expression of all cytR alleles is identically regulated and both wild-type and CID proteins are functional, stable repressors. The response to induction was compared using these two strains. To ensure that steady-state transcriptional activity was being compared as opposed to steady-state enzyme levels governed by protein turnover, the differential rate of CytR-controlled β-galactosidase synthesis from a udp-lac fusion was measured in exponentially growing cells.

The differential rate of CytR regulated β-galactosidase synthesis in bacteria that co-express wild-type and CID CytR subunits should depend on the response of CID/wild-type heterodimers to cytidine. There are three possibilities. First, if the heterodimer is induction-defective, then both CID/CID homodimers and CID/wild-type heterodimers would repress udp-lac expression. Because the wild-type allele is plasmid-encoded, while the CID allele is chromosomal, these bacteria have excess wild-type subunits, hence excess wild-type homodimers. However, under inducing conditions, these homodimers no longer bind cooperatively to the promoter; their affinity is reduced by 2 orders of magnitude (Fig. 3). Consequently, even a severalfold excess of wild-type homodimers would be insufficient to compete effectively with induction refractive heterodimers and
the small proportion of CID homodimers. The differential rate of β-galactosidase synthesis would approximate that of CID homozymous bacteria, even under inducing conditions. Second, if CID and wild-type subunits do not associate to form heterodimers, then no CID subunits expressed must be present as CID homodimers. Again, the level of noninducible CytR, now comprised of the CJD/CID homodimers, would be sufficient to yield a noninducible phenotype. Third, if (and only if) cytidine binding by the wild-type subunit of CID/wild-type heterodimers is sufficient for induction, then the differential rate of β-galactosidase synthesis under inducing conditions would be much greater than that observed for CID homozymous bacteria, even approaching that observed for wild-type CytR homozymous bacteria. Full wild-type levels of induction would not be expected because of competition by the nonresponsive CID/CID homodimeric CytR, even when present as a small fraction of the total repressor.

The rates of bacterial growth and β-galactosidase synthesis for the individual strains are shown in Fig. 6. These rates were constant throughout the entire experiment, demonstrating that cytidine induction is at equilibrium and that the enzyme levels represent steady-state production in all cultures. The differential rates of β-galactosidase synthesis were calculated from these curves. Under inducing conditions, the rate for bacteria expressing both wild-type and CID CytR subunits was found to be 5-fold greater than that measured for CID homozymous bacteria (2543 units versus 499 units). It was slightly greater than half of that found for wild-type homozymous bacteria (4827 units). These results can be explained if wild-type and CID subunits do associate to form heterodimers, which in turn do respond to induction by cytidine.

DISCUSSION

CRP and CytR mediate coordinate regulation of the unlinked genes that encode the proteins necessary for nucleoside util-

zation in E. coli. The interplay between these regulatory proteins, comprised of both cooperative and competitive interactions, appears to direct both multistage activation and multistage repression of transcription of individual cistrons (12). The critical role of CytR-CRP cooperativity is highlighted by the mechanism of cytidine-mediated induction. This gene regulatory mechanism relies on loss of cooperativity, not on reduction in DNA binding affinity as found with other LacI family members. Understanding the allosteric coupling between cytidine binding and CytR-CRP cooperativity is necessary to understanding the coordinate regulation of this gene family.

The specific question addressed herein is whether induction is a concerted process coupled to the quaternary state of the CytR dimer or a sequential process coupled to the ligated state of the individual subunits. The former holds for LacI and PurR, which undergo an MWC-type allosteric transition between quaternary T and R states (4–6). CytR presumably does not experience the same global conformational change upon ligand binding as LacI and PurR, since inducer binding is not coupled to DNA binding. Moreover, CytR-CRP cooperativity appears to be pairwise and complementary, in nature (12). A multistage induction such as would result from a sequential coupling
between binding of cytidine to an individual subunit, and a
tertiary conformational change affecting only that subunit’s
cooperative interaction, could play a significant role in differ-
tential expression of the unlinked genes.

Three allosteric mechanisms can be considered: first, a clas-
se MWC mechanism featuring an equilibrium between two
symmetric quartenary states, one that interacts cooperatively
with CRP and one that does not (Fig. 1A); second, a strictly
sequential KNF mechanism in which the tertiary confor-
mation of each subunit switches to a noncooperative state concomitant
with cytidine binding to that subunit (Fig. 1B); third, a sequen-
tial but concerted mechanism in which distinct quartenary
states are formed as each cytidine site is filled (Fig. 1C). Elimina-
tion of CytR-CRP cooperativity and induction of transcrip-
tion might occur when the first cytidine binds, when the second
binds, or in part when both bind in proportion to the overall
fractional saturation.

We investigated the cytidine-mediated transition from coop-
erative to noncooperative CytR binding to CRP-saturated
deoP2 to distinguish among these possibilities. The data were
analyzed according to a general formulation (Scheme I; Equa-
tion 1) that encompasses all three allosteric mechanisms. Only
two numerical solutions of Equation 2 were found to be con-
sistent with the data. Two common features of these solutions
both point to the third allosteric mechanism (Fig. 1C) and are
inconsistent with MWC and KNF mechanisms (Fig. 1A and
B). First, the analysis suggests that the transition from coop-
erative to noncooperative CytR binding is coupled to a single
cytidine binding event. Second, cytidine binding is character-
ized by negative cooperativity.

That loss of cooperativity is coupled to only a single cytidine
binding step is reflected by the characteristic shape of the
transition curve. Other mechanisms, such as a cooperative
transition between pre-existing states (MWC; Fig. 1A) or se-
quential coupling to each cytidine binding step (e.g. KNF; Fig.
1B) yield either sharper or shallower transitions. These are
inconsistent with the data. The finding of negative cooperativ-
ity in cytidine binding to CytR is also inconsistent with an
MWC allosteric mechanism. A concerted transition between
pre-existing states necessarily yields positive cooperativity in
ligand binding.

The two numerical solutions to Equation 2 do differ in detail
regarding the negative cooperativity. When both cytidine bind-
ing steps are considered in the analysis, the cytidine binding
constants estimated indicate approximately a 7-fold effect.
When Equation 2 is truncated to consider only one cytidine
binding step, this is equivalent to the assumption that linkage
reflects only the first cytidine binding step. This would mean
that binding of the second cytidine is insignificant over the
concentration range investigated, suggesting a much higher
degree of negative cooperativity.

We cannot distinguish between these possibilities, even
based on the titration data for cytidine binding to free CytR
(Fig. 2). These data were reanalyzed by considering cytidine
binding as comprising two steps. This analysis (Fig. 1) esti-
ated an intrinsic cytidine binding affinity equal to 0.4 μM,
neary identical to that identified as being coupled to loss of
cooperativity in DNA binding (0.5 μM; Fig. 3) and negative
cooperativity accounting for greater than an 80-fold affect. It
also yielded a limiting ratio of cytidine retained to CytR dimer
equal to 1.60, consistent with a stoichiometry of two per dimer.
According to this model, the transition in Fig. 1 corresponds
primarily to the first cytidine binding step; the plateau con-
 tinues sloping upward, reflecting the second binding step that
occurs at higher cytidine concentration. These features mirror
the result obtained from the DNA binding data in Fig. 3, when
the latter are analyzed simply by assuming linkage to only the
first cytidine binding step.

However, the cytidine titration data in Fig. 2 are also rea-
sonably described by the alternative analysis of the DNA bind-
ing data. The data in Fig. 2 were analyzed using the stepwise
cytidine binding constants, $K_1$ and $K_2$, obtained from analysis
of the DNA binding data in Fig. 3 as fixed input parameters.
According to this interpretation, cytidine binding in Fig. 2 looks
like a single transition, because the negative cooperativity is
very moderate to produce separate binding transitions. This
analysis estimates a limiting ratio of cytidine retained to CytR
dimer equal to 0.9. While this is quite low compared with the
model’s stoichiometry of two per dimer, the discrepancy could
reflect poor CytR retention efficiency in the filter assay.

Despite uncertainty in details, these analyses support three
conclusions: first, that cytidine binding to CytR is negatively
cooperative; second, that cooperative binding of CytR to
deoP2<CRPcAMP> is primarily coupled to only one of the two
cytidine binding steps; and third, the intrinsic cytidine binding
affinity in free CytR that is coupled to this transition is 0.2–0.5
μM. Thus, cytidine binding must switch CytR between three
conformational states, one corresponding to each cytidine liga-
tion state, as represented by Fig. 1C. However, these data do
not identify which conformational change eliminates CytR-CRP
cooperativity. To address this issue, it is necessary to evaluate
the behavior of the intermediate state with only one subunit
liganded. For this, wild-type and CID CytR alleles were co-
expressed, thus allowing assembly of hybrid CytR dimers,
which have only one subunit capable of binding cytidine. The
behavior of these hybrids in vivo was used to assess the induc-
tion competency of the intermediate ligation state.

For this approach, it was necessary to find a CytR mutant
whose only defect is inability to bind cytidine. We focused the
search on a region of the CytR sequence in which CID mutants
had been identified previously (20). The newly identified CID
mutants expressed wild-type levels of protein, and most had
repressor activity equal to wild-type (Table II). One allele,
cytRN281N, was found independently both in the previous
screen following random mutagenesis of the entire CytR gene
(20) and in the present screen following targeted mutagenesis.
CytRD281N was shown to require cooperative interaction with
CRP for repression as does wild-type CytR (Table III). From
this we infer that repression remains coupled to the mecha-
nism that underlies induction. Cytidine binding assays demon-
strated the only defect found, a 2000-fold or greater reduction
in cytidine binding affinity (Table IV).

The finding that cytidine binding affinity is reduced as a
result of amino acid substitutions for Asp281 is consistent with
observations on other LacI proteins. This is a conserved aspar-
tate in the sequences of many of the LacI repressors as well as
the E. coli periplasmic binding proteins (3). The equivalent
aspartate is essential for ligand binding in LacI, PurR, and the
periplasmic sugar-binding proteins for glucose, ribose, and
arabinose (43–45). The substitutions for CytR Asp281 that yield
the CID phenotype (Asn, Ala, Glu, and Ile) are understandable
if Asp281 participates directly in cytidine binding as a hydrogen
bond partner to sugar hydroxyls. Perhaps, the decreased affin-
ity of CID mutants for cytidine is functionally equivalent to the
inability of 2'- or 3'-deoxycytidine to compete with cytidine for
binding to wild-type CytR. The substitutions that yield the CID
phenotype suggest that charge (D281N) and side chain size
(D281E) as well as hydrogen bonding potential (D281A) are
important to this interaction. Whether this interaction pro-
vides only affinity and specificity, or is also important to the
coupling between cytidine binding and CytR-CRP cooperativ-
ity, cannot be determined from our data. However, we note that
in LacI, only affinity is affected (46).

Evaluation of the capacity of the hybrid dimers to support induction in vivo assumes that the steady-state levels of wild-type and CID subunits are proportional to the gene copy number. Since the wild-type and CID alleles are identically regulated, this assumption is supported by the finding of comparable CytR levels in extracts made from bacteria expressing either wild-type or mutant CytR. Thus, for bacteria expressing both alleles and assuming a plasmid copy number of 6–8 per cell (41, 42) and random assortment of subunits, then CytR dimer is proportioned about 75% as wild-type homodimer, 23% as heterodimer, and the remainder as CID homodimer. Uncertainty in plasmid copy number has a negligible effect on this distribution. If heterodimers respond to cytidine, this distribution yields a 50-fold excess of inducible over non-inducible dimers. When offset by the higher affinity of non-induced dimers, some reduction of the extent of de-repression is expected, perhaps in line with what was observed (Fig. 6). If heterodimers do not respond to cytidine binding, then only a 3-fold excess of inducible over noninducible dimers results. This is insufficient to compete effectively for DNA binding under inducing conditions and de-repression should not be observed. Similarly, if CID and wild-type subunits do not assemble as heterodimers, then the CID subunits must be assembled as CID homodimers to account for the fact that CID CytR is functional repressor. Again, the excess of inducible over noninducible dimer would not be sufficient to support significant de-repression. Therefore, the in vivo data support the conclusion that hybrid dimers respond to cytidine. Presumably, half-saturated wild-type dimers behave in the same manner.

These results, obtained by monitoring the effect of cytidine on CytR-regulated gene expression in vivo, are remarkably consistent with those obtained by in vitro investigation of allosteric coupling between cytidine binding to CytR and cooperative interaction of CytR and deoP2 (CRPcAMP). This consistency supports a molecular model in which cytidine binding switches CytR between three states and binding of cytidine to the first subunit of CytR dimer yields complete induction. Because cytidine binding to CytR is negatively cooperative, the third CytR state, that generated by ligation of both subunits by cytidine, forms only at very high cytidine concentrations. Such concentrations are not normally attained in E. coli nor were they attained in our experiments. We note the similarity to CRP, which also has three distinct conformational and functional states corresponding to its cAMP ligation states (47).

Why does CytR differ from other LacI family members for which ligand binding affects the poise of an equilibrium between DNA binding and non-DNA binding conformations? We envision two possibilities. First, perhaps the CytR inducer binding core domain does undergo a similar conformational change when cytidine binds, but this is uncoupled from the structure of the DNA binding domain. Comparing the sequences of the Lac repressors, we note that where other family members have a pair of conserved alanines in the hinge helix sequence, CytR has proline (Pro57) and glycine (Gly59) (3). Perhaps, instead of hinge helices, CytR has an extended coil such that DNA binding is unaffected by the conformation of the core dimer. Second, perhaps cytidine binding is uncoupled from the T-state to R-state transition, but is instead coupled to transitions between subconformations belonging to the quarter- nary R-state. With this scenario, it is interesting to speculate whether saturation by cytidine might induce a T-state transition and whether this would have an effect on DNA binding.