Role of Multiple CytR Binding Sites on Cooperativity, Competition, and Induction at the Escherichia coli udp Promoter

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Stacey A. Gavigan, Tulan Nguyen, Nghia Nguyen, and Donald F. Senear‡

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92697

The CytR repressor fulfills dual roles as both a repressor of transcription from promoters of the Escherichia coli CytR regulon and a co-activator in some circumstances. Transcription is repressed by a three-protein complex (cAMP receptor protein (CRP)-CytR-CRP) that is stabilized by cooperative interactions between CRP and CytR. However, cooperativity also means that CytR can recruit CRP and, by doing so, can act as a co-activator. The central role of cooperativity in regulation is highlighted by the fact that binding of the inducer, cytidine, to CytR is coupled to CytR-CRP cooperativity; this underlies the mechanism for induction. Similar interactions at the different promoters of the CytR regulon coordinate expression of the transport proteins and enzymes required for nucleoside catabolism but also provide differential expression of these genes. A fundamental question in both prokaryotic and eukaryotic gene regulation is how combinatorial mechanisms of this sort regulate differential expression. Recently, we showed that CytR binds specifically to multiple sites in the E. coli deoP promoter, thereby providing competition for CRP binding to CRP operator site 1 (CRP1) and CRP2 as well as cooperativity. The effect of the competition at this promoter is to negate the role of CytR in recruiting CRP. Here, we have used quantitative footprint and mobility shift analysis to investigate CRP and CytR binding to the E. coli udp promoter. Here too, we find that CytR both cooperates and competes for CRP binding. However, consistent with both the distribution of CytR recognition motifs in the sequence of the promoter and the regulation of the promoter, the competition is limited to CRP2. When cytidine binds to CytR, the effect on cooperativity is very different at the udp promoter than at the deoP2 promoter. Cooperativity with CRP at CRP1 is nearly eliminated, but the effect on CytR-CRP2 cooperativity is negligible. These results are discussed in relation to the current structural model of CytR in which the core, inducer-binding domain is tethered to the helix-turn-helix, DNA-binding domain via flexible peptide linkers.

The Escherichia coli CytR regulon comprises at least nine unlinked transcriptional units that encode enzymes and transport proteins required for nucleoside catabolism and recycling. CRP1 activates transcription of these units in response to intracellular cAMP levels. Transcription is repressed by CytR, a member of the LacI family of bacterial repressors, and is induced when CytR binds cytidine. These features are common to all of the unlinked transcriptional units that comprise the regulon and serve together to coordinate their regulation (1). However, a key feature of the CytR regulon is that extents of activation, repression, and induction vary substantially among the different transcription units (cf. Refs. 2 and 3).

An intriguing question is how the interplay among these two transcriptional regulatory proteins and the various promoters yields differential regulation. Presumably, combinatorial mechanisms that rely on local features of the promoters, such as different arrangements of the control elements, are involved. Similar combinatorial mechanisms also appear to be important in the regulation of cell growth and differentiation, processes that also often involve a small number of key regulatory proteins. Thus, the CytR regulon has general significance as a model for understanding gene regulatory processes. Our goal is to understand how functional, multi-protein, transcription complexes form at different promoters with different arrangements of protein binding sites.

CytR has two features that are not observed in other LacI family members and that appear to be important to its role as a differential transcriptional regulator. First, CytR and CRP bind cooperatively to form a three-protein complex on the DNA. In this complex, CytR binds to an operator site (usually referred to as CytO) that is flanked by tandem CRP operators, CRP1 and CRP2, as are found in most CytR-regulated promoters. CytR forms a protein bridge between the bound CRP dimers. The importance of this cooperativity is highlighted by the fact that expression is induced because the cooperativity is lost when CytR binds cytidine; cytidine binding to CytR has no effect on the intrinsic DNA binding of CytR (4, 5). Therefore, it is the three-protein complex that is the functional repressor, not CytR alone. Second, CytR exhibits lower DNA binding specificity than other LacI family members and most other bacterial repressors. As a consequence, as described below, CytR does not bind only to CytO but also binds to additional binding sites whose number and arrangement appear to differ among the promoters. Given these facts, we wish to address two questions. First, how does binding of CytR differ in different promoters? Second, how do these differences affect cooperativity and its modulation by cytidine?

A key to how CytR binds to different promoters is its relatively broad DNA sequence specificity. Like other LacI family members, the basic DNA binding unit of CytR is a homodimer (6). As expected, based on this quaternary structure, CytR binding sites contain tandem recognition motifs. However, the exact recognition motif has proven difficult to define. It has been reported as both TGCAA (7) and, more recently, as GTT-GCAT (8), based on different systematic evolution of ligands polymerase; bp, base pair(s); bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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‡ To whom correspondence should be addressed. Tel.: 949-824-8014; E-mail: dfsenear@uci.edu.

The abbreviations used are: CRP, cAMP receptor protein; CRP1 and CRP2, CRP operator sites 1 and 2, respectively; RNAP, E. coli RNA polymerase; bp, base pair(s); bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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by exponential enrichment experiments conducted by the same group. Based on where CytR binds specifically to the CytR-regulated deoP1 and deoP2 promoters, we proposed that TTGCAA, a symmetric variant of these sequences, is the recognition sequence (5). CytR is also unusually tolerant of variation in spacing between recognition motifs. The preferred spacing is 2–3 base pairs (7,8), but CytR-mediated regulation of gene expression has been demonstrated on synthetic promoters in vivo with spacing up to three helical turns (9).

In this context, it is important to note that most CytR-regulated promoters feature multiple degenerate repeats of the (TTTGCAAA sequence motif with variable spacing between them. Depending upon the spacing between such repeats, additional CytR binding sites might exist at these promoters. In fact, when we investigated CytR binding to the deoP2 promoter, we found that CytR does bind specifically to multiple sites (5). CytR and CRP bind cooperatively when CytR binds CytO. However, CytR also binds to separate, specific sites at deoP2, one of which overlaps CRP1, and another that overlaps CRP2. In this situation, CytR competes directly for CRP binding to CRP1 and CRP2.

This special mode of protein-DNA interaction in which CytR can either enhance CRP binding or compete for CRP binding affects both repression and activation. Repression by the cooperative CytR-CytR-CRP complex results from competition between CytR and RNAP, both of which are recruited by CRP to bind to the DNA sequence flanked by CRP1 and CRP2 (10). However, competition between CytR and CRP for binding to CRP1 and CRP2 provides a second mode of CytR-mediated repression. In activation, one consequence of the competition between CytR and CRP is to facilitate configurations in which CRP is bound either to CRP1 or to CRP2, but not to both. This is significant because CRP1 and CRP2 are thought to mediate different mechanisms of activation (11, 12). Based on their sequences (Ref. 5; Fig. 1), we expect the different promoters to vary as to whether CytR competes for CRP binding to CRP1, to CRP2, or to both sites. The primary function of CytR may be differential modulation of CRP1-mediated versus CRP2-mediated activation. In this way, different patterns of CytR binding at different promoters might provide differential gene regulation.

The broad DNA binding specificity of CytR might also result in different contributions to the stability of the three-protein repression complex at the different promoters. It has been shown recently that when CytR and CRP are used together to select DNA sequences that are preferred for formation of the three-protein complex, the sequences most commonly selected are CytR recognition motifs separated by 10–13 base pairs and almost centered between CRP1 and CRP2 (8). This result is surprising because it does not match what is found in the natural promoters, in which CytO is usually located significantly off-center, adjacent to either CRP2 (as in deoP2) or CRP1 (as in cdd, nupG, and udp) with a 2–5-bp spacing between recognition motifs. Nevertheless, a structural model has been proposed in which the DNA is wrapped smoothly around the three-protein complex, and the two CytR DNA binding domains (one per subunit) bind to DNA sequences that are arranged centrosymmetrically and are separated by 11 base pairs (13).

For a centrosymmetric three-protein complex to form as envisioned in the model, CytR would have to dissociate from CytO and instead bind more widely spaced and symmetrically arranged DNA sequences. Alternatively, CytR, CRP, and/or the DNA would have to be distorted from the symmetric arrangement proposed in the model to accommodate off-center binding by CytR to CytO. Either of these situations would necessarily contribute unfavorably to the stability of the three-protein complex, the former as a result of a decrease in CytR-DNA binding affinity, and the latter as a result of an unfavorable conformational change. Which of these two possible accommodations a particular promoter uses may depend on what alternative, relatively high affinity, CytR-DNA binding sites are provided by the local array of CytR recognition motifs.

In these ways, site-specific CytR binding, cooperativity, and competition are inextricably linked. Because cytidine is an effector of CytR-CRP cooperativity, its effect should also be linked to how CytR binds at the various promoters. This linkage might underlie the observed differences between the promoters in effectiveness of induction.

To test these hypotheses and to assess how linkage might be involved in differential regulation of transcription, we have investigated cooperative and competitive CytR and CRP binding to the udp promoter. We compare these interactions to those that we investigated previously at the deoP2 promoter. We chose udp for comparison to deoP2 because these promoters differ substantially in regulatory properties. The ranges of regulated rates of transcription in vivo are about 30-fold for the udp promoter (3) versus only about 5- to 6-fold for the deoP2 promoter (2). CRP is a more effective activator of udp than of deoP2, and CytR is a more effective repressor of udp than of deoP2. Whereas the two promoters do not differ significantly in the arrangement of CRP sites, they do differ in the arrangement of putative CytR binding sites (Fig. 1). Most prominently, the udp promoter contains no putative CytR binding site that occludes CRP1.

The results we present here demonstrate that CytR binds specifically to multiple sites at the udp promoter. The results also confirm the expectation that CytR and CRP compete for binding to CRP2 but not for binding to CRP1. However, the most interesting result we found is that cytidine binding to CytR has very different effects on the pattern of CytR-CRP cooperativity at udp from those observed by us at deoP2. Whereas cytidine binding essentially eliminates all CytR-CRP cooperativity in binding to deoP2, it has a very selective effect on CytR-CRP cooperativity in binding to udp. Cytidine binding to CytR largely eliminates pairwise cooperativity between CytR and CRP bound to CRP1 of udp. In contrast, it has a negligible effect on pairwise cooperativity between CytR and CRP bound to CRP2. The net effect on cooperativity in the CytR-CytR-CRP complex is moderate.

These results indicate that CytR is highly adaptable to different arrangements of CytR and CRP sites in the absence of cytidine in order to form the three-protein repression complex. However, much of this adaptability appears to be lost when CytR binds cytidine. Thus, the arrangement of the operators might have a substantial influence on induction. An interesting explanation for this behavior can be found in what is known about the structure of CytR and the allosteric mechanism of induction.

MATERIALS AND METHODS

Reagents and Enzymes—Crystalline eAM (>99% pure as free base) was purchased from Sigma. Crystalline cytidine (>99% pure as free acid) was purchased from ICN. Stock concentrations in 50 mM Bis-Tris, pH 7.0, and 1 mM EDTA were determined, and purity was assessed spectrophotometrically as described previously (5). DNase I (code D from Worthington) was treated as described previously (14, 15). [α-32P]Deoxyxynucleotide triphosphates (3,000 Ci/mmol) were purchased from NEN Life Science Products; unlabeled deoxyxynucleotide triphosphates were obtained from Life Technologies, Inc. Buffer components and reagents were electrophoresis grade, if available; otherwise, they were reagent grade.

CRP and CytR Purification—The CRP preparation used has been described previously (5). CRP overexpressed from plasmid pFLcCRP1 (16) in E. coli strain K12 was isolated to at least 98% purity. The CRP
Electrophoretic Mobility Shift Analysis of CytR Binding to udpP

Cooperativity and Competition in Cytidine-mediated Induction of udpP

Dried gels were imaged using a Molecular Dynamics PhosphorImager and amino acid residues in a protein (17, 18). This calculated value is about 11,300 L/mmol (Max). In Equation 1, $Y_i$ represents the fraction of CRP bound to site $i$, $P_o$ is the baseline fractional protection for a given promoter, $P_{max}$ is the maximum fractional protection for a given promoter, $k$ is the association constant, and $L$ is the size of the promoter DNA fragment used for footprinting. Equation 1 is given by:

$$ Y_i = P_o + (P_{max} - P_o) \cdot \frac{k \cdot L}{1 + k \cdot L} $$

In Equation 1, $Y_i$ is the fractional saturation of binding site $i$ at the free protein ligand concentration, $L$ is the association constant, and $P_o$ and $P_{max}$ are the baseline and maximum fractional protection for a given titration (14). For simple binding of either CRP or CytR alone, Equation 1 gives the intrinsic free energy change for local binding, $\Delta G_i$. For binding experiments in which both CytR and CRP are present, analysis according to Equation 1 provides an accurate estimate of the individual site loading free energy change, $\Delta G_i$, and its confidence limits (26, 27).

Subsequently, global analysis of the individual site CytR and CRP binding data was conducted using equations that describe cooperative and competitive binding of CRP and CytR according to the model defined by the promoter configurations specified in Table II. Equations to describe the binding to each of the individual sites were derived by considering the relative probability of each promoter configuration as given by the following equation:

$$ f_i = e^{-\Delta G_{iRT}} \cdot [CRP(cAMP)]^2 \cdot [CytR]^j $$

For the specific CRP binding motif in Figure 1, the following equation was used:

$$ f_i = e^{-\Delta G_{iRT}} \cdot [CRP(cAMP)]^2 \cdot [CytR]^j $$

$\Delta G_i$ is the sum of free energy contributions for configuration $s$ (Table III). $i$ and $j$ are the stoichiometries of bound CRP(cAMP) complexes and CytR dimers in configuration $s$. Summation of the relative probabilities for all configurations in which protein is bound to any given site derives the binding equation for that site. For reduced crp1 and crp2, configurations in which CRP(cAMP)1, is bound to the mutated site were excluded from the summation.

Global numerical analysis was conducted as described previously (5, 28) using the nonlinear least squares parameter estimation program NONLIN (29). Variances obtained from the initial separate analysis of each binding curve using Equation I were used to calculate normalized weighting factors. Goodness of fit and internal consistency were evaluated based on two criteria: (i) comparison of the loading free energy changes for each of the individual sites in wild type and mutant operators as calculated from the global fitting parameters to the values that were determined experimentally; and (ii) comparison of the ratio of variances for separate and global analyses of binding to each individual site to the F-statistic.

Mobility Shift Titrations—Mobility shift titrations were conducted as described previously (5) using 3.5% acrylamide gels (29:1 acrylamide: bisacrylamide) and 0.5× Tris/borate/EDTA electrophoresis buffer (23).

Proteins and udp DNA (10 pg of the 498-bp fragment) were incubated for 40–60 min at 20 °C (±0.1 °C) in the DNase I footprint binding buffer plus 2 µg/ml calf thymus DNA. 1.5% Ficoll was added to facilitate gel loading. Aliquots (20 µl) of equilibrated binding reaction mixtures containing 500–600 dpm of 32P were loaded onto 1.5-mm minigels in a Bio-Rad Mini Protein II device that had been pre-electrophoresed for 5 min. Gels were electrophoresed at a constant 150 V for 35 min. As a control, samples were loaded in some gels with the current on and in others with the current off. These yielded indistinguishable results.

Dried gels were imaged as described above, and the images were analyzed as described previously (5, 27) to determine the fraction of DNA in each electrophoretic band, $\theta_i$. Whenever applicable, $\theta_i$ was related to the macroscopic product association equilibrium constant by $K_{eq} = e^{-\Delta G_i} / [CytR]^j$.

The value of $N$ in $\theta_i$ was treated as an adjustable parameter in the analysis, representing the average CytR stoichiometry of the higher order complexes.

RESULTS

Our goal is to understand how CytR and CRP mediate differential regulation of CytR-regulated promoters. In particular, why is CRP a more effective activator and CytR a more effective repressor of udp than of deoP2, two promoters with similar regulatory elements (Fig. 1)? The arrangements of regulatory elements in these promoters differ from one another in two respects. First, whereas both promoters contain CytR recognition motifs that overlap CRP2, the udp promoter lacks CytR recognition motifs overlapping CRP1 and downstream to the 10 promoter element, which are present in deoP2. Do these motifs in udp constitute separate, specific CytR binding sites? Do differences between CytR binding sites in udp and deoP2 result in different patterns of competition between CytR and CRP? Second, CytO in the udp promoter is located directly adjacent to CRP1, whereas it is located more equidistant between CRP1 and CRP2 in the deoP2 promoter. Does this affect CytR-CRP cooperativity?
sites to which CytR can bind simultaneously, we first conducted titrations of CytR binding alone using electrophoretic mobility shift analysis. In addition to the unliganded udp DNA band, at least three distinct lower mobility bands are observed as a function of increasing CytR concentration in these experiments (Fig. 2A). The bands were quantitated (see “Materials and Methods”) in each lane to assess the CytR concentration dependence of each band (Fig. 2B).

An important result of this analysis is that the maximum fraction of DNA for each successive mobility-shifted band occurs at a successively higher CytR concentration. This fact identifies these bands unambiguously as representing higher order ligation states (27). It is possible for species to separate electrophoretically because they differ in topology rather than in CytR stoichiometry. However, any such species must have the same CytR concentration dependence as one another. No such species are observed. Therefore, each successive decrement in mobility represents binding of additional CytR.

Calf thymus DNA was present in these experiments at approximately a 1,000-fold gram excess over udp DNA (see “Materials and Methods”) and at a concentration sufficient to provide a ratio of nonspecific binding sites to the highest CytR concentrations used of almost 1:1. This had no effect on the concentration dependence for appearance of the first several mobility-shifted bands. Therefore, these bands represent site-specific binding.

The band distribution in Fig. 2B was analyzed according to Equation 3 to estimate the free energy changes corresponding to macroscopic product equilibrium association constants for binding of one or more CytR dimers to udp. Equation 3 assumes that each successive mobility-shifted band results from the binding of exactly one additional CytR dimer. This assumption is supported by the close correspondence between the shapes of the fitted curves, which are sensitive to the binding order, and the experimental data. The results of this analysis (Fig. 2B) indicate that the first two CytR dimers that bind, bind to sites that have similar CytR binding affinity. Accounting for the statistical factors embedded in the macroscopic product association constants and allowing for some uncertainty in the total number of sites, the intrinsic free energy changes for binding to the two highest affinity sites are approximately −10 and −9.5 kcal/mol. Thus, the specificity for the preferred operator site is only a fewfold. In contrast, under the same reaction conditions, the specificity for the preferred deoP2 operator is 10-fold as compared with the next highest affinity site.

**Footprint Titration Analysis of CytR Binding**—Next, CytR binding to udp was investigated by DNase I footprint titration. This analysis allows us to connect the affinities estimated from the mobility shift titrations to individual DNA sites. When CytR binds to udp, a region extending from about 25 to 110 bp upstream from the start site for transcription is protected from cleavage (Fig. 3). This region encompasses CRP1, CRP2, and the intervening DNA. It is evident by inspection that protection occurs first, i.e. at the lowest CytR concentrations, in the intervening DNA where CytO is located; only at higher CytR concentration is comparable protection observed at CRP1 and CRP2. It is not clear by inspection to what extent the protection at CRP1 is distinct from overall protection of the entire DNA fragment. Overall protection is due to completely nonspecific CytR binding, and occurs at only slightly higher CytR concentrations.

The protection in blocks of contiguous DNA bands in CRP1, CRP2, and CytO (shown in Fig. 3A) was quantitated to produce the individual site binding isotherms shown in Fig. 3B. Analysis of the curves for CytO and CRP2 using Equation 1 yielded free energy changes equal to −10.9 ± 0.2 and −10.1 ± 0.2 kcal/mol, respectively. These values indicate that the specificity of CytR for CytO as compared with the next highest affinity site in the udp regulatory region (CRP2) is only a fewfold, thus confirming the results of the mobility shift analysis. Analysis of
individual site loading free energy changes equal to values from used in are plotted as a function of the log of the CytR dimer concentrations shown in curves for CytR binding to the left (CRP1).

D kcal/mol, and 0.03. This yielded a free energy change equal to 2 kcal/mol, indicating about 100-fold lower affinity than that for CytO, whereas the affinity for CytR binding to sites overlapping CRP1 is similar to that for nonspecific DNA.

Analysis of Cooperative and Competitive Binding of CytR and CRP—Cooperative interactions between CytR and CRP and competition between CytR and CRP for binding to CRP2 were evaluated by considering the thermodynamic cycles for simultaneous binding of both proteins. We have described this analysis previously (5, 27). Two types of binding experiments are necessary. Each type is conducted using wild type udp and also using each of the mutant promoters. In the first type of binding experiment, each protein, either CRP or CytR, is titrated alone; in the second, each protein, either CRP or CytR, is titrated in the presence of a constant, near the saturating concentration of the other protein (Fig. 4). As practical approximations to the limit of saturating concentrations, 0.1 μM CRP (total dimer) and 75 nM CytR were used here. This concentration of CRP yields 0.98 and >0.99 fractional saturation of CRP1 and CRP2, respectively. The CytR concentration yields 0.91 fractional saturation of CytO, about 0.72 fractional saturation of the region overlapping CRP2, and only about 0.09 fractional saturation of CRP1.

Results of these experiments are represented by the loading free energy changes evaluated for each of the titrating binding sites (Table I). For the CRP1- and CRP2- mutants, no specific binding of CRP to either mutated CRP site was observed, and intrinsic CRP binding to the nonmutated site was indistinguishable from what was observed for wild type udp. Similarly, CytR titrations of wild type, CRP1-, and CRP2- udp yielded indistinguishable binding curves for CytO. These results indicate that mutating either CRP1 or CRP2 has no effect on the intrinsic binding to any of the remaining sites. However, CytR binding to CRP2 is affected by the bp substitutions that eliminate site-specific CRP binding to this site. The affinity of CytR for the mutant DNA sequence is 4- to 5-fold higher than that for the wild type sequence.

One effect of competition between CytR and CRP for binding to CRP1 and CRP2 of deoP2 is that whereas CRP, whether bound to CRP1 alone, to CRP2 alone, or to both together, is a net recruiter of CytR to CytO; the reverse is not so. CytR is not a net recruiter of CRP to either CRP1 or CRP2 (5). This is because the favorable effect due to cooperativity with CytR binding to CytO is offset by the unfavorable effect of CytR binding to and competing for CRP1 and CRP2. The values in Table I demonstrate a similar behavior, although with a different pattern of effects, for CytR and CRP binding to udp. Here too, CRP bound to either CRP1 or CRP2 or both is a net recruiter for CytR binding. In contrast, whereas CytR is a net recruiter for CRP binding to CRP1, it is not a net recruiter for CRP binding to CRP2.

The pattern of interaction suggested by this result is entirely consistent with the protection pattern observed in DNase I footprints. It forms the basis for the molecular model represented by the udp promoter configurations listed in Table II. The salient features of this model are that CytR binds separately to CytO and to a site at CRP2. CytR binds separately to CRP1 and to CRP2. In this model, CRP and CytR compete for binding to mutually exclusive sites at CRP2, but CRP alone binds to CRP1 with no competition from CytR. CytR binding to
Fig. 4. Individual site binding of CytR and CRP(cAMP), to the udp promoter. A, CytR and CRP(cAMP), binding alone, each in the absence of the other. B—D, CytR and CRP(cAMP), binding together, i.e. the concentration of one protein is varied in presence of a fixed concentration of the other. B, binding to the udp promoter CRP1; C, binding to the wild type udp promoter. D, binding to udp promoter CRP2. Symbols denote the different operator sites: ▼, CRP2; ▲, CRP1; ●, CytR; ○, CytR binding to CRP2. The curves represent the global analysis of all data shown according to the model defined in Table II. Residuals from this analysis are shown below each panel. Parameter values are in Table III.

TABLE I

| udp valence | Titrant | Effector(s) | No. of experiments | Operator site |
|-------------|---------|-------------|--------------------|---------------|
| All CRP     | None    | 4           | CRP2               | −13.5 ± 0.3   |
| All CytR    | None    | 13          | CRP2               | −10.7 ± 0.4   |
| WT/CRP1−    | CytR    | 8           | CRP2               | −10.7 ± 0.2   |
| Wild type   | CytR    | 2           | CRP2               | −13.9 ± 0.1   |
| CRP1−       | CytR    | 2           | CRP2               | −13.7 ± 0.4   |
| CRP2        | CytR    | 2           | CRP2               | −13.5 ± 0.1   |
| Wild type   | CytR    | 3           | CytR               | −12.8 ± 0.2   |
| CRP1        | CytR    | 3           | CytR               | −12.4 ± 0.3   |
| CRP2−       | CytR    | 4           | CytR               | −12.7 ± 0.2   |
| Wild type   | CytR    | 3           | CRP, cytidine      | −12.2 ± 0.4   |
| CRP1        | CytR    | 2           | CRP, cytidine      | −11.9 ± 0.3   |
| CRP2        | CytR    | 2           | CRP, cytidine      | −11.2 ± 0.1   |

* Effector concentrations: CRP, 0.1 μM (total dimer); cAMP, 150 μM (present in all experiments); CytR, 75 nM (dimer); cytidine, 1 mM.

υ ΔGo values shown are the means of multiple determinations (± S.D.). The number of separate experiments represented in the means is indicated.

CRP1 and CRP2 and CytR binding to CytO are cooperative.

Fig. 4 shows the binding curves that result from a global analysis of the experiments represented in Table I conducted according to this model. These curves describe the data pertaining to each different titrant and different operator well, as indicated by residuals that are nearly randomly distributed in every case. Table III lists the parameter values. This analysis estimated the free energy change for CytR binding to CRP2 (ΔGo in Table II) based on the effect of the resulting CytR-CRP binding competition on the remaining fitting parameters. It did not fit the protection data for CytR binding to CRP2. In this way, the additional CytR site is defined thermodynamically but is not defined in relation to any particular sequence of DNA. The value of ΔGo obtained in this manner (−10.5 ± 0.4 kcal/mol; Table III) can be compared with that obtained independently from analysis of the protection data for CytR binding to CRP2 (−9.9 ± 0.5 kcal/mol; Table I) to assess how well the model represents the molecular interactions. There is no statistically significant difference between the two values, thus supporting the accuracy of our model.

As a control, we conducted an analysis in which ΔGo was fixed as equal to 0. This analysis addresses the question of whether a model that does not include competition between CytR and CRP for binding CRP2 can adequately describe the individual site binding data. When ΔGo was fixed in this manner, the result from the global analysis was a large increase in the variance of the fit. This increase was more than 4-fold greater than what is statistically significant at the 65% confidence level. This result confirms that competition between CRP and CytR for binding CRP2 is a significant feature of the interaction of these proteins with udp, just as it is for deoP2. It is a result that is consistent with both the extended DNase I protection pattern and the distribution of species in the mobility shift experiments.
When we analyzed CytR and CRP binding to the deoP2 promoter (5), we also considered an alternative explanation for the competition between CytR and CRP for CRP1 and CRP2. In this alternative view, CytR binding to CRP1 and to CRP2 would be nonspecific but would be nucleated by cooperative interactions with CytO bound to CytO. When we analyzed the deoP2 binding data according to this alternative model, we found that only negative cooperativity between CytR binding to CytO and CytR binding to flanking, nonspecific sites could account for the data (5). That is, according to this model, the nonspecific CytR sites flanking CytO would be the last nonspecific sites to fill, contradicting the direct observation that these sites fill first. In considering such a model as an explanation for CytR binding to deoP2, another contradiction is also evident. That is, if binding of CytR to sites flanking CytO is nonspecific, then protection of CRP1 and protection of CRP2 should have the same CytR concentration dependence, which they do not. For these reasons, nonspecific binding is not a viable explanation for CytR binding to CRP2 of deoP2.

**Effect of Cytidine Binding to CytR on Cooperativity**—CytR has a unique mechanism of induction among LacI family repressors. Whereas inducer or co-repressor binding to other family members controls a switch between DNA-binding protein and non-DNA-binding protein conformations, cytidine binding to CytR affects only cooperative interactions between CytR and CRP. Previously, we showed that the conformational change in CytR that eliminates its cooperative binding to CRP-liganded deoP2 occurs when the first of the two subunits of the CytR dimer binds cytidine. To ascertain whether this result of the conformational change is widespread or unique to deoP2, we also assessed the effect of cytidine binding to CytR on CytR-CRP cooperativity in binding to udp.

Fig. 5 shows binding of CytR to CRP-liganded udp in the presence of a saturating concentration (3) of cytidine. Control experiments showed that cytidine has no effect on intrinsic CytR binding to udp, confirming results obtained with other promoters (3–5). For comparison, Fig. 5 also shows the binding curve corresponding to the loading free energy (Table I) for CytR binding (cooperative) in the absence of cytidine. Loss of cooperativity is apparent in the rightward shift of the curve when CytR binds cytidine. However, this loss accounts for only a 0.6 kcal/mol effect on cooperativity according to the loading free energy changes listed in Table I. Thus, in contrast to other promoters, binding of the inducer has only a modest effect on CytR-CRP cooperativity at udp.

To assess whether a similar modest effect pertains to pairwise cooperativity, we also investigated the effect of cytidine on cooperative CytR binding to CRP1 and CRP2 promoters (Fig. 5). The results obtained using these two promoters were very different. Whereas the effect of cytidine on pairwise cooperativity between CytR and CRP bound to CRP2 is modest, similar to that for wild type udp, pairwise cooperativity between CytR and CRP bound to CRP1 is nearly eliminated, similar to that for deoP2 (3).

To assess these effects together with competition between CytR and CRP for binding CRP2, we extended the model defined in Table I to include terms to describe the effects of cytidine on cooperativity. Parameter values obtained in this analysis are shown in Table III. Consistent with the binding curves in Fig. 5, the fitted ΔG_{ij(k)} values indicate nearly complete elimination of CytR-CRP2 cooperativity, but only negligible effects on CytR-CRP1 cooperativity, resulting in modest effects on cooperative assembly of the three-protein complex. An interesting consequence of this pattern of effects is that cooperativity is still complementary pairwise when CytR is liganded by cytidine, just as in the absence of cytidine.

**DISCUSSION**

The most significant aspect of the CytR regulon is that it illustrates how different patterns of expression of different
intense interest (direct different kinetic mechanisms of activation from different CRP as an activator of transcription, particularly its ability to bacteria, whereas CytR is a regulon-specific factor. The role of gene regulatory proteins. Most of the CytR-regulated genes can be achieved using the same small complement of gating their cooperative binding to discovered competition between CytR and CRP while investigated by only 2- to 3-fold in udp, the binding curves for these sites overlap extensively; hence, CytR binding to CRP2 is necessarily of physiological relevance. In addition, it is only by including competition between CytR and CRP for binding to CRP2 in the global analysis of the footprint data that we can accurately account for the experimentally determined loading free energy changes (Table I). However, it is evident that this simple model (Table II) does not fully explain all six mobility-shifted species observed in the mobility shift assays. These species suggest a hierarchy of CytR binding from specific to nonspecific. The lower affinity of these species are probably not relevant physiologically because they are abundant only at very high CytR concentrations that may not reached in the bacteria. We have not addressed these weaker interactions in our model.

This simplification has a small but still observable effect on the accuracy of the fits to the titrations when CytR and CRP bind DNA together, with one protein in the presence of a near saturating concentration of the other. The loading free energy changes predicted by the fitted parameters differ from the experimentally determined values by 0.3 kcal/mol, on average, for these cases. These differences are small, accounting for only 1.5× in binding affinity. They are identifiable only because of the unusually precise underlying data.

Two significant features of the fitted parameters are not affected by this slight uncertainty. First, the relative affinities for CRP binding to CRP1 and CRP2 and for CytR binding to CytO are very similar in udpP2. Second, the free energy changes for pairwise cooperativity between CytR and CRP are the same whether CRP is bound to CRP1 or to CRP2 and are the same for udp as for deoP2. In both cases, the cooperative free energy change when all three sites are filled is greater than either of the individual pairwise terms and is similar to their sum. We referred to this pattern previously as complementary pairwise cooperativity (5). However, there is a small but significant decrease in cooperative free energy for the three-protein com-
plex CRP-CytR-CRP when bound to udp as compared with that seen when bound to deoP2. This suggests a structural inhibition to forming the three-protein repression complex on udp that is not present in deoP2.

An important result of our study is that whereas cytidine is an effector of CytR interactions with CRP bound to udp-CRP1, cytidine is not an effector of CytR interactions with CRP bound to udp-CRP2. A similar effect was noted also for the nupG promoter (32) but was not assessed quantitatively. At first glance, such a pattern of cytidine-mediated effects appears to make regulatory sense if CRP1 is the primary activating site, as may be the case for udp. However, the result of this pattern of effects is that the free energy change for formation of the ternary protein complex of CRP-CytR-CRP bound to DNA is only slightly smaller in the presence of cytidine than in its absence (Table 1). Thus, CytR occupancy of CytO is little affected by cytidine binding to CytR. Whereas the effect might be amplified to some extent by competition with RNAP for DNA binding, it seems unlikely that the change in CytO occupancy alone could explain induction. In this context, the suggestion that the primary competition between CytR and RNAP is for interaction with CRP (31) is attractive. If so, elimination of CytR-CRP interactions in response to cytidine binding might allow the CRP-RNAP interaction necessary for full activation even in the presence of DNA-bound CytR.

Recently, we analyzed the allosteric mechanism of cytidine-mediated induction (3). This analysis considered both the thermodynamic linkage between cytidine binding to CytR and the cooperative binding of CytR to deoP2 and also considered the inducibility of heterodimeric CytR composed of one wild type subunit and one mutant subunit that is unable to bind cytidine. Given the complementary pairwise CytR-CRP cooperativity noted above, the immediate question was whether induction is a stepwise process in which each subunit responds individually to cytidine binding or a concerted, all-or-nothing process in which the dimer responds, similar to other LacR repressors. Our results indicated that induction is a concerted process, but one in which both subunits respond when cytidine binds to either one of them.

Because this allosteric mechanism is a property of CytR rather than the DNA to which it binds, the same mechanism must apply to other promoters. Although not explicitly stated, our expectation was that cytidine binding would produce a CytR conformation that would not interact with CRP. The results obtained here force a modification of this view. Presumably, either the spacing between the CytR and CRP sites or something inherent in the CytR site, such as the spacing between recognition motifs, is responsible for controlling whether interactions between CytR and CRP are still possible when cytidine is bound.

The challenge then is to explain both the very broad specificity of CytR for DNA binding and for CytR-CRP cooperativity when cytidine is not bound, together with the control of cooperative interactions when cytidine is bound. For two other LacR family members, PurR and LacR, the conformational transitions that accompany ligand binding have been investigated by x-ray crystallography (33–35). Ligand binding to these two proteins controls a change in tertiary structure that substantially alters the dimer interface. In one configuration, hinge helices that connect the helix-turn-helix motif to the globular core domain of each subunit are destabilized, and the hinge-helix motifs from the different subunits are thought to no longer be held in proper register with successive DNA major grooves. Ligand binding to the protein is linked to DNA binding by the protein via this mechanism. Because CytR couples ligand binding not to DNA binding but to cooperativity, allostery must have a different structural basis than that in other LacR/PurR proteins.

To explain how CytR uncouples ligand binding from DNA binding, CytR is thought to connect the helix-turn-helix motifs to the globular core domain via peptide linkers in an extended conformation, rather than via hinge helices (3, 9). The basis for this prediction is the observation that CytR has the helix breakers proline (Pro57) and glycine (Gly59), whereas the other family members have conserved helix maker alanines in the hinge helix (36). Flexibility of the peptide linkers is inferred from their protease sensitivity (9). This structure is expected to accommodate similar conformational transitions of the globular core domain as for other LacR family members without affecting DNA binding. This highly flexible connection between the DNA-binding domains and the ligand-binding core dimer is thought to explain how CytR can tolerate widely variable spacing between DNA recognition motifs. It has even been suggested that the helix-turn-helix, DNA-binding feet of CytR might be able to turn 180° to accommodate binding to recognition motifs arranged as either tandem or inverted repeats (8).

A prominent feature of the ligand-induced conformational change in PurR and LacR is a relative rotation of the ligand-binding core, N-terminal subdomains around an axis lying roughly along their interface. The twist amounts to about 40° in PurR and 25° in LacR (33, 35). If CytR also undergoes a similar ligand-linked change in quaternary structure, then this must change the orientation of the CytR core dimer relative to the DNA to which the core dimer is tethered at two locations. This would be so, even accounting for flexibility in the tether. The analogy is to a ship anchored in the harbor by both bow and stern. By tensioning spring lines attached to the anchor lines, the orientation of the ship can be controlled, even against the action of the wind and current.

We speculate that relative rotations of the subunits of the CytR dimer in response to ligand binding might similarly control the alignment of the dimer with the DNA axis as necessary to orient protein-protein contacts with flanking CRP dimers. In such a model, both the spacing between the CytR and CRP sites and the spacing between recognition motifs of the CytR site would impose additional constraints on orientation that might account for different patterns of cooperativity. A crystallographic study of the effect of cytidine binding on CytR core domain conformation should provide a direct approach to address these issues.

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