Kinetic and Thermodynamic Studies of Purine Repressor Binding to Corepressor and Operator DNA*

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The kinetic and thermodynamic parameters for purine repressor (PurR)-operator and PurR-guanine binding were determined using fluorescence spectroscopy and nitrocellulose filter binding. Operator binding affinity was increased by the presence of guanine as demonstrated previously (Choi, K. Y., Lu, F., and Zalkin, H. (1994) J. Biol. Chem. 269, 24066–24072; Rolfe, R. J., and Zalkin, H. (1990) J. Bacteriol. 172, 5637–5642), and conversely guanine binding affinity was increased by the presence of operator. Guanine enhanced operator affinity by increasing the association rate constant and decreasing the dissociation rate constant for binding. Operator had minimal effect on the association rate constant for guanine binding; however, this DNA decreased the dissociation rate constant for corepressor by ~10-fold. Despite significant sequence and structural similarity between PurR and LacI proteins, PurR binds to its corepressor ligand with a lower association rate constant than LacI binds to its inducer ligand. However, the rate constant for PurR-guanine binding to operator is ~3-fold higher than for LacI binding to its cognate operator under the same solution conditions. The distinct metabolic roles of the enzymes under regulation by these two repressor proteins provide a rationale for the observed functional differences.

The biosynthesis of IMP,$^1$ a branch point in the synthesis of AMP and GMP, involves ten enzymatic steps (1, 2). Purine repressor (PurR) binding to multiple pur operon sequences regulates expression of the enzymes involved in IMP synthesis, the conversion from IMP to GMP and AMP, and other related biochemical pathways (1, 2). PurR recognizes a series of conserved and partially symmetric DNA sequences upon binding to a corepressor ligand, guanine or hypoxanthine (1,3–18). In the presence of PurR-purine corepressor, RNA polymerase transcription of enzymes from the associated promoter region is inhibited, and biosynthesis of purine nucleotides correspondingly diminishes (1,3–20).

PurR consists of 341 amino acids and is a member of the LacI family of DNA binding proteins (21–24). PurR is a homodimer, and each monomer consists of a helix-turn-helix DNA binding domain connected to a core purine binding domain by a hinge region (19, 21, 25, 26). The x-ray crystallographic structures of the cognate PurR-guanine binding domain (amino acids ~60–341) have been determined (26–29). The DNA binding domain and a helix formed by the hinge sequence make direct contact with the operator DNA to recognize features of the specific cognate sequence (26). The N-terminal helix-turn-helix domain binds in the major groove of the DNA, whereas the hinge helix, which appears to be disordered in the free protein, contacts the DNA sequence in the minor groove and introduces a bend of 45–50 ° in the DNA (26,30). Each core domain contains N- and C-subdomains, designations based on similarity to the family of periplasmic sugar binding proteins (21,26,31). The corepressor binding pocket is located in the cleft between the N- and C-subdomains. Upon corepressor binding, the two N-subdomains appear to change their relative positions to generate the proper conformation for DNA binding (26,27). Amino acids responsible for monomer-monomer interactions are dispersed across the two subdomains to form the subunit interface (26).

The key role of PurR in regulating a central biosynthetic pathway motivates a thorough understanding of the biochemical properties and the relationship of structure to function in this protein. Although corepressor binding in the absence of operator (19) and operator binding affinity in the presence and the absence of guanine (19, 20, 31) have been measured, detailed parameters for PurR interactions with ligands have not been established. In this study, we have determined PurR binding constants to both operator DNA and the corepressor guanine under a consistent set of conditions, allowing a more complete analysis of the thermodynamics and kinetics of regulation in this system.

MATERIALS AND METHODS

Buffers and Guanine Solutions

PurR was purified in Buffer A, which contained 10 mM HEPES-KOH, 0.1 mM EDTA, 5% glycerol, 0.1 mM diethiothreitol, pH 7.6. All reactions for the equilibrium and kinetic analyses were conducted in Buffer E, based on modifications of the previously described Buffer II (20,31). Buffer E contained 100 mM HEPES-KOH (pH 7.5), 250 mM potassium glutamate, 150 mM sodium chloride, 10 mM magnesium acetate, and 1 mM EDTA. The binding of PurR to operator is responsive to purine in Buffer E, with (a) saturation in Buffer E by stirring overnight with mild heating (~40 °C) or (b) dissociation of guanine to 40 mM in 1 M NaOH. These stock solutions were diluted to the appropriate concentrations for specific experiments, and the concentrations were confirmed by absorbance. The pH of final PurR and guanine solutions was not altered by the latter preparation process, and no differences in results between the methods of guanine dissolution were noted.

Purification of PurR

Purine repressor was overexpressed and purified according to previously published procedures (20) with the following modifications. E. coli BL21 cells were lysed by sonication instead of French press;
phosphocellulose was used for further purification following DEAE chromotography instead of heparin agarose; and the gradient for the phosphocellulose column was 30–400 mM KCl in Buffer A. PurR was eluted from phosphocellulose at 300–350 mM KCl. The binding activity of the protein was determined by titration under stoichiometric conditions to be >90% for DNA and for guanine.

**Intrinsic Fluorescence**

The intrinsic fluorescence changes of PurR upon guanine binding were recorded within either 1-cm² or 0.2 × 1.0-cm pathlength quartz cuvettes at room temperature in an SLM-8100 fluorescence spectrometer. The excitation wavelength for emission spectra was 290 nm. In some experiments, a cut-off filter (Corning, O-52) was used to record the total fluorescence change at wavelengths longer than 340 nm. Two methods were employed to correct for the fluorescence decrease caused by guanine absorption at the excitation wavelength (inner filter effect). The first method used the absorbance of each sample at 290 (A$_{290}$) and 350 nm (A$_{350}$), using the equation $F = F_0 + (A_{290} + A_{350})/2$ for correction. The second method employed the effect of guanine on the fluorescence of N-acetyltryptophanamide. The fluorescence of the sample was corrected by the ratio of $F/F_0$, for N-acetyltryptophanamide, where $F_0$ corresponds to the initial fluorescence and $F$ corresponds to the fluorescence value at each guanine concentration. These methods resulted in similar correction values. PurR concentration was fixed at 2 × 10$^{-7}$ M monomer; guanine (1 × 10$^{-3}$ to 3 × 10$^{-5}$ M) was added for binding assays with operator, whereas a higher concentration range (5 × 10$^{-7}$ to 5 × 10$^{-5}$ M) was added for assays without operator. When present, operator concentration was 2 × 10$^{-7}$ M. All samples were incubated at room temperature for 1–2 h before the fluorescence measurements were taken. Corepressor binding affinity was determined by globally fitting the data using a 2 guanine:1 PurR dimer noncooperative binding model with the software package BIOEQS (32). Binding affinity was linked across data sets, while all other parameters were allowed to float.

**Filter Binding**

**Operator Binding Assay**—Operator DNA binding in the absence and the presence of corepressor, guanine (1 × 10$^{-5}$ M), was measured by nitrocellulose filter binding. Operator DNA (30 bp) was synthesized by the Great American Gene Company. The sequences of the two single-stranded DNAs were: 5’ GAAATCCCTACGCAAACGTTTGCGTTTGTTTTCGTTAACATCGCAAACGTTTGCGTTTTCGACGAGATTGCAAGCTTGGTTTGCCTTGGATCC 3’, 5’ CAGACAGATACGAGGCGAGATTGCAATCGTTATGTTGGTCG 3’.

These oligonucleotides were hybridized to form double-stranded operator fragments with 2-bp 5’-overhangs at either end. The operator fragments were then labeled by polynucleotide kinase using [γ-32P]ATP. The incubation buffer for these measurements was Buffer E with 50 μg/ml bovine serum albumin (BSA). Operator concentration was 2 × 10$^{-12}$ M. PurR was added in concentrations from 1 × 10$^{-12}$ M to 5 × 10$^{-6}$ M dimer in the absence of guanine and from 1 × 10$^{-12}$ M to 1 × 10$^{-7}$ M dimer in the presence of guanine. PurR was incubated with 1 × 10$^{-5}$ M guanine in Buffer E with BSA for 20 min before operator DNA was added. Samples were incubated at room temperature for 20 min before filtration through a 0.45-μm nitrocellulose membrane (Schleicher & Schuell). Samples were filtered through the membrane, and the retention of radioactivity on each filter was determined by scintillation counting. Operator affinity was determined by fitting the data to Equation 1.

$$ Y = Y_m[PurR]/K_s + [PurR] $$

where $Y$ is fractional saturation, $Y_m$ is a factor that allows $Y_{max}$ (the saturation value) to float, $K_s$ is the equilibrium dissociation constant, [PurR] is PurR concentration in monomer, and $n$ is the Hill coefficient. Values for $n$ were near unity in all cases.

**Corepressor Binding Assay**—Guanine binding affinities in the absence and the presence of operator DNA were measured by nitrocellulose filter binding. [1C]Guanine concentration was 3 × 10$^{-7}$ M with or without operator present. When present, operator concentration was 1 × 10$^{-6}$ M protein concentration < 1 × 10$^{-6}$ M and was equimolar at protein concentrations ≥ 1 × 10$^{-6}$ M. Samples were incubated at room temperature for 2–3 h before filtration through a nitrocellulose membrane. The retention of radioactivity on the filter was determined by scintillation counting. Guanine binding affinity was determined by fitting the data to Equation 2.

$$ Y = Y_m[PurR]/K_s + [PurR] $$

where $Y$ is the fractional saturation, $Y_m$ is a factor that allows $Y_{max}$ (the saturation value) to float, $K_s$ is the equilibrium dissociation constant, [PurR] is PurR concentration in monomer, and $n$ is the Hill coefficient. Values for $n$ were near unity in all cases.

**Filter Binding**

**Operator Binding Kinetics**

The association and dissociation rates for operator binding in the presence and the absence of guanine were measured by nitrocellulose filter binding. For association reactions, PurR at three different concentrations (Buffer E plus 50 μg/ml BSA) was incubated with 8.3 × 10$^{-11}$ to 1 × 10$^{-12}$ M 32P-labeled operator DNA. Samples were filtered through nitrocellulose membranes following different incubation times. The amount of PurR-operator complex was determined by scintillation counting of each individual filter. The observed association rate ($k_{obs}$) was determined by fitting the data at each individual protein concentration to Equation 3.

$$ F = A + \Delta A e^{-k_{obs}t} $$

where $F$ is the amount of PurR-operator, A is the amount of operator-PurR at equilibrium, $\Delta A$ is the total change in operator-PurR between zero time and equilibrium, $k_{obs}$ is the observed rate constant, and $t$ is time. $F$ was measured by scintillation counting. The association rate constants were determined by fitting $k_{obs}$ at different protein concentrations into Equation 4.

$$ k_{obs} = k_{max}[PurR] + k_{max} $$

where $k_{obs}$ is the observed rate, $k_{max}$ is the association rate constant, $k_{max}$ is the dissociation constant, and [PurR] is the protein concentration in dimer. Dissociation rates were measured in a similar manner, except that 5 × 10$^{-9}$ M PurR (dimer concentration) was incubated with 2.5 × 10$^{-12}$ M 32P-labeled operator DNA at room temperature for 10 min before 3 × 10$^{-7}$ M cold operator DNA was added. When guanine was present, PurR (5 × 10$^{-5}$ M dimer) was incubated with 1 × 10$^{-5}$ M guanine at room temperature for 20 min prior to operator addition. The amount of complex present after different times following addition of cold operator was measured by filtering samples through nitrocellulose membranes. The dissociation rate constants were determined by fitting data into Equation 5.

$$ F = A + \Delta A e^{-k_{dissoc}t} $$

where $F$ is the amount of 32P-labeled operator-PurR, $A$ is the amount of 32P-labeled operator-PurR at equilibrium, $\Delta A$ is the total change of 32P-labeled operator-PurR between zero time and equilibrium, $k_{dissoc}$ is the dissociation rate constant, and $t$ is time.

**Guanine Binding Kinetics by Stopped Flow**

The association and dissociation rates of guanine binding to PurR in the presence and the absence of operator DNA were measured using a stopped flow system in an SLM-8100 fluorescence spectrometer. For association rates, PurR (4 × 10$^{-7}$ M) was mixed with an equal volume of various concentrations of guanine in Buffer E. When present, operator concentration was equimolar to PurR dimer concentration. For dissociation rates, PurR (4 × 10$^{-8}$ M monomer) and guanine (8 × 10$^{-6}$ M) were incubated in Buffer E at room temperature for 1 h before being diluted 3.5-fold with Buffer E. The dissociation rate in the presence of operator was measured by manually diluting a solution containing PurR (4 × 10$^{-6}$ M monomer), operator DNA (4 × 10$^{-6}$ M), and guanine (4.5 × 10$^{-5}$ M) into a 20-fold volume of Buffer E in a 1-cm² quartz cuvette. Fluorescence signal changes during the association or dissociation processes were monitored using an excitation wavelength of 290 nm and following emission using a 340 nm cut-off filter. The association rate constants and dissociation rate constant in the absence of operator were determined by fitting data to Equation 6.

$$ F = A + \Delta A e^{-k_{dissoc}t} $$

where $F$ is the amount of PurR-operator, $A$ is the amount of PurR-operator at equilibrium, $\Delta A$ is the total change of PurR-operator between zero time and equilibrium, $k_{dissoc}$ is the dissociation rate constant, and $t$ is time.
RESULTS

Guanine Binding Affinity—Guanine binding affinity for PurR in the presence and the absence of operator DNA was determined by nitrocellulose filter binding. Guanine concentration was fixed well below the equilibrium constant, and PurR concentration was varied. There are two guanine binding sites in each dimer of PurR, and the end point of this titration is a single guanine bound to each dimer. Therefore, the affinity for the first binding site was the value measured in this assay (Fig. 1). The results in the presence and the absence of operator DNA are summarized in Table I.

Alternatively, the affinities were measured by fluorescence titration (Fig. 1), which monitors both binding sites for purine. PurR contains four tryptophans in each monomer of PurR (22, 26). Trp<sup>147</sup> is positioned near the opening to the corepressor binding pocket in aporepressor (27). Based on the crystal structures, the environment of Trp<sup>147</sup> is less aromatic in the purine-PurR-operator complex than the free core binding domain (26, 27). This environmental change for Trp<sup>147</sup> presumably generates the observed increase of the intrinsic fluorescence signal and the slight red shift of the emission spectrum from 340 to 342 nm (19). Using this intrinsic fluorescence signal, the effects of different concentrations of guanine were recorded (Fig. 1). Due to the absorbance of guanine at the excitation wavelength, correction of the observed fluorescence as reported under “Materials and Methods” was essential. The results from both filter binding and fluorescence measurements demonstrated that the presence of operator DNA increased PurR affinity for guanine by 7–17-fold (Table I).

Operator Binding Affinity—A DNA sequence derived from the purF operator was used in operator binding assays for convenient comparison with previously published results (31). PurR exhibits higher affinity for this optimized sequence over a 30-bp purF operator but lower affinity compared with a 270-bp purF operator sequence (19, 20, 31). Operator affinities in the presence and the absence of guanine were determined by filter binding. As shown in Fig. 2, guanine increased operator affinity significantly. According to the equilibrium dissociation constants derived from these titrations, the presence of guanine increases PurR affinity for operator by ~210-fold.

Thermodynamic Analysis—Two paths exist from aporepressor to purine- and operator-bound holorepressor as shown in Fig. 3. As predicted by thermodynamic principles, the total free energy for route 1 (ΔG<sub>1</sub> = ΔG<sub>A</sub> + 2ΔG<sub>G</sub>) should equal that of the alternative path, route 2 (ΔG<sub>2</sub> = 2ΔG<sub>G</sub> + ΔG<sub>C</sub>). These equations assume equivalent affinity for both purine binding sites in a dimer, as indicated by the binding studies. The equilibrium dissociation constants and ΔG values calculated for different conditions are listed in Table I. Using values derived from filter binding experiments, ΔG<sub>C</sub> and ΔG<sub>G</sub> were ~27.5 kcal and ~27.2 kcal, respectively, taking the stoichiometry for guanine into account. The 0.3-kcal difference between the total free energy changes for these two paths may derive from experimental limitations, in particular difficulty with filter binding at high protein concentrations. Values of ~26.5 and ~27.2 kcal are calculated for ΔG<sub>C</sub> and ΔG<sub>G</sub>, respectively, using K<sub>F</sub> values determined by fluorescence for the guanine binding free energy determination. The difference between ΔG<sub>C</sub> values calculated from filter binding and fluorescence measurements may be ascribed to the high error for the fluorescence measurements.

Kinetic Measurements of Guanine Binding—Kinetic constants for guanine binding were determined by monitoring the fluorescence signal change in PurR as described under “Materials and Methods.” As shown in Fig. 4, the association rate constants in the presence and in the absence of operator were very similar, ~1 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1</sup>. However, these values were 10-fold lower than the corresponding rate constant for the homologous LacI protein to its ligand IPTG (Table II). The purine-PurR dissociation rate constant was decreased ~10-fold in the presence of operator (Fig. 5 and Table II), demonstrating that operator binding elicits increased guanine binding affinity primarily by affecting the dissociation rate for the corepressor.

Kinetic Measurements of Operator Binding—The kinetics for PurR-operator binding were determined by nitrocellulose filter binding. As shown in Figs. 6 and 7, guanine increased the association rate constant for operator by ~50-fold and decreased the dissociation rate constant by ~20-fold, resulting in substantial stabilization of the PurR-operator complex. The dissociation rate and equilibrium constants for LacI binding to its operator were determined in the same buffer. Interestingly, the association rate constant for PurR holorepressor binding to its operator is greater than that for LacI binding to its operator at the ionic strengths employed for this assay, as shown in Table III.
Thermodynamic Studies—All the equilibrium dissociation constants for guanine and operator binding to the purine repressor, constituting a complete thermodynamic loop, were determined in this study. The results demonstrated that guanine increased the operator binding affinity for PurR by 210-fold, a value greater than found previously (14-fold) (31). Furthermore, these measurements demonstrated for the first time that the presence of operator increases PurR affinity for guanine. PurR, as a master regulator for expression of genes whose products are involved in purine and pyrimidine nucleotide biosynthesis, must be able to respond appropriately to in vivo environmental changes, specifically alterations in the level of purine available. In the absence of purine, the expression of biosynthetic enzymes is unimpeded, and free purine levels rise from nucleotide/nucleoside degradation as production exceeds demands. As purine levels approach the $K_d$ for binding to PurR, the holorepressor binds to the operator and diminishes expression for the genes associated with that operator site. If PurR binds to the operator in the absence of purine, its affinity for available purine is increased, and the sensitivity of the system...
to repression is enhanced.

Exogenous sources and endogenous degradation of high levels of nucleosides and nucleotides provide sufficient free purine to bind PurR and promote pur operator binding. PurR holorepressor exerts tighter control over the pathway from phosphoribosylpyrophosphate to IMP than the conversion from IMP to AMP or GMP (1, 4, 6, 9). When de novo IMP synthesis is decreased in response to PurR-purine binding to pur operon sites, salvage pathways are used for production of IMP, AMP, and GMP, using the available free bases (33). In this way, E. coli cells avoid the complicated and energy-expensive de novo pathway from phosphoribosylpyrophosphate to AMP and GMP when free purine is available. As free purine concentrations decrease, pur operators are released from PurR repression until the levels of free purine rise again. These mechanisms not only ensure that purine mononucleotides are available but also that the pool of free purine is depleted rapidly, thereby avoiding the accumulation of potentially toxic levels (33).

Kinetic Studies—We have measured the association and dissociation rates for PurR binding both to operator DNA sequences and to a corepressor, guanine. The equilibrium binding constants determined from the kinetic values are consistent with those measured by fluorescence titration (see Table I).

**TABLE II**

| Ligand Binding Parameters for Purine Repressor |
|---------------------------------------------|
| PurR parameters are for binding to guanine, whereas LacI parameters are for binding to IPTG. All the measurements were carried out in Buffer E. The values reported for association rate constants for PurR and PurR-operator are the weighted averages of two separate sets of replicate measurements, one set of which is shown in Fig. 4. |
| $k_{\text{association}}$ | $k_{\text{dissociation}}$ | $k_{\text{dissociation}}/k_{\text{association}}$ | $K_d$ |
|--------------------------|--------------------------|--------------------------|----------|
| PurR + operator | $1.2 \pm 0.02 \times 10^{-2}$ | $1.3 \pm 0.08 \times 10^{4}$ | $9.2 \times 10^{-7}$ | $9.8 \times 10^{-7}$ |
| PurR | $1.2 \pm 0.02 \times 10^{-1}$ | $1.2 \pm 0.04 \times 10^{4}$ | $10 \times 10^{-6}$ | $7.0 \times 10^{-6}$ |
| LacI | $2.1 \pm 0.1 \times 10^{-1}$ | $1.2 \pm 0.09 \times 10^{3}$ | $1.8 \times 10^{-6}$ | $0.8 \times 10^{-6}$ |

$a$ $K_d$ values correspond to those measured by fluorescence titration (see Table I).
with the direct measurements of affinity (Tables II and III). Guanine increases the affinity of PurR for its operator by (a) increasing the association rate and (b) decreasing the dissociation rate. When the cellular purine concentrations are low, PurR aporepressor interacts with its operator sites with a slower association rate constant and a faster dissociation rate constant, minimizing occupancy of operator sites and avoiding inhibition of necessary downstream gene expression. When cellular purine levels increase, PurR-purine complex binds to its cognate operator sites with an increased association rate constant and dissociates more slowly from the operator, thereby blocking downstream gene expression.

In the presence of operator, PurR binds to its corepressor ligand with lower rate constants for both association and dissociation compared with the homologous LacI protein (Figs. 4 and 5). In the presence of guanine, PurR binds to its operator with a faster association rate constant than LacI and a diminished dissociation rate constant. In the absence of guanine, PurR binds to its operator with a lower association rate constant, and a similar dissociation rate constant as observed for LacI. These differences can be rationalized by the different physiological roles for these proteins. Although both proteins are members of the LacI family of proteins, the role of PurR in the metabolism of the cell is distinct from that of LacI. Specifically, the lactose operon encodes proteins involved in the catabolism of sugars. LacI controls the expression of mRNA encoding β-galactosidase, lactose permease, and thiogalactoside transacetylase, enzymes involved in the transport and breakdown of lactose and other β-galactosides to generate an energy source. LacI affinity for operator is diminished upon binding to inducer ligands (e.g., IPTG or allolactose). The release of operator allows transcription of the downstream genes and production of the enzymes for lactose transport and digestion. LacI must both bind to its ligand rapidly ($\sim 1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ for LacI-operator binding to IPTG and $\sim 1 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for LacI binding to IPTG) and release operator quickly ($>0.1 \text{ s}^{-1}$ for LacI-IPTG dissociation from lac operator) to free the promoter for transcription. This process generates the requisite enzymes in a short time frame to ensure rapid utilization of environmentally available lactose as a carbon source.

In contrast, PurR controls the expression of multiple enzymes involved in the biosynthesis of nucleotides and other related anabolic pathways. PurR represses the expression of these genes upon binding to its corepressor ligand (guanine or hypoxanthine). In the absence of purine, the operator is primarily in the free state, and biosynthesis of mRNA and encoded biosynthetic enzymes can proceed. As purine levels accumulate, either from nucleoside/nucleotide breakdown or from environmental sources, binding to PurR occurs but with a lower association rate constant than observed for the LacI protein. The PurR-guanine complex then binds with increased affinity to the operator sequence. With respect to the dissociation process, rate constants for release of DNA are lower than for the corresponding states of LacI, and the rate constant for release of guanine in the presence of operator DNA is also significantly diminished.

The kinetic differences observed for PurR compared with LacI parallel the distinct metabolic roles of the enzymes controlled by these regulatory proteins. LacI must respond rapidly to the presence of lactose in the environment, and the enzymes must be generated quickly to ensure capture of this energy source. In contrast, PurR effectively “integrates” the concentration of available purine over a period of time before generating a transcriptional response to alterations in purine levels. Minor

![Figure 7](image.png)

**FIG. 7. Determination of dissociation rate constants for operator binding.** The dissociation rates for operator binding were determined by filter binding. All the reactions were carried out in Buffer E with 50 μg/ml BSA. PurR (5 $\times$ 10$^{-5}$ M dimer) was incubated with 2.5 $\times$ 10$^{-12}$ M 32P-labeled operator DNA at room temperature for 10 min before 3 $\times$ 10$^{-7}$ M cold operator DNA was added. PurR (5 $\times$ 10$^{-9}$ M dimer) was incubated with 1 $\times$ 10$^{-5}$ M of guanine, when present, at room temperature for 20 min prior to initiation of dissociation. The amount of complex present after different times following addition of cold operator was measured by filtering samples through a nitrocellulose membrane. The data from multiple determinations were fit as described under “Materials and Methods.” A, in the presence of guanine. B, in the absence of guanine.

**Table III**

Summary of operator binding constants

|                | $k_{\text{dissociation}}$ | $k_{\text{association}}$ | $k_{\text{dissociation}}/k_{\text{association}}$ | $K_d$ |
|----------------|---------------------------|---------------------------|-----------------------------------------------|-------|
| PurR           | 2.8 ± 0.4 $\times$ 10$^{-2}$ | 3.2 ± 0.6 $\times$ 10$^5$ | 8.8 $\times$ 10$^{-8}$                   | 4.0 $\times$ 10$^{-8}$ |
| PurR + guanine | 1.2 ± 0.2 $\times$ 10$^{-5}$ | 1.5 ± 0.2 $\times$ 10$^7$ | 0.8 $\times$ 10$^{-10}$                   | 1.9 $\times$ 10$^{-10}$ |
| LacI           | 3.7 ± 1.3 $\times$ 10$^{-2}$ | [5.1 $\times$ 10$^6$]$^a$ | [7.2 $\times$ 10$^{-5}$]$^a$            |       |

$^a$ $K_d$ values were measured by nitrocellulose filter binding.

$^b$ Value calculated from the ratio of $k_{\text{dissociation}}/k_{\text{association}}$. Measurement of association rate constant for LacI at a single protein concentration in Buffer E yielded values of $\sim 5$–$8 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ but with significant error; for this reason, these values are not reported in the table.
changes in the pool of free purine do not elicit an immediate alteration in the production of biosynthetic enzymes; rather, an enduring rise in the levels of free purine results in the shutdown of mRNA for the enzymes in this pathway. Once the PurR-corepressor-operator complex forms, the release of corepressor from PurR-operator and PurR-corepressor from its operator are both slowed by 10-fold and are slower than the corresponding reactions for LacI. The lower dissociation rate constant for PurR holorepressor-operator results in concomitantly slow generation of enzymes for de novo synthesis when purines are available for salvage.

These kinetic differences in proteins with high sequence, structure, and functional similarity (21–24, 26, 34, 35) mirror the distinctions in the metabolic roles of the enzymes under their regulation. Such differences presumably arise from subtle alterations in structure that control rates for the respective binding reactions. Previous work has shown that a single amino acid change (K84L) in LacI can significantly decrease the inducer binding rate constants for this protein by >100-fold and consequently alter the rate of release for operator DNA substantially (36). Thus, small changes in sequence can be presumed to have dramatic effects on the functional properties of even closely related proteins. Despite high levels of sequence similarity and structural alignment (21–27, 34, 35), the sequence differences observed between PurR and LacI result in significant divergence in equilibrium and kinetic properties. Further comparison of structure and function relationships in these closely related proteins may provide insight into the mechanisms used broadly to modulate protein binding parameters for ligands.

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