Role of the Purine Repressor Hinge Sequence in Repressor Function†

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A protease-hypersensitive hinge sequence in *Escherichia coli* purine repressor (PurR) connects an N-terminal DNA-binding domain with a contiguous corepressor-binding domain. Binding of one molecule of dimeric repressor to operator DNA protects the hinge against proteolytic cleavage. Mutations in the hinge region impair repressor function in vivo. Several nonfunctional hinge mutants were defective in low-affinity binding to operator DNA in the absence of corepressor as well as in high-affinity corepressor-dependent binding to operator DNA, although binding of corepressor was similar to binding of the wild-type repressor. These results establish a role for the hinge region in operator binding and lead to a proposal for two routes to form the holoPurR-operator complex.

Escherichia coli purine repressor (PurR) functions to regulate nine *pur* operons (12-14, 23) involved in de novo synthesis of purine nucleotides as well as several other genes with functions related to purine nucleotide synthesis (2, 11, 17, 28, 31, 36). PurR contains a 6-kDa N-terminal DNA-binding domain joined to a 32-kDa corepressor-binding domain (3). Guanine or hypoxanthine or both are the corepressors that are required for high-affinity binding of PurR to a 16-bp operator sequence (24, 27). Amino acid sequence similarities suggest homology of the corepressor-binding domain and a subgroup of periplasmic sugar-binding proteins that includes ribose-binding protein (9), arabinose-binding protein (30), and glucose/galactose-binding protein (21). Likewise, amino acid sequence similarities place PurR in a LacI family of likely homologous bacterial repressors (26, 34).

PurR amino acid residues that are required for binding corepressors have been identified (4). These results provide evidence for similar binding interactions of purine corepressors to PurR and sugars to periplasmic binding proteins, thus supporting the evolutionary relationship between these proteins (1, 4, 29). In an effort to understand the role of corepressor in high-affinity binding to operator DNA, we have investigated the region that joins the DNA-binding and corepressor-binding domains. A peptide sequence that functions as a hinge has been identified by hypersensitivity to cleavage by proteases and by correspondence to a previously identified hinge in LacI (7). Mutational analysis indicates an essential role of this hinge in binding of PurR to operator DNA. These results and the properties of corepressor-binding mutants (4) are used to formulate a model for PurR function.

Protease cleavage sites identify an interdomain hinge. Specific cleavage of PurR by trypsin after Arg-52 separates the 6-kDa N-terminal DNA-binding domain from the contiguous 32-kDa corepressor-binding domain (3). Single cleavages by trypsin were not detected after Lys-41 or Lys-55, suggesting that the exposed region is confined within these boundaries. Similar 32- and 6-kDa fragments were produced by mild digestion with chymotrypsin (Fig. 1, compare lanes 1 and 2). Five cycles of N-terminal amino acid sequence analysis (20) of

the 32-kDa fragment gave a single sequence corresponding to residues Lys-55 to Thr-59, indicative of a specific cleavage after Leu-54. Thus, Arg-52 and Leu-54 are exposed and accessible to protease cleavage whereas neighboring residues including Lys-55 are inaccessible. Arg-52, Ser-53, and Leu-54 therefore constitute the central segment of the interdomain hinge (Fig. 2).

Binding to operator DNA inhibits hinge cleavage. Binding of corepressor provokes a conformation change in the 32-kDa C-terminal domain that does not influence the accessibility of Arg-52 to trypsin (3). However, a hinge structure that connects DNA-binding and corepressor-binding domains may undergo changes in conformation upon binding to DNA. Therefore, the accessibility of Arg-52 was determined for PurR bound to operator DNA. For these experiments we used a synthetic 30-mer containing an idealized 16-bp *pur* regulon operator flanked by nucleotides corresponding to the *purF* control site (4). Binding of operator DNA to PurR largely inhibited hinge cleavage by trypsin (Fig. 1, compare lanes 4 and 5). The kinetics for hinge cleavage by trypsin are shown in Fig. 3. Under the conditions used, binding of PurR to operator DNA essentially abolished hinge cleavage. An approximately 1.2-fold molar excess of operator DNA was required for the

FIG. 1. Proteolytic cleavage of PurR by chymotrypsin and trypsin. PurR (6.6 μ M dimer), purified as previously described (3), was digested for 15 min with 0.4% chymotrypsin or trypsin in buffer II (27) containing 30 μ M guanine (3). Lanes: 1, no protease; 2, chymotrypsin; 3, no protease; 4, trypsin; 5, 8 μ M 30-bp operator included, digested with trypsin. Digestions with chymotrypsin and trypsin are from separate experiments.

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FIG. 2. Diagrammatic representation of the PurR hinge. Highlighted amino acids were mutated to residues shown below. The method of Kunkel et al. (19) was used for mutagenesis. Replacements of Ser-53 and Leu-54 were obtained by using mutagenic oligonucleotides with a mixture of bases at two positions of the target codon. Arrows mark positions for cleavage by proteases.

maximal protection (Fig. 4). This indicates high-affinity binding with 1:1 molar stoichiometry for PurR dimer with operator DNA. Data in Fig. 3 and 4 show the DNA specificity for hinge protection. Poly(dI-dC:dI-dC) or nuclease-digested operator had a partial protective effect that was perhaps due to inhibition of trypsin (Fig. 3). Figure 4 shows that the 30-mer DNA fragment in which the 16-bp operator sequence was randomized did not inhibit hinge cleavage. PurR does not bind this nonoperator 30-mer (4). The overall conclusion from these data is that binding of PurR to operator DNA sequesters two hinge residues, Arg-52 and Leu-54, against cleavage by proteases.

For the preceding experiments the PurR concentrations were more than 100-fold higher than used in the standard gel retardation assay for repressor-operator interaction (4). As a consequence of the high PurR concentration, inclusion of corepressor was not required for formation of the specific

FIG. 3. Kinetics of hinge cleavage by trypsin. PurR (6.6 μ M dimer) was incubated for 30 min in 65 μ l of buffer II (27) containing 30 μ M guanine. When present, the concentration of 30-mer operator DNA was 8 μ M and that of poly(dI-dC:dI-dC) was 0.2 μ g/ μ l. Samples were digested with 0.4% (wt/wt) trypsin at room temperature, and 10- μ l aliquots were removed at different times, quenched with buffer (3), and stored at -20°C awaiting electrophoresis. Following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, Coomassie blue-stained bands were quantitated by densitometer scanning and the results were plotted against the time of proteolysis. Symbols: ●, no DNA; ○, operator DNA; ▲, DNase-treated operator; ■, poly(dI-dC:dI-dC).

FIG. 4. Effect of DNA concentration on hinge cleavage. Reaction mixtures of 20 μ l containing 6.6 μ M PurR, buffer II (27), 30 μ M guanine, and various concentrations of operator or nonoperator 30-mer were equilibrated at room temperature for 30 min and then digested with 0.4% trypsin for 20 min. The percentage of intact PurR was plotted against the DNA concentration. Symbols: ○, operator DNA; ●, nonoperator DNA.

repressor-operator complex (4) and corepressor was not required for hinge protection by operator DNA (data not shown). To determine whether the corepressor-dependent interaction with operator DNA sequesters the PurR hinge from protease digestion, we used a Western immunoblot assay to increase the sensitivity of PurR detection. For this experiment, whose results are shown in Fig. 5, the PurR concentration was 40 nM, similar to that used for corepressor-dependent interaction with operator DNA (4). The anti-PurR antibody reacted with the 32-kDa corepressor-binding domain but did not detect the 6-kDa N-terminal domain. The data in Fig. 5 show that hypoxanthine corepressor was required for the operator to protect the PurR hinge against proteolysis by trypsin. Similar results were obtained with guanine as the corepressor (results not shown). This result confirms that the

FIG. 5. Effect of corepressor-dependent operator interaction on PurR hinge cleavage. PurR (60 ng, 0.8 pmol) was incubated in 20 μ l of buffer II (27) for 30 min at room temperature. When present, the concentration of guanine was 50 μ M and that of operator DNA was 0.46 μ M. Hyp is hypoxanthine. Samples were digested with 0.4% (wt/wt) trypsin for 20 min. Reactions were quenched, and digestion was monitored by SDS-polyacrylamide gel electrophoresis (33) and Western blotting (32). Rabbit antiserum was partially purified (10) and diluted 1:10,000. Goat anti-rabbit immunoglobulin G coupled with alkaline phosphatase was diluted 1:3,000.

TABLE 1. In vivo regulation of the *purF-lacZ* fusion gene by PurR hinge mutants^a

Plasmid	β -Galactosidase activity (Miller units)	Regulation (fold)
pUC119	43.3	1.0
pPR1005-2	3.1	14.0
pR52A	13.8	3.0
pR52E	30.1	1.4
pS53I	28.9	1.5
pS53R	26.4	1.6
pS53C	5.9	11.1
pS53A	2.4	18.2
pS53V	26.8	1.6
pS53G	5.3	8.2
pL54K	18.7	2.3
pL54S	19.8	2.2
pL54W	19.4	2.2
pL54T	16.9	2.6
pL54R	18.3	2.4
pL54M	3.4	12.7
pK55A	10.0	4.3
pK55E	28.2	1.5

^a β -Galactosidase was assayed (4) in *E. coli* TX 773 (*purR purF-lacZ*) (12) carrying one of the indicated plasmids. The parental plasmid, pPR1005-2, encodes wild-type *purR* in pUC119 (27). Other plasmids are named according to the PurR mutation.

high-affinity corepressor-dependent interaction with operator sequesters the hinge from protease.

Construction and analysis of hinge mutations. A series of single-amino-acid substitutions at the central region of the hinge were constructed by site-directed mutagenesis. Lys-55, the first residue of the C domain, was also replaced. These mutations are listed in Fig. 2. Each residue was replaced by a small amino acid and by one having very different properties. The in vivo function of each mutant repressor was determined and is given in Table 1. In this assay there was 14-fold repression of *purF-lacZ* by wild-type PurR, similar to the 17-fold repression obtained previously by the chromosomal *purR*⁺ gene (12). For most of the mutants except for S53A, S53C, S53G, and L54M there was decreased repression of *purF-lacZ*. In this survey we did not determine the repressor level and therefore cannot distinguish between decreased repressor level due to turnover and defective repressor func-

	49	50	51	52	53	54	55	56	57
PurR	A	V	A	R	S	L	K	V	N
RibaR	A	L	A	R	S	L	K	L	N
ScrR(S.t.)	I	H	A	R	S	L	R	N	N
EndR(B.p.)	E	V	A	R	S	L	K	Q	K
RafR	T	Q	A	R	R	L	K	T	G
CytR	P	M	G	R	N	V	K	R	N
CcpA(B.s.)	A	V	A	R	G	L	A	S	K
GalR	A	N	A	R	A	L	A	Q	Q
EbgR	S	-	A	R	K	L	Q	T	G
FruR	A	V	A	A	G	L	R	A	G
MaiI	R	Q	A	S	A	L	R	G	G
RbtR(K.a.)	R	Q	A	S	M	L	R	S	K
GalS	A	N	A	Q	A	L	A	T	Q
LacI	R	V	A	Q	Q	L	A	G	K
	51	52	53	54	55	56	57	58	59

FIG. 6. Alignment of putative hinge sequences in LacI family repressors. Sequences were taken from reference 34. Numbers at the top are for PurR, and those at the bottom are for LacI. Sequences are for *E. coli* proteins unless designated otherwise. Abbreviations: S.t., *Salmonella typhimurium*; B.p., *Bacillus polymyxa*; B.s., *Bacillus subtilis*; K.a., *Klebsiella aerogenes*.

tion. Nevertheless, there are two main conclusions from the data in Table 1. (i) Replacements with a small residue such as alanine were least disruptive. For Leu-54 the best function was with another hydrophobic amino acid, methionine. (ii) Drastic replacements, Arg or Lys to Glu, or Ser to a hydrophobic or charged residue, were most disruptive.

One defective amino acid replacement at each position was chosen for further characterization. Mutant *purR* genes encoding R52E, S53V, L54K, and K55E were transferred to a T7 vector for overexpression and protein purification (3). The structure and function of the mutant repressors were assessed by four assays: chemical cross-linking to determine quaternary structure, trypsin digestion to search for perturbations in hinge structure, DNA binding, and corepressor binding (3). The results are presented in Table 2. The R52E, L54T, and K55E mutants were structurally similar to the wild-type repressor and were cross-linked into stable dimers. The hinge in the L54T and K55E repressors was cleaved by trypsin, similar to the wild type. For the R52E mutant, in which the trypsin cleavage site was replaced, a 32-kDa fragment was not produced but there was a low rate of multiple cleavages having a complex pattern. The S53V repressor, on the other hand, was structurally perturbed. It was monomeric as determined by

TABLE 2. Characteristics of PurR hinge mutants

Protein	Purity (%)	In vivo function	DNA binding ^a	Corepressor affinity ^b (μ M)		Oligomer state ^c	Hinge cleavage ^d
				Hyp	Gua		
PurR	>98	Yes	Yes	9.3	1.5	Dimer	Normal
R52E	>95	No	No	8	1.5	Dimer	Slow ^e
S53V	>75	No	No	ND ^f	ND	Monomer	None ^g
L54T	>95	No	No	9.3	1.3	Dimer	Normal
K55E	>95	No	No	7.5	1.4	Dimer	Normal

^a Determined by gel retardation assay (3).

^b Determined by equilibrium dialysis (3).

^c Determined by chemical cross-linking (3).

^d Limited proteolysis with 0.4% trypsin after Arg-52.

^e Slow cleavage at less than 10% of wild type to give uncharacterized ~34- and ~32-kDa fragments.

^f ND, no detectable corepressor binding.

^g No cleavage by 0.4% trypsin in 30 min at room temperature.

FIG. 7. Model for formation of PurR-operator complex. Species 1 is the aporepressor, species 2 is the holorepressor, species 3 is the aporepressor bound to operator (Op), species 4 is the repressed gene, and species 5 is the active gene ready for transcription by RNA polymerase (RNAP). Binding of apoPurR (species 3) or holoPurR (species 4) to operator results in a change in hinge conformation. Binding of guanine corepressor (species 2) results in a high-binding-affinity conformation (species 4) different from that of apoPurR.

cross-linking, Arg-52 in the hinge was not accessible to cleavage by trypsin, and the protein did not bind corepressor.

The R52E, L54T, and K55E repressors bound corepressors with K_d values indistinguishable from that of the wild-type PurR (Table 2), but specific binding to operator DNA in corepressor-dependent or -independent modes was not detected by the gel retardation assay. Corepressor-independent binding has been interpreted as the capacity for a low-affinity interaction with operator DNA at higher protein concentration (4). These results demonstrate that although the corepressor-binding domain was intact in these three mutants, the repressors were disabled by the hinge mutations and did not recognize operator DNA.

Conserved hinge structure in the LacI family of repressors.

The results in this report extend our previous identification of a protease-sensitive interdomain hinge connecting the PurR DNA-binding and corepressor-binding domains. The hinge is inferred to be approximately 9 amino acids in length, extending from residues 49 to 57 (Fig. 6). This assignment is based on the correspondence to the hinge in LacI (7) and is in agreement with alignments of periplasmic sugar-binding proteins (29). Sugar-binding proteins (9, 21, 30), homologous to the PurR corepressor-binding domain, start immediately following the hinge at residues 58 and 59. The LacI hinge, shown in Fig. 6, is cleaved specifically by proteases after Arg-51, Leu-56, and Lys-59 (5-7). Although the PurR and LacI hinge sequences share only three identical residues, both are predicted α -helices (29) that join DNA-binding and effector-binding domains and are accessible to specific proteolytic cleavages. As shown in Fig. 6, there is substantial similarity in this region in other LacI family repressors. Most have conserved Ala and Leu residues at PurR positions 51 and 54, respectively, and most have two basic residues. It therefore appears likely that hinge function is conserved in LacI family repressors.

PurR hinge function. Two main conclusions about hinge function were derived from the present work. (i) The hinge functions in concert with the helix-turn-helix motif (26) and a hydrophobic core (15) for specific binding to operator DNA. (ii) Upon binding of PurR to operator DNA, the hinge is no longer susceptible to protease digestion. Analyses of three mutants, R52E, L54T, and K55E, indicate a role of the hinge

in DNA binding. These mutants were clearly disabled from binding to operator, although corepressor binding was indistinguishable from that of the wild type and these repressors were dimeric, similar to the wild type. The overall conformation of the hinge was perhaps not grossly perturbed, on the basis of the normal cleavage of Arg-52 by trypsin in the L54T and K55E mutants. A role for the PurR hinge in DNA binding is not unexpected on the basis of previous studies with LacI. Every position in the LacI hinge region is subject to missense mutation, and, along with numerous other sites in the DNA-binding domain, these mutations lead to an I^{-d} *trans*-dominant constitutive phenotype (8, 18). This phenotype is due to altered repressor subunits that have lost the capacity to bind operator but can aggregate with wild-type subunits to form inactive tetramers. Although there is controversy about a role for the effector-binding domain in operator binding (16, 22, 35), the LacI hinge in conjunction with the helix-turn-helix structure and residues required to form a hydrophobic core structure (15, 16) is clearly needed for binding to operator. Although analogous mutational and structural studies have not been reported for PurR, similarities in amino acid sequence (26, 29, 34) lead to the expectation for similar modes of operator binding by PurR and LacI.

Although binding of PurR to operator DNA sequesters Arg-52 and Leu-54 from protease digestion, the physical basis for this result is not known. A change in hinge structure accompanying binding of PurR to DNA and direct interaction of hinge residues with DNA are among the possibilities.

Model for PurR function. The properties of the hinge mutants described here and the mutations in residues required for corepressor binding (4) lead to a working model for PurR function, which is outlined in Fig. 7. According to this model, there are two routes to form the repressor-operator complex, structure 4, that represses gene transcription. In one route, apoPurR, species 1, binds corepressor to form holoPurR, species 2, which is conformationally altered and can bind with high affinity to operator and repress gene transcription. A second route to the repressed gene may involve specific low-affinity binding of apoPurR to operator, species 3. Low-affinity binding to operator DNA is dependent on helix-turn-helix and hinge sequences in the DNA-binding domain of

dimeric aporepressor. This interaction of apoPurR with operator leads to a change in hinge structure. Binding of corepressor to aporepressor-operator DNA complex results in a corepressor-binding domain conformation change that leads to high-affinity interaction with operator that is associated with gene regulation. Modeling of PurR to the ribose-binding protein structure (25) predicts a two-domain structure for the corepressor-binding domain (4). By direct analogy to ribose-binding protein (25), the two domains may equilibrate between open and closed conformations that bind operator DNA with low affinity and high affinity, respectively. According to this model, corepressor binding locks the protein into the closed active (high affinity) conformation, by analogy with ribose-binding protein (25).

Specific binding of apoPurR to operator DNA has been demonstrated *in vitro*, and mutations which impair corepressor binding have little effect on low-affinity operator interaction (4). This corepressor-independent binding of PurR to operator DNA may reflect a function derived from a progenitor LacI/RbsR type of repressor which binds to operator DNA with high affinity in the absence of effectors. Binding of corepressor is accompanied by a conformation change in the corepressor-binding domain (3), although it is not known how this change contributes to the enhanced stability of the repressor-operator interaction.

PurR hinge mutants R52E, L54T, and K55E lost the capacity for low-affinity binding to operator DNA, thus supporting a role of the hinge region in DNA binding. Earlier experiments with LacI headpiece fragments 1 to 51 and 1 to 59 have suggested that all contacts with *lac* operator are made by residues 1 to 51 of the DNA-binding domain and that hinge residues 52 to 59 function to maintain the DNA-binding structure (15, 16). As a result, it is useful to consider a model in which the conformation change that results from binding of corepressor to apoPurR either facilitates access of operator DNA or in some other way generates a high-affinity binding structure. The applicability of the model shown in Fig. 7 depends on the existence of species 3 *in vivo*. The existence of species 3 *in vivo* remains to be established.

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