Mutant Tryptophan Aporepressors With Altered Specificities of Corepressor Recognition

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Manuscript received November 20, 1990
Accepted for publication January 19, 1991

ABSTRACT

The Escherichia coli trpR gene encodes tryptophan aporepressor, which binds the corepressor ligand, L-tryptophan, to form an active repressor complex. The side chain of residue valine 58 of Trp aporepressor sits at the bottom of the corepressor (L-tryptophan) binding pocket. Mutant trpR genes encoding changes of Val58 to the other 19 naturally occurring amino acids were made. Each of the mutant proteins requires a higher intracellular concentration of tryptophan for activation of DNA binding than wild-type aporepressor. Whereas wild-type aporepressor is activated better by 5-methyltryptophan (5-MT) than by tryptophan, Ile88 and other mutant aporepressors prefer tryptophan to 5-MT as corepressor, and Ala86 and Gly88 prefer 5-MT much more strongly than wild-type aporepressor in vivo. These mutant aporepressors are the first examples of DNA-binding proteins with altered specificities of cofactor recognition.

Many regulatory proteins are allosteric, and bind small metabolic intermediates that modify their activities, allowing the intracellular pattern of gene expression to respond to physiological change. For example, the Escherichia coli trp gene encodes tryptophan aporepressor, which binds the corepressor ligand, L-tryptophan, to form an active repressor complex. The side chain of residue valine 58 of Trp aporepressor sits at the bottom of the corepressor (L-tryptophan) binding pocket. Mutant trpR genes encoding changes of Val58 to the other 19 naturally occurring amino acids were made. Each of the mutant proteins requires a higher intracellular concentration of tryptophan for activation of DNA binding than wild-type aporepressor. Whereas wild-type aporepressor is activated better by 5-methyltryptophan (5-MT) than by tryptophan, Ile88 and other mutant aporepressors prefer tryptophan to 5-MT as corepressor, and Ala86 and Gly88 prefer 5-MT much more strongly than wild-type aporepressor in vivo. These mutant aporepressors are the first examples of DNA-binding proteins with altered specificities of cofactor recognition.

Although mutations that alleviate the requirement of DNA binding proteins for their allosteric cofactors have been described (Adhya and Garges 1990; Menon and Lee 1990), no mutant repressors or activators with altered specificities of cofactor recognition have been found to date. In this paper, we describe the first such mutants, Trp aporepressors altered in their interactions with L-tryptophan and 5-methyltryptophan (5-MT), a toxic corepressor analog.

The product of the E. coli trp gene is a monomer of 107 amino acids that assembles as a dimer (Gunsalus and Yanofsky 1980; Joachimiak et al. 1983; Arvidson, Bruce and Gunsalus 1986). The crystal structure of Trp aporepressor (Zhang et al. 1987) shows that each monomer is a bundle of six a-helices, A–F. Four of the six a-helices, A, B, C, and F are interwoven in the dimer, and multiple, symmetric intermonomer interactions stabilize its extraordinary hydrophobic core. The aporepressor dimer denatures at a very high temperature (>90°C) (Baek et al. 1988).

Comparisons of the crystal structures of repressor (Schevitz et al. 1985; Lawson et al. 1988; Otwinowski et al. 1988), and aporepressor (Zhang et al. 1987) suggest that activation of the TrpR dimer involves repositioning a-helices D and E with respect to the rigid hydrophobic core determined by a-helices A, B, C, and F. Crystal structures of the Trp repressor complex show that each of the two symmetric corepressor binding pockets in a repressor dimer is formed by side chains from both monomers (Schevitz et al. 1985; Lawson et al. 1988; Otwinowski et al. 1988). In these structures, tryptophan participates in hydrophilic and hydrophobic interactions with these side chains (Figure 1). Both the carboxylate and a-ammonium groups of tryptophan are involved in hydrophobic bonding networks. The carboxylate group forms hydrogen bonds (H-bonds) with the guanidino group of Arg84 from one monomer, and the hydroxyl group of Thr117 from its partner monomer (1). The ammonium group forms H-bonds with the Ser88 hydroxyl and the main-chain carbonyls of Leu411 and Leu457. The indole ring makes multiple hydrophobic interactions with side chains that mold the binding pocket. The plane of the indole ring is sandwiched between the parallel, extended aliphatic side chains of Arg84 and Arg86, and the C5-C7 edge of the indole ring packs against the branched hydrophobic side chains of Ile57 and Val98. The corepressor, L-tryptophan, must act like a keystone in the hydrophobic core of aporepressor. Its hydrophobic indole ring is buried in an "argi-
nine sandwich™ and framed by hydrophobic interactions, and its hydrophilic functional groups are on the surface of the repressor complex.

However, by no means do the crystal structures of tryptophan repressor give us a complete understanding of how tryptophan binds as corepressor and elicits the allosteric change between aporepressor and repressor. In part, this is because structural differences between aporepressor and repressor are obscured by lattice interactions in the crystals. For example, the sum of RMS (root mean square) differences in the coordinates of two different crystal forms of repressor is greater than the sum of differences between the same form of aporepressor and repressor (LAWSON et al. 1988).

In addition, the pattern of specific weak bonds that stabilizes tryptophan binding in the crystal structures of repressor must be different than the pattern in vivo. The crystal structures do not explain two things. First, Thr44 appears to make an important bond with the tryptophan carboxylate in vivo, the change Thr44 → Ala has only a subtle effect on tryptophan binding in vitro (He and Matthews 1990). Second, the distance between carbon-5 of the tryptophan indole and the γ-methyl carbons of Val58 in the repressor crystal structures (ca. 3.8Å) (Schevitz et al. 1985; Lawton et al. 1988) is too short to accommodate an additional methyl group. This is disturbing because the related Enterobacter aerogenes and Enterobacter cloacae tryptophan aporepressors are predicted to have isoleucine, not valine, at this position (our unpublished results). Furthermore, aporepressor binds 5-methyltryptophan (5-MT) more tightly than tryptophan as corepressor (Marmorstein et al. 1987; D. N. Arvidson and R. P. Gunsalus, unpublished results).

To investigate the nature of corepressor binding, we have examined the effects of all possible amino acid changes of residue valine 58. These mutants allow us to explore the role of this residue in a manner independent of the conclusions we may draw from a structure in vitro. Several amino acid changes should shorten or lengthen the Val58 side chain without compromising its hydrophobic character. Therefore, if the carbon-5 of tryptophan is close to the γ-methyls of Val58 in the repressor complex, then a subset of Val58 changes (including Ile58 and Ala58) should alter the preference of aporepressor for 5-MT and tryptophan. Indeed, we show that many of these mutant aporepressors have altered specificities of corepressor recognition. Even more surprisingly, we find that a wide variety of amino acid changes are tolerated at this residue position, suggesting that the corepressor binding pocket is much less rigid than the structures represented in the crystals.

MATERIALS AND METHODS

Bacteria, phage and plasmids: Bacterial strains are derivatives of S. typhimurium 1T2 or E. coli K12. Salmonella strain MS1363 (leuA-am414 supE) (Susskind 1980) was used for the permissive growth of challenge phages; MS168/F°lacF° (Bass et al. 1987) carrying plasmid pPY2000 was used for the permissive growth of challenge phages. The “Trp” challenge phage used to assay DNA-binding in vivo, P22 Kn9 O-ref1 arc-amH1605 (Bass et al. 1987) carries a minimal reference-type trp operator, 5′ GAACAGTTAATAGTTTC 3′, controlling ant expression. Plasmid pPY2000 is a derivative of
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Quantitation of mutant proteins: E. coli CG103 with each mutant derivative of plasmid pPY2000 was grown in Trp “drop-out” medium (Bass et al. 1987) with 50 pg/ml prototrophy and 100 pg/ml thiamine and 480 pg/ml carbenicillin, to a density of 2 x 10^9/ml. Proteins were resolved by SDS polyacrylamide gel electrophoresis (Schagger and von Jagow 1987) and were transferred to nitrocellulose electrophoretically (Tsang, Peralta and Simons 1983). Nitrocellulose-bound TrpR was detected by incubation with rabbit anti-aporepressor antibody (the gift of R. P. Gunsalus) (Gunsalus, Miguel and Gunsalus 1986), biotin-conjugated goat anti-rabbit antibody, and streptavidin-conjugated alkaline phosphatase (Harlow and Lane 1988). In Salmonella, Gly^s^ is unstable without or with 2 pg/ml L-tryptophan, but may be produced stably in the presence of 5-MT (data not shown). Thus, the addition of a methyl group to the 5 position of the indole ring of corepressor, or to the α-carbon of Gly^s^, confers stability on this mutant aporepressor/corepressor complex. Rapid β-galactosidase assays: Rapid β-galactosidase assays were performed as described by Gardella, Moyle and Süsskind (1993) with three modifications. Each CG103 strain with a pPY2000 derivative was grown in media with or without 2 μg/ml tryptophan before assay; cells were grown in wells of microtiter plates in 250 μl volumes without active aeration at 37°C; and, a high multiplicity of T-even phage was used to lyse cells from without (Doerrmann 1952). Activities are given in Barrick units. Data varied less than 20% from experiment to experiment.

Challenge phage assays: Overnight cultures of MS1868/F^+/lacD^0 carrying plasmid pPY2000 and each of its mutant derivatives were diluted 100-fold into LB medium with 50 μg/ml ampicillin, and grown to a density of 4 x 10^9/ml. Aporepressor expression was induced by the addition of isopropyl-β-thiogalactoside (IPTG) to 10^{-3} M; cultures were incubated at 37°C for 30 min, and chilled to 4°C. Each culture was infected with challenge phage P22 K9 O-raf arc-AMH1605 at a multiplicity of 25 phage/cell. After adsorption of the phage for 15 min at 25°C, infected cells were diluted in the wells of microtiter plates, and 4 μl of each serial dilution was spotted on green tryptophan drop-out plates with ampicillin, kanamycin, IPTG (10 μM), and tryptophan (or tryptophan plus 5-MT). The efficiency of survival is the number of surviving cells divided by the number of input cells (assayed on green plates with ampicillin); averages of at least three independent measurements are shown.

RESULTS

Mutant aporepressors with Val^58^ changes: Plasmid pPY2000 expresses wild-type Trp aporepressor (Fig. 2). On this plasmid, the lacUV5 promoter directs transcription of the trpR^* gene, under the control of Lac repressor. Codon 58 of trpR was mutagenized with a mixed oligonucleotide, used to prime the resynthesis of the trpR sense strand on single-stranded template pPY2000 DNA (Taylor, Ott and Eckstein 1985; Bass, Sorrells and Youderian 1988). Mutagenized DNA was transformed into strain X90/F^+/lacD^0 which makes high levels of Lac repressor to prevent the expression of potentially lethal levels of mutant aporepressors (Bass, Sorrells and Youderian 1988). Transformants were pooled, and plasmid DNA was extracted from the pool. Because site-directed mutagenesis results in a high frequency of untargeted second-site mutations, a small restriction fragment with the target codon was generated by cleavage of the plasmid DNA at unique SalI and MluI sites, purified, and subcloned into wild-type pPY2000 backbone. Sequences of this fragment in the subclones show that 47 mutant plasmids represent 20 different
plasmid missing the 434-bp otherwise isogenic with pPY2000. The percentage of cross-reacting material (CRM) relative to wild-type aporepressor produced from parentheses; codons with asterisks were recovered from a "clean-codons for 17119 possible amino acid changes at position 58 (Table 1). Plasmids producing aporepressors with the two remaining amino acid substitutions were made using a second, less degenerate primer.

Aporepressor made by each of the mutant plasmids was determined from the analysis of immunoblots. β-Galactosidase activity is given in Table 1. Three of the 19 mutant proteins, Pro, Asp, and Arg, are made in modest amounts (14-38%). The remaining 13 mutant aporepressors are made at more than 45% of wild-type levels.

The ability of each of these mutant aporepressors to repress the E. coli trp operator was measured in E. coli host CG103. This strain has deletions of both the chromosomal trpR and lac genes, and an integrated λ prophage with a trpE/lacZ gene fusion (D. N. Arvidson and C. G. Arvidson, unpublished results). In CG103, synthesis of the LacZ fusion protein is regulated by the plasmid-encoded Trp aporepressor (Figure 2). As shown in Table 1, wild-type aporepressor produced by pPY2000 represses LacZ expression about 50-fold without added tryptophan; with tryptophan, lower levels of LacZ activity are made. An otherwise isogenic plasmid with a deletion of trpR does not repress the gene fusion. Five mutant aporepressors (Cys > Ile > Ala = Ser > Thr) retain near wild-type activity, and seven mutant proteins (Met > Phe > His > Asn = Tyr > Gln > Leu) have intermediate activities. Even in the presence of tryptophan, the seven most labile mutant aporepressors (Gly > Glu > Trp > Lys > Asp > Pro > Arg) effect little repression of the gene fusion.

Activation of mutant aporepressors by corepressors: The interaction of Trp repressor with the wild-type E. coli trp operator involves the binding of multiple repressor dimers to tandem sites within operators (Kumamoto, Miller and Gunsalus 1987; Bass et al. 1987; Elledge and Davis 1989; Staacke et al. 1990). Mutations in trpR may have pleiotropic phenotypes, affecting not only the ability of individual dimers to bind single sites, but also the ability of multiple dimers to bind tandem arrays of sites. To exclude pleiotropic effects of trpR mutations on the tandem binding of dimers, we studied the binding of each mutant protein to a single operator site, using the challenge phage assay (Benson et al. 1986).

A P22 challenge phage will lysogenize host cells that produce a repressor able to bind a phage-borne operator. After infection of a sensitive subh host, a challenge phage with a trp operator will establish lysogeny if and only if its trp operator is bound by Trp repressor (Figure 2). Once integrated, the P22 prophage confers a kanamycin-resistant phenotype upon its surviving host. Alternatively, if the challenge phage operator is free of repressor, P22 will make antirepressor, develop lytically, and kill its host. Because the fractional occupancy of an operator increases with increasing repressor concentration, the efficiency of lysogeny of a challenge phage with a trp operator increases in response to increasing levels of both aporepressor and the corepressor, L-tryptophan (Bass et al. 1987).

The Salmonella host for challenge phage infection assays carries the Trp aporepressor-producing plasmid (pPY2000) and the F'lac episome, which produces Lac repressor. In the presence of high levels of IPTG, an inducer of Lac repressor, this host produces high levels of aporepressor. Under these conditions, the efficiency of lysogeny of a challenge phage depends on the amount of exogenous corepressor, because the amount of active Trp repressor complex is limited by the amount of added corepressor. As shown
in Figure 3, the fraction of lysogenic survivors among infected cells producing wild-type aporepressor is low in the absence of exogenous tryptophan and increases dramatically (more than 1000-fold) upon the addition of tryptophan.

The frequency of lysogeny of each aporepressor-producing host depends on added tryptophan concentration; differences in this dependence allow us to rank the requirement of each mutant aporepressor for tryptophan in vivo. Each of the mutant Trp aporepressors requires more tryptophan than Val58 aporepressor for activity (Figure 3). Seven mutant aporepressors permit the efficient lysogenic development of challenge phage with 100 μg/ml tryptophan. These are ranked in decreasing order of activity: Ile > Cys > Thr > Ser > Ala > Leu > Met. This order agrees well with that of the ability of mutant aporepressors to control the trpE/lacZ fusion regulated by the wild-type E. coli trp operator. The five mutant aporepressors that repress the fusion best are the most active in the challenge phage assay.

Four mutant aporepressors permit lysogenic development with maximum efficiencies less than wild type, and are ranked by activity in the order: His > Phe > Asn > Gln. Both His58 and Asn58 proteins require more tryptophan for activity than wild-type aporepressor; addition of tryptophan does not increase their maximum ability to support lysogeny. Presumably, when these proteins are complexed with tryptophan, they have lower intrinsic affinities for the trp operator than the wild-type complex in vivo. The eight mutant aporepressors that are least active have changes of Val58 to Pro or Gly, to each of four charged amino acids (Asp, Glu, Lys and Arg), or to residues with larger side chains (Lys, Tyr, Trp and Arg).

The tryptophan analog, 5-MT, can substitute for L-tryptophan as corepressor in vivo (COHEN and JACOB 1959), and is bound by wild-type aporepressor with a four-fold higher affinity in vitro (MARMORSTEIN et al. 1987; D. N. ARVIDSON and R. P. GUNSALUS, unpublished results). This analog is toxic to both S. typhimurium and E. coli, either because incorporation of 5-MT into essential proteins renders them inactive, or because 5-MT both causes feedback inhibition of anthranilate synthase and acts as corepressor, the combined effect of which is lethal tryptophan starvation (SOMERVILLE 1983).

The toxic effect of 5-MT is antagonized by small amounts of added tryptophan; these amounts do not permit the efficient lysogenization of cells by challenge phage. Thus, we measured the frequency of lysogeny of a challenge phage as a function of 5-MT concentration in the presence of 1 μg/ml added tryptophan (Figure 3). As expected, wild-type aporepressor requires less added 5-MT (0.4 μg/ml 5-MT + 1 μg/ml tryptophan) than tryptophan (3 + 1 μg/ml) to permit efficient lysogeny. Seven mutant aporepressors permit the Trp challenge phage to lysogenize with wild-type efficiencies in the presence of 1 μg/ml tryptophan and 10 μg/ml 5-MT. These are ranked in order of their increasing dependence on 5-MT: Ala > Cys > Ser > Leu > Thr > Ile = Met. Four mutant aporepressors, in the presence of high levels of 5-MT, permit lysogeny with efficiencies less than observed with wild-type aporepressor, and are ranked in the order: Gly > Asn > Gln > Lys. The remaining eight mutant aporepressors (Pro, Asp, Glu, His, Phe, Tyr, Trp and Arg) do not permit efficient lysogeny at any 5-MT concentration.

**DISCUSSION**

All of the changes at residue Val58 of E. coli Trp aporepressor reduce either the stability or activity of this protein. The Val58 side chain is located in the carboxyl-terminal half of α-helix C. It is no surprise that the change, Val58→Pro, predicted to disrupt this secondary structure, results in an unstable protein. The electrostatic character of the residue 58 side chain is perturbed by the introduction of a hydrophobic group at position 58. The electrostatic character of the residue 58 side chain is perturbed by the introduction of a hydrophobic group at position 58.
chain is also a critical determinant of corepressor recognition. Changes of the hydrophobic Val58 side chain to charged side chains (e.g., Arg, Lys, Asp, Glu) result in unstable mutant aporepressors.

Ile58 is the most active mutant aporepressor. Ile58 requires much more 5-MT for activity than wild type. In this case, the addition of a single methyl group to the side chain of residue 58 (Val → Ile) changes the preference of aporepressor from a corepressor with a 5-methyl group to one without. Presumably, steric exclusion of the additional 6-methyl of Ile58 by the 5-methyl of 5-MT distorts the structure of the repressor complex. In contrast, Ala58 aporepressor requires more tryptophan than Val58 (wild type) for activation, yet is activated by 5-MT as well as the wild type. Whereas lengthening the Val58 side chain by the addition of a 6-carbon impairs the interaction of aporepressor with 5-MT, shortening the Val58 side chain by the subtraction of 5-carbons impairs the interaction of aporepressor with tryptophan. Gly58 aporepressor can be activated in the challenge phage assay only by 5-MT. The volume of the residue 58 side chain is one of the most critical determinants of corepressor recognition specificity.

The geometry of the residue 58 side chain is also an important determinant of corepressor recognition. APOrepressors with more extended residue 58 side chains fare better with 5-MT as corepressor than with tryptophan in the challenge phage assay. Thus, whereas Ile58 prefers tryptophan, Leu58 prefers 5-MT; in addition, Lys58 is activated only by 5-MT. Of the four mutant repressors with large aromatic or side chains, only repressors with the smallest two side chains (Phe and His) are activated by tryptophan, and none are activated by 5-MT.

Substitution of polar residues for Val58 weakens the interaction between aporepressor and corepressor. Thus, the Ser58 and Thr58 aporepressors require both more tryptophan and more 5-MT for activity than wild type. However, the volume of the side chain is more important than polarity, because these mutant proteins bind tryptophan in the order of affinity: Val > Thr > Ser > Ala.

The hydrophobic interaction of residue Val58 of Trp aporepressor with the indole ring of bound corepressor is highly specific. All of the changes at Val58 reduce the activity of Trp aporepressor. Nonetheless, a subset of Val58 changes has new specificities of corepressor activation in vivo. These mutant aporepressors prefer tryptophan to 5-MT, or prefer 5-MT much more strongly than wild-type aporepressor, suggesting that the side chain of Val58 interacts directly with carbon-5 of the indole ring in vivo, as predicted from the interpretation of the crystal structures of Trp repressor (SCHEVITZ et al. 1985; LAWSON et al. 1988; OTWINOWSKI et al. 1988).

The most surprising result to emerge from this study is that many different amino acid changes at Val58 do not result in a major loss of repressor activity. In a related study, WELLS et al. (1987) examined all possible changes of residue Met222, located in the interior of the substrate binding pocket of subtilisin. All of the changes of Met222 that increase the specific volume of the residue 222 side chain (e.g., Phe222) result in dramatic decreases in enzyme activity. Unlike the substrate-binding pocket of subtilisin, the corepressor-binding pocket of tryptophan aporepressor must be less rigid to accommodate both a wide variety of indole analogs (MARMORSTEIN et al. 1987), and a wide variety of amino acid substitutions at Val58. Although the X-ray crystal structures of Trp repressor protein may provide us with an initial picture of how activation by corepressor might work, it is likely that more detailed spectroscopic analyses of the motion of this flexible substructure, the tryptophan binding pocket, will be necessary to fill in the details of how tryptophan activates Trp aporepressor.

We thank Cindy Grove Arvidson, Fred Eiserling, Andy Kumamoto, Todd Lane, Dennis Sarai, Mimi Suskind, Gary Stormo and Gary Trump for advice and helpful discussions. We thank Paul Sigler for permission to reprint Figure 1 from Schevitz et al. (1985). This work was supported by National Institutes of Health grant GM34150 to P. Y. and GM12629 to D.N.A.

LITERATURE CITED


ELLEDGE, S. J., and R. W. DAVIS, 1989 Position and density effects


Communicating editor: G. Mosig