The binding of l-tryptophan to Escherichia coli tryptophan aporepressor enables the holorepressor complex to bind operator DNA tightly. The side chain of residue alanine 77 is located in one of the most flexible regions of Trp repressor, between residues critical for binding DNA. Codon-directed mutagenesis was used to make genes encoding mutant Trp repressors with each of the 19 naturally occurring amino acid changes of Ala77. The 19 mutant proteins are made at the same steady-state levels as wild type. Sensitive challenge phage assays show that 7 of the 19 mutant proteins (Cys, Ser, Val, Leu, Thr, Ile, and Lys) are more active than wild-type protein when tryptophan is limiting in vivo. Among these 7 mutant super-aporepressors, proteins with Cys and Ser changes also are super-holorepressors, because they repress better than wild-type holorepressor when tryptophan is in excess. These results and others suggest that super-aporepressors associate more poorly than wild-type aporepressor with nonspecific DNA. Consistent with this idea, these 7 changes are predicted to disrupt the tertiary structure of aporepressor, but have more limited effects on the structure of holorepressor.

Trp aporepressor binds 2 molecules of the corepressor ligand, l-tryptophan, to form a holorepressor complex with increased affinity for specific DNA sites on the Escherichia coli genome. Trp repressor is a symmetric dimer formed from two identical monomers 107 amino acid residues in length (Gunsalus and Yanofsky, 1980). Comparisons of the crystal structures of aporepressor and holorepressor (Schevitz et al., 1985; Zhang et al., 1985; Lawson et al., 1985; Otwonowski et al., 1985) suggest that activation of the TrpR dimer involves the repositioning of flexible DNA "reading heads" (α-helices D and E) with respect to its rigid hydrophobic core (α-helices A, B, C, and F). The flexible reading heads in aporepressor are about 25 Å apart, and become separated by 30–34 Å in holorepressor, enabling them to contact successive major grooves of operator DNA (Fig. 1; Schevitz et al., 1985; Luisi and Sigler, 1990; Arrowsmith et al., 1991). The turn between α-helices D and E (Leu75-Gly76-Ala77-Gly79) shows the greatest variation in coordinates for defined residues in the orthorhombic structures of aporepressor and holorepressor (Lawson et al., 1988).

Several mutations that change amino acids in the flexible D/E turn have been isolated. Whereas changes of residue Gly76 to Ser and Asp result in a loss of repressor function, the change Ala77 → Val improves function. Expression of the mutant Val77 protein in the absence of exogenous tryptophan inhibits the growth of a host with a mutation in the traA gene that decreases but does not abolish tryptophan synthase activity. In contrast, expression of wild-type repressor in an otherwise isogenic host does not inhibit growth (Kelley and Yanofsky, 1983). Also, the Ala77 → Val change has been isolated as a second-site revertant of a change (Gly86 → Arg) that decreases TrpR activity (Klig et al., 1988). However, purified Val77 protein has the same operator-binding activity as wild-type TrpR (Ala77), both in the presence and absence of tryptophan. Even more puzzling, the Val77 protein aporepressor requires as much tryptophan to be activated for operator binding as wild-type repressor in vitro (Hurlburt and Yanofsky, 1990).

To understand why the mutant Val77 protein appears to be more active than wild type in vivo, but not in vitro, we have examined its phenotype in vivo, using challenge phage assays (Benson et al., 1986; Bass et al., 1987; Bass et al., 1988; Arvidson et al., 1991a). In addition, we have asked whether other changes of this residue may help elucidate the role residue Ala77 plays in TrpR function. Here we describe the construction and phenotypic characterization of plasmids producing mutant aporepressors with each of the possible amino acid changes at residue Ala77. Although 12 of these 19 changes decrease TrpR activity, 7 result in mutant proteins with super-aporepressor phenotypes. When tryptophan is limiting, lower levels of these proteins are required for repression. Two changes, to cysteine and serine, also have super-holorepressor phenotypes. Lower levels of these proteins are required for repression when tryptophan is present in excess. Phenotypic differences imply that these 7 changes affect different combinations of equilibria between free aporepressor, free repressor, and their complexes with both specific (operator) and nonspecific DNA.

**EXPERIMENTAL PROCEDURES**

*Bacteria, Phage, and Plasmids—* Bacterial strains are derivatives of E. coli K12 and Salmonella typhimurium LT2. E. coli strain CJ236 (dut-1, ung-1, thi-1, relA-1 (pCJ105); Bio-Rad Laboratories) was used as the host for preparing uracil-containing single-stranded plasmid templates for site-directed mutageneses. Plasmid pCJ05 is a derivative of the F episome that permits phage M15 adsorption and confers resistance to chloramphenicol. E. coli X90/F' lacQ4 (recA argE am thi-1 ΔproAB-lac), NalR, RifR; Amann et al., 1983), carrying an F episome that produces high levels of Lac repressor, was used as the transformation recipient for mutant plasmids. E. coli CG103/RSF2001 (lac-pro ΔApR-504 thi-1 recA-56 srl: Tn10 (AACL45)) was used as the host for rapid β-galactosidase assays. It carries a specialized transducing λ prophage with a fusion of the wild-type E. coli trp promoter/operator, attenuator, and start of trpE to lacZ. The episome RSF2001 is a derivative of F with an insertion of a small
related to the repair of apyrimidinic sites produced in the dut+ host.
To ensure that our results do not reflect the phenotypes of secondary, mutated targets, the small 180-bp Sall-MluI fragment from each original mutant plasmid was subcloned into an otherwise wild-type plasmid Sall-MluI backbone from plasmid pPY2000S prior to characterization. To construct plasmid pPY2000S, the oligonucleotide 5'-CTCAGAGTTAAATAGTACATCCGGCGCG make a silent change of codon 74 on plasmid pPY2000 from GAA to GAG, which creates a unique SacI site overlapping codons 73 and 74 of trpR. The small 180-bp Sall-MluI fragment from one mutant plasmid with the desired change was subcloned into an otherwise wild-type pPY2000 backbone. The unique SacI site in pPY2000, the unique SacI and MluI sites, restriction enzymes and phage T4 DNA ligase (Stratagene Cloning Systems) were used in the mutagenesis and subcloning procedures under conditions specified by the supplier.

Quantitation of Mutant Proteins—Minimal tryptophan drop-out (TDO) medium (Bass et al., 1987) consists of M9 medium (Smith and Levine, 1964) supplemented to 20 μg/ml L-arginine HCl, L-aspartic acid, t-histidine HCl, L-isoleucine, L-leucine, L-lysine HCl, L-methionine, L-phenylalanine, L-tryptophan, and L-tyrosine (Sigma), and to 0.4% glucose (Difco). Relative content of derivatives of pPY2000 was grown in TDO medium supplemented with 1 μg/ml tryptophan, 50 μg/ml proline, 100 μg/ml thiamin, and 400 μg/ml carbenicillin to a density of 2 × 10^6/ml. Cells (1 ml) were pelleted by centrifugation, resuspended in 70 μl of sample buffer with 2% sodium dodecyl sulfate, and heated for 5 min at 100°C. Proteins in cell lysates (10 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose electrophoretically (Arvidson et al., 1991a). Bound repressor was detected by sequential incubation with a 1:1,000 dilution of primary rabbit anti-aporepressor antibody (Gonzales et al., 1986), the kind gift of Rob Gonzales, and a secondary goat anti-rabbit antibody conjugated with alkaline phosphatase, using the Picoblue screening reagent kit (Stratagene Cloning Systems). Under these conditions, TrpR represents about 0.1% of the total cell protein.

Modified Challenge Phage Assays—Challenge phages are derivatives of temperate Salmonella phage P22 that afford a direct selection for proteins that bind specific DNA sites (Benson et al., 1986, 1988; Arvidson et al., 1991a). After injection of a sensitive sup+ host, a challenge phage with the trp operator will establish lysisogeny if and only if its trpR operator is bound by active Trp repressor produced by the host. If the challenge phage operator is free of repressor, the infecting P22 genome will be produced and the cell will develop lytically, and result in death of the infected cell. If the operator is bound by repressor, the infecting P22 genome will lysogenize and confer a kanamycin-resistant phenotype upon its surviving host. Because the fractional occupancy of the trp operator increases with increasing concentrations of holorepressor, the relative level of lysisogeny of a host injected with a trp challenge phage increases in response to both increasing levels of aporepressor and increasing levels of the corepressor, t-tryptophan (Bass et al., 1987, and Fig. 3, below). The Salmonella host we use for challenge phage assays carries two plasmids, pPY2000, which produces Trp repressor from the lacUV5 promoter, and the f lacP episome. The level of TrpP expression in this host is low in the absence of IPTG, an inducer of Lac repressor, because a high level of F-encoded Lac repressor inhibits transcription of trp from the lacUV5 promoter.

Challenge phage assays were modified from previous procedures (Bass et al., 1987, 1988) to permit determination of serial dilutions of phage-infected cells were made using a programmable automatic pipetting device to transfer multiple aliquots simultaneously to the wells of a sterile microtiter dish. In addition, multiple aliquots of these serial dilutions were spotted simultaneously onto solid culture media in large (150-mm diameter) Petri dishes.

To measure repression as a function of the amount of plasmid-encoded Trp repressor required for the efficient lysisogenization of the challenge phage, overnight cultures of MS1868/F'lacP carrying pPY2000 or each mutant plasmid were diluted 100-fold into 5 ml of LB medium with 50 μg/ml ampicillin and grown to a density of 2.5 × 10^7/ml at 37°C. IPTG was added to a final concentration of 1 × 10^{-3} M to induce the expression of trp repressor, and cells were grown for an additional 30 min at 37°C, then placed on ice for 15 min. To an aliquot (135 μl) of each culture, an equal volume of challenge phage P22 Kn9 O-ref2 arc-amH1605 at a titer of 10^{10}/ml was added, to give a multiplicity of infection of approximately 25 plaque/cell. After

The abbreviations used are: bp, base pair(s); IPTG, isopropyl β-D-thiogalactopyranoside.
Adsoption of the phage for 15 min at 25 °C, infected cells were diluted seven successive 5-fold dilutions in the wells of microtiter plates, and 4 μl of each 5-fold serial dilution was spotted on green tryptophan drop-out plates with various concentrations of IPTG, ampicillin (100 μg/ml), kanamycin (25 μg/ml), and with or without tryptophan (100 μg/ml).

To measure repression as a function of the amount of tryptophan (corepressor) required for the efficient lysogenization, 4 μl of each serial dilution was spotted on green tryptophan drop-out plates with 10−4 M IPTG, ampicillin (100 μg/ml), kanamycin (25 μg/ml), and tryptophan at various concentrations.

The rationale whether mutant repressors have new binding specificities, cultures of MS1865 F− lacIΔ8 carrying each mutant plasmid were grown in exponential phase in TDO medium with 10−8/ml at 37 °C, IPTG was added to 10−4 M, and cells were grown for an additional 30 min at 37 °C. Cultures were infected with each of the eight different challenge phages derived from P22 Kn9 O-rep2 arc-amH1065 that carry symmetric changes in the consensus trp operator which prevent the binding of wild-type repressor (Bass et al., 1987). 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 10−4 M IPTG, ampicillin (100 μg/ml), kanamycin (25 μg/ml), and tryptophan (40 μg/ml).

For each challenge phage assay, the efficiency of survival is the titer of surviving cells divided by the titer of input cells (assayed on green plates with ampicillin); averages of the results from at least three independent experiments are shown. Efficiencies of survival varied less than 5-fold from experiment to experiment.

Rapid β-Galactosidase Assays — Rapid β-galactosidase assays were conducted as described by Arvidson et al. (1991b). Each CG103 strain with plasmid pMS421 and a derivative of pPY2000 was grown in TDO medium with 400 μg/ml carbenicillin and with or without 1 μg/ml tryptophan in the wells of microtiter plates. Exponential cultures were lysed from without with phage T4 in 8-galactosidase assay buffer, and liberation of β-nitrophenol from o-nitrophenyl-β-D-galactoside was monitored as A414 over time with a microtiter plate spectrophotometer. Activities were calculated using the equation:

\[
\text{Activity} = \left(\frac{1000}{b}\right)(A_{492})^{-1} \left(\text{f} \right)^{-1} \quad \text{(Eq. 1)}
\]

where b is the slope of linear least squares fit to the plot of A414 versus time (min), and f (0.2 in our assays) is the fraction of cells added to the total volume of lysate. In all cases, averages of three or more independent determinations varied less than 20%.

A high concentration of the relatively slowly hydrolyzed penicillin analog, carbenicillin, was chosen for these assays, because hosts carrying some of the mutant pPY2000 derivatives (in particular, those producing more active aporepressors or holorepressors) segregate a high fraction of daughter cells that have lost the plasmid in parallel assays with ampicillin. This choice of β-lactam improves but does not solve the problem of plasmid loss. Assays with carbenicillin in the presence of tryptophan concentrations greater than 4 μg/ml or the absence of plasmid pMS421 also result in a high frequency of loss of some pPY2000 derivatives and yield higher, more variable β-galactosidase activities.

**RESULTS**

Site-directed Mutagenesis of Codon 77 — To change codon 77 of the trpR gene, we used a degenerate oligonucleotide to mutate the restriction site of pPY2000, a plasmid which expresses Trp repressor from the lac promoter. The mutated template was transformed into E. coli host X90/F’ lacIΔ8. This E. coli host produces a high level of Lac repressor from its F’ episome that can turn down the potentially lethal expression of mutant trpR genes.

As summarized in Table I, we sequenced plasmid DNA isolated from 8 transformants and found mutant plasmids with 14 different codons representing 12 different amino acids at position 77. To make the remaining 7 possible amino acid changes, we used 3 additional synthetic oligonucleotides to prime the synthesis of the sense strand of pPY2000, as with the more degenerate primer.

**Mutant Repressors Are Made at Normal Levels in Vivo —** To measure the relative steady-state levels of mutant repressors with Ala77 changes in vivo, derivatives of the E. coli host strain, CG103, with each of the mutant plasmids were grown to exponential phase in TDO medium with 1 μg/ml tryptophan, and the relative amounts of intracellular TrpR protein were quantitated using a Western immunological assay. As shown in Fig. 2, all of the mutant repressors are made in amounts similar to that of the wild type, indicating that none of these changes results in a protein significantly less stable than the wild type in vivo.

Two Mutant Repressors Are Super-holorepressors — To rank the relative activities of mutant TrpR proteins with Ala77 changes, we have used the challenge phage assay under three different conditions: with excess exogenous tryptophan (100 μg/ml) and varying levels of IPTG, with limiting (no) exogenous tryptophan and varying IPTG, and with varying levels
phage in the presence of excess tryptophan increases with increasing amounts of IPTG, an inducer of Lac repressor. Without IPTG, this host makes such low levels of E. coli Trp repressor that it cannot be lysogenized efficiently. Presumably, the low background level of Trp repressor encoded by the single-copy trpR gene on the host Salmonella chromosome is not sufficient to promote efficient lysogeny, although it accounts for a significant background frequency. With high IPTG, this host produces enough Trp repressor to allow efficient lysogenic development of the challenge phage.

Under this first set of conditions, two mutant repressors, Cys$^{77}$ and Ser$^{77}$, are more active than wild-type repressor, and four mutant repressors, Val$^{77}$, Leu$^{77}$, Thr$^{77}$, and Ile$^{77}$, have activities close to that of the wild type (Fig. 3a). The mutant Met$^{77}$, Lys$^{77}$, Asn$^{77}$, and Gly$^{77}$ repressors have lower activities; however, when they are made in sufficient amounts, they permit efficient lysogenization (Fig. 3b). The mutant His$^{77}$ repressor has barely detectable activity, and all other mutant repressors are inactive in this assay. These results rank active mutant holorepressors in the order: Cys > Ser > Val, Leu, Ala > Ile, Thr > Lys, Met > Asn > Gly > His.

**Seven Mutant Repressors Are Super-holorepressors**—Under a second set of conditions, two mutant repressors, Cys$^{77}$ and Ser$^{77}$, are more active than wild-type repressor, and four mutant repressors, Val$^{77}$, Leu$^{77}$, Thr$^{77}$, and Ile$^{77}$, have activities close to that of the wild type (Fig. 3a). The mutant Met$^{77}$, Lys$^{77}$, Asn$^{77}$, and Gly$^{77}$ repressors have lower activities; however, when they are made in sufficient amounts, they permit efficient lysogenization (Fig. 3b). The mutant His$^{77}$ repressor has barely detectable activity, and all other mutant repressors are inactive in this assay. These results rank active mutant holorepressors in the order: Cys > Ser > Val, Leu, Ala > Ile, Thr > Lys, Met > Asn > Gly > His.

**Fig. 3.** Two mutant repressors with Ala$^{77}$ changes are super-holorepressors. The efficiency of survival of cells infected with a high multiplicity of tryptophan is a measure of how the phage-borne reference trp operator is occupied by plasmid-encoded Trp repressor. In this experiment, repressor binding in vivo is measured as a function of the increasing concentration of intracellular trp repressor protein in the presence of excess tryptophan (100 µg/ml). Because corepressor is present in excess under these conditions, these dose-response curves allow us to rank the relative values of $K_{on}$ for each mutant holorepressor. (Identical results were obtained with higher concentrations of exogenous tryptophan.) Mutant proteins with higher activities than wild type (Cys$^{77}$, Ser$^{77}$) are super-holorepressors.

of tryptophan in the presence of a moderate concentration of IPTG (10 µM).

Challenge phage assays done with an excess of exogenous tryptophan and varying amounts of Trp repressor allow us to measure the relative DNA-binding activities of mutant holorepressors to an idealized, minimal trp operator in vivo (Bass et al., 1988). This is the case for mutant TrpR proteins with increased activities arising from each of three different Glu → Lys changes. Fig. 3 shows that the efficiency of lysogeny of the host MS1868/F' lac$^{b}$ (pPY2000) with a trp challenge

$^{2}$ D. N. Arvidson, J. Pfau, M. Shapiro, and P. Youderian, unpublished results.
FIG. 4. Seven mutant Trp repressors with Ala\textsuperscript{77} changes are super-aporepressors. The DNA-binding activities \textit{in vivo} for mutant repressors depleted for the corepressor, tryptophan, are shown (see legend to Fig. 3). In this experiment, repressor binding \textit{in vivo} is measured as a function of the increasing concentration of intracellular Trp repressor protein in the absence of exogenous tryptophan. Because the defined (TDO) medium used for the growth of survivors of challenge phage infection has an excess of both phenylalanine and tyrosine, but no tryptophan, the concentration of free intracellular tryptophan is further limited by the slower synthesis of 3-deoxy-D-arabino-heptulosonate 7-phosphate (a common precursor of the aromatic amino acids) under these conditions (Brown and Somerville, 1971). TrpR protein is expected to exist predominantly as aporepressor, not holorepressor, in this assay; consequently, the 7 mutants with activities higher than the wild type under these conditions are called "super-aporepressors." However, we do not wish to imply that mutant aporepressor species can effect repression under these conditions (see text).

fuson operon carried by an integrated \(\lambda\) prophage in \(E.\ coli\). Unlike the challenge phage assays, which measure repression of a minimal symmetric TrpR binding site, this assay measures repression of the wild-type \(E.\ coli\) trp operon, a more complex binding site composed of multiple, tandem repressor dimer binding sites (Kumamoto et al., 1987). To maximize the sensitivity of this assay, each of the plasmids with codon 77 changes was transformed into \(E.\ coli\) host CG103 carrying a second plasmid, pMS421, which produces high levels of Lac repressor to turn down transcription of \(trpR\) (Gardella et al., 1989). In the absence of pMS421, constitutive expression of wild-type and many mutant Trp repressors in this host results in the near complete repression of the \(trp/lac\) fusion operon (see Arvidson et al., 1991a, for example) and makes it even more difficult to observe phenotypic differences between mutant and wild-type Trp repressors.

Table 1 shows that the 19 mutant proteins with Ala\textsuperscript{77} changes are roughly grouped into three different classes on the basis of their ability to repress the \(E.\ coli\ trp/lacZ\) fusion operon in CG103(pMS421) (Table I). Six of the mutant repressors (Cys > Val > Ala, Thr, Ser > Ile, Leu) turn down expression of the fusion operon at least as efficiently as the wild type. Five mutant repressors (Met, Gly, Lys, Asn, His) have intermediate activities, and eight mutant repressors with changes of Ala77 to Gln, Pro, Arg, Asp, Glu, Phe, Tyr, and Trp, show little or no activity. The results of this assay also
show that all of the active, mutant repressors have increased activities in the presence of added corepressor; none is tryptophan-independent.

The relative activities of mutant repressors determined by this β-galactosidase assay agree with those determined by the challenge phage assay, although the relatively insensitive β-galactosidase assay reveals the super-repressor phenotype of only two of the mutants, Cys and Val. The six mutant repressors with activities better than, or comparable to, wild type in the challenge phage assay (Cys, Ser, Val, Leu, Thr, Ile) are the most active in this β-galactosidase assay. Five mutant repressors (Lys, Met, Asn, Gly, His) show intermediate levels of activity, and the remaining mutants show little or no detectable activity in both assays.

**DISCUSSION**

Residue alanine 77 is located in the center of the turn of the “helix-turn-helix” DNA-binding motif of E. coli tryptophan repressor and is critical for TrpR function. The majority (12/19) of Ala77 changes result in either inactive (Arg, Asp, Gln, Glu, Phe, Pro, Trp, and Tyr) or only partially active (Asn, Gly, His, and Met) proteins. Neither amino acids with acidic side chains (Asp, Glu) nor amino acids with the four largest side chains (Arg, Trp, Tyr, Phe) are acceptable at this position. Because the Pro77 protein has almost undetectable activity, the conformation of the turn between α-helices D and E also must be critical for function.

Changes of Ala77 that impair TrpR activity do not alter its steady-state level of production. All of the 19 different mutant proteins with Ala77 changes are made at similar steady-state levels as the wild-type protein. Presumably, all single amino acid changes affecting residue Ala77 permit folding to a protease-resistant state. In contrast, all of the changes of residue Val77, which frames the tryptophan binding pocket, result in repressors that are made at lower steady-state levels in vivo (Arvidson et al., 1991a).

When tryptophan is limiting, 7 mutant aporepressors with Ala77 changes are more active than wild-type aporepressor (Figs. 4 and 5). At high tryptophan concentrations, the 7 super-aporepressors fall into three groups on the basis of their relative activities in the challenge phage assay. Under these conditions, Cys and Ser holorepressors are more active than wild type; Val, Leu, Ile, and Thr are about as active as wild type (Fig. 3a); and Lys holoholorepressor is less active than wild-type (Fig. 3b). In contrast, none of the single amino acid changes of residue Val77 improves TrpR function (Arvidson et al., 1991a).

How do these changes affect TrpR function? As modeled thermodynamically in Fig. 6a, TrpR protein is distributed among six different states in vivo, related by a series of linked equilibria involving aporepressor (A), tryptophan (T), nonspecific DNA (D), and operator DNA (O) (Arvidson 1988; adapted from von Hippel, 1979). When tryptophan is saturating, TrpR protein populates only 3 of the 6 states, free holorepressor (AT), holorepressor bound to nonspecific DNA (ATD), and holorepressor bound to operator DNA (ATO) (Fig. 6b). The activity of a mutant holorepressor in the presence of excess tryptophan reflects the competition between its ability to bind nonspecific and operator DNAs and involves only two equilibrium dissociation constants, KATD and KATO. The ability of holorepressor to bind operator DNA measured in vivo is related to these constants by the equation

\[
K_{ap} = K_{ATO} + \frac{K_{ATD}}{D} \quad \text{(Eq. 2)}
\]

Because nonspecific DNA competes with operator for repressor binding, the apparent equilibrium dissociation constant (Kap) is equal to the actual equilibrium dissociation constant (KATO) plus a dominant term that depends on the ratio of specific and nonspecific binding constants and increases with nonspecific DNA concentration [D] (Segel, 1976). For experiments in vivo in which [D] is held constant, differences in apparent binding reflect differences in this ratio.

The results of challenge phage infections with excess tryptophan rank the activities of four mutant holorepressors, Glu13 → Lys, Glu18 → Lys, Glu4′ → Lys, and Ala77 → Val in vivo, in the same order as the magnitudes of Kapo determined for these holorepressors in vitro (Hurlburt and Yanofsky, 1990); these assay conditions provide us with a relative measure of Kap in vivo. Therefore, Cys77 and Ser77 holorepressors must have higher Kap values than wild type; Ile77, Leu77, Thr77, and Val77 holorepressors have Kap values similar to wild type; and Lys77 and Met77 holorepressors must have higher Kap values.

One of these mutant super-repressors, Val77, has been purified and examined in vitro. Using a nitrocellulose filter-binding assay, Hurlburt and Yanofsky (1990) have found that KATD, KATO, and KATD + KATO for Val77 protein are indistinguishable from those for wild-type TrpR. These results agree well with our physiological data; we see no effect of the Ala77 → Val change on Kap in vivo (Fig. 3a). Because the pathways A → AT → ATO and A → AO → AOT involve the same net change in free energy, if KATD and KATO are the same for Ala77 and Val77, KATD must be the same. Therefore, the super-aporepressor phenotype of Val77 must be due to depletion of the AD state. If KATD and KATO are the same for Val77 and Val77, KATD must be the same. Therefore, the super-aporepressor phenotype of Val77 must be due to depletion of the AD state. If KATD and KATO are the same for Val77 and Val77, only the equilibrium that could be affected by the Ala77 → Val change are described by KAD and KADT. The Val77 change must result in an increase in KAD, and, because the pathways A → AT → ATO and A → AO → AOT involve the same free energy change, a commensurate decrease in KADT. Together, these effects will depopulate [AD], which, in turn, will result in an increase in [ATO]. A corollary of this deduction is that a significant fraction of wild-type aporepressor.

![Fig. 6. Macroscopic description of the linked equilibria involved in the assembly of trp holorepressor/operator complexes. Symbols: A, aporepressor; D, nonspecific DNA; O, operator DNA; T, tryptophan. The last letter in subscripts of each of the equilibrium dissociation constants (K values) indicates the species being bound; for example, KAD refers to the binding of operator DNA (O) by aporepressor (A), and KATD refers to the binding of nonspecific DNA (D) by holorepressor (AT). When the concentration of intracellular tryptophan is saturating, the cycle of linked equilibria diagrammed in a collapses to the “bottom line” description shown in b. Unfortunately, because tryptophan is an essential amino acid, conditions described by the corresponding “top line” may be approximated in vitro, but never realized in vivo.](image-url)
must be associated with nonspecific DNA in vivo.

Mutations that increase the activity of a repressor by affecting the interaction of a repressor–ligand complex with nonspecific DNA are not without precedent. Super-repressor mutants with Ala\textsuperscript{77} changes, which show tighter apparent binding to the trp operator with decreased corepressor concentrations, are analogous to a subset of lac\textsuperscript{r} mutants, which show tighter apparent binding to the lac operator with increased inducer concentrations (Jobe et al., 1974; von Hippel, 1979).

If we assume that other Ala\textsuperscript{77} changes, like the Ala\textsuperscript{77} → Val change, do not have pronounced affects on \(K_{AT}\) (Marmorstein and Sigler, 1989), then the effects of each change can be dissected into effects on \(K_{app}\), the population of the AD state, or both. This simplifying assumption is reasonable because residue 77 plays no direct role in tryptophan binding in the structures of repressor. According to this model, the change from Ala\textsuperscript{77} to Cys decreases both \(K_{app}\) and [AD]; the change to Ser decreases \(K_{app}\) and [AD] less dramatically; changes to Val, Leu, Ile, and Thr decrease [AD] without affecting \(K_{app}\) significantly; and the change to Lys must decrease [AD] (Fig. 4), yet also increase \(K_{app}\) (Fig. 3). Consistent with this model, we can define conditions for Lys\textsuperscript{77} under which these opposing effects on aporepressor and repressor activities balance each other, and Lys\textsuperscript{77} behaves much like the wild type (Fig. 5b).

This interpretation is supported directly by a comparison of the orthorhombic crystal structures of aporepressor and holorepressor (Fig. 1). In both forms, the side chain of Ala\textsuperscript{77} points toward the interior of the protein and away from the DNA-binding surface. In the aporepressor structure, the helix-turn-helix motif is collapsed against the hydrophobic core; in the repressor structure, the motif is rotated away from the core. In aporepressor, the side chain of Ala\textsuperscript{77} is almost completely buried; in repressor, it is more solvent-exposed. Thus, the Ala\textsuperscript{77} → Val change should cause a steric clash in the aporepressor crystal structure, but not in the holorepressor crystal structure (Fig. 1; Marmorstein and Sigler, 1989; Luisi and Sigler, 1990). Lengthening the Ala\textsuperscript{77} side chain by mutation should result in proteins that cannot assume this aporepressor conformation.

Consistent with this picture, there is a correlation between the specific volumes of mutant side chains at position 77 and phenotype. Changes to Ser and Cys involve the smallest increases in side chain volume and result in proteins that are super-holorepressors. The side chains of Ile, Leu, Thr, and Val are somewhat larger; mutant proteins with these changes are super-aporepressors, but have holorepressor activities similar to Ala\textsuperscript{77}. These intermediate-size residues are predicted to disrupt the aporepressor tertiary structure, yet are tolerated by the holorepressor structure. The Lys\textsuperscript{77} protein, with the largest functional side chain, is a better aporepressor, but a worse holorepressor, than wild type. This large side chain should interfere with folding of both aporepressor and holorepressor (Fig. 1). The Met\textsuperscript{77} protein, with a side chain almost as long as lysine, also has decreased holorepressor activity (Fig. 3). Mutant repressors with larger side chains (Arg, Phe, Tyr, Trp) have little detectable holorepressor activity.

An alternative explanation of the Val\textsuperscript{77} phenotype has been suggested by Marmorstein et al. (1991). Using an assay that measures the ability of TrpR protein to protect an idealized trp operator from dephosphorylation by alkaline phosphatase, they find different results: for Val\textsuperscript{77} repressor, \(K_{AT}\) is 2.5-fold higher and \(K_{app}\) is 8.5-fold lower than wild type. However, their data cannot explain why the Val\textsuperscript{77} protein requires less tryptophan for activation in vivo, because they find that \(K_{AT}\) is the same for Val\textsuperscript{77} as for wild type (Marmorstein 1989), and the pathways A → AO → AOT and A → AT → ATO must involve the same free energy change. Their data argue that assembly of the repressor/operator complex should be impaired 2.5-fold by either route; i.e., an 8-fold increase in \(K_{app}\) must be accompanied by an 18-fold decrease in \(K_{AT}\), because \((K_{AT})(K_{app}) = (K_{AO})(K_{AOT})\). A mutation that increases \(K_{AO}\) 8-fold should not have a significant effect on repression by increasing the relatively small AO population, unless this complex prevents transcription initiation. More important, these experiments were done under conditions in vitro that do not reflect the physiological state in vivo. At higher salt concentrations, the super-repressor phenotype of the mutant Glu\textsuperscript{40} → Lys repressor observed both in vivo (Kelley and Yanofsky, 1983) and in vitro (Klig and Yanofsky, 1988; Hurlburt and Yanofsky, 1990) is also revealed by the phosphatase inhibition assay (Marmorstein et al., 1991).

In addition to the 7 super-repressors we have described in this paper, only 4 other single amino acid changes are known to increase TrpR activity. All 4 of these changes arise by single base pair transitions and are predicted to make the DNA-binding surface of the repressor dimer more basic (Kelley and Yanofsky, 1983; Klig et al., 1988). In contrast, genes encoding 4 of the 7 mutant super-aporepressors with Ala\textsuperscript{77} changes (including Cys\textsuperscript{77}, the most active super-repressor) can arise from the wild-type trpR gene by mutation of a single nucleotide base pair. Because classical methods of chemical mutagenesis give rise to mutant codons with tandem double or triple base pair changes only very rarely, such methods would preclude the isolation of the majority of mutant repressors with Ala\textsuperscript{77} changes that increase activity. Thus, our results suggest both that a significant fraction of possible single amino acid changes throughout the protein will increase repressor activity and that our more biochemically thorough approach of isolating mutants with each of the possible single changes at each residue position of Trp repressor will yield dividends that classical genetic approaches cannot.

We have used an assay in vivo based on a genetic selection to deduce that mutant Trp aporepressors have decreased affinities for nonspecific DNA. Such affinities are likely to be in the millimolar range, a range that precludes direct and accurate measurement in vitro by many standard biochemical methods. It is clear that novel combinations of biochemical and genetic methods will be necessary to probe the relationship between Trp repressor structure and function.

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