A Site-Specific Endonuclease Derived from a Mutant Trp Repressor with Altered DNA-Binding Specificity†

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ABSTRACT: Site-directed mutagenesis was used to construct mutant Trp repressors with each of the 38 possible single amino acid changes of the first 2 amino acid residues (Ile79 and Ala80) in the second "recognition" α-helix of the helix–turn–helix DNA-binding motif. Eight of these mutant repressors with Ile79 and Ala80 changes are more active than the wild-type protein when tryptophan is limiting, and are super-*apo*repressors. Eleven mutant repressors have extended DNA-binding specificies *in vivo*, and bind operators which the wild-type repressor cannot. One mutant repressor, Lys79, has a classical altered specificity phenotype *in vivo*, and binds the wild-type *trp* operator less well than wild-type repressor, yet binds a mutant operator better than wild-type repressor. A site-specific nuclease was derived from Lys79 repressor by constructing a double-mutant protein with Lys79 and a sole cysteine residue, Cys49, and alkylating this cysteine with a 1,10-phenanthroline–copper adduct. This nuclease has an altered specificity of DNA binding *in vitro*. When activated by the addition of thiol and hydrogen peroxide, the Lys79 nuclease cleaves operator DNA within its new recognition sequence with high efficiency.

Specific DNA-binding proteins comprise one of the largest families of proteins with related function; more than 10% of all sequenced genes encode DNA-binding proteins. Like the family of proteases, DNA-binding proteins may be grouped into classes on the basis of mechanism. One large class of prokaryotic and eukaryotic regulatory proteins shares the helix–turn–helix (HTH) secondary structural motif (a pair of α-helices separated by a short, surface turn) which interfaces with DNA target sites. Co-crystals of seven HTH proteins complexed with their double-stranded DNA recognition sites have been refined, revealing the atomic nature of the interactions at the protein–DNA interface (Anderson et al., 1987; Aggarwal et al., 1988; Jordan & Pabo, 1988; Beamer & Pabo, 1992; Wolberger et al., 1988, 1991; Schultz et al., 1991; Kissinger et al., 1990). In four of the co-crystal structures of prokaryotic proteins, the second α-helix in the HTH motif, or "recognition" helix, penetrates the major groove of the DNA target, and direct bonds are formed between side chains of the protein and nucleotide functional groups. Phages 434 and λ repressors, phage 434 Cro protein, and *Escherichia coli* GAP protein use the first two residues in this recognition helix to recognize critical bases in their target (operator) sites (Anderson et al., 1987; Aggarwal et al., 1988; Jordan & Pabo, 1988; Beamer & Pabo, 1992; Wolberger et al., 1988; Schultz et al., 1991).

In contrast, the two co-crystal structures of Trp holorepressor bound to its operator suggest that TrpR protein recognizes its target site primarily by a pattern of water-mediated contacts (Otwinowski et al., 1988; Lawson & Carey, 1993). These models are controversial, because they do not agree with the results of biochemical and genetic studies of the complex in solution. For example, the majority of contacts observed in the co-crystals involve the unesterified oxygen atoms of six phosphates in each operator half-site; multiple contacts are made with four of these six oxygens. Ethylnitrosourea alkylates these oxygens, and should prevent the formation of critical H-bonds with these atoms. However, ethylation of three of the six phosphates in a half-site contacted in the Trp co-crystals does not interfere with repressor binding in solution (Kumamoto, 1989). In contrast, ethylation interference experiments with five other specific protein–DNA complexes identify sets of critical operator phosphates which are proper subsets of the phosphates contacted directly by amino acids in co-crystals of these complexes (Bushman et al., 1985; Anderson et al., 1987; Jordan & Pabo, 1988; Beamer & Pabo, 1992; Wolberger et al., 1988; Schultz et al., 1991; Ellenberger et al., 1992). In only one case, the co-crystal structure of the GAL4 complex with DNA, is an imperfect correspondence observed: this involves only one phosphate of five, which occurs near the end of the co-crystallized oligonucleotide and is proximal to a lysine side chain (Marmorstein et al., 1992). In this case, however, lattice interactions which force oligonucleotides into a linear array in the co-crystal may perturb a direct interaction between this lysine and phosphate which occurs *in vivo*.

Replacement of operator base pairs with natural and synthetic bases shows that particular nucleotide functional groups are critical for repressor binding (Bass et al., 1987; Mazzerelli et al., 1992). Only a subset of these critical functional groups participates in (water-mediated) contacts in the co-crystal complexes. For these and other reasons, the co-crystal structures may represent nonspecific complexes (Brennan & Matthews, 1989). In contrast with the crystal structures, the simplest interpretation of the NMR solution structures of the holorepressor–operator complex is that recognition involves direct hydrogen bonds between amino

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acid side chains and base pair functional groups (Zhang et al., 1994).

In the crystal structures, the first two residues of the recognition helix play a limited role. The side chains of Ile79 and Ala80 do not participate in direct contacts with the chemical groups found on the edges of critical bases. Instead, the main chain amide nitrogens of residues Ile79 and Ala80 appear to make two water-mediated contacts with operator bases (Otwinowski et al., 1988; Lawson & Carey, 1993). In the solution structures, the side chains of Ile79 and Ala80 play more active roles. Protons of the Ile79 and Ala80 side chains participate in nuclear Overhauser effects (NOE's) with protons of base pairs 8 and 4 of the operator DNA, as well as with protons of other amino acid side chains involved in intermolecular (protein–DNA) NOE's (Zhang et al., 1994).

To explore the contribution of individual amino acid residues to the DNA-binding specificity of Trp repressor, we have combined genetic and biochemical approaches. Mutations that change amino acids involved directly or indirectly in operator recognition can result in mutant proteins with changes in DNA-binding specificity. Such changes identify amino acid–base pair interactions critical for the formation of these novel mutant–mutant protein–DNA complexes (Youderian et al., 1983; Vershon et al., 1985; Wharton & Ptashne, 1987; Anderson et al., 1987; Aggarwal et al., 1988; Ellenberger et al., 1992; Kim et al., 1993), and may provide clues for understanding the wild-type interaction. Independent measurements of the affinities of wild-type and mutant proteins for wild-type and mutant operators *in vitro* can confirm the presence or absence of new protein–DNA interactions predicted by mutation (Youderian et al., 1983).

This report describes the properties of mutant Trp repressors with each of the single amino acid changes of residues Ile79 and Ala80, the first two residues of the recognition helix in the HTH motif. Many members of the family of HTH proteins (including those related to *E. coli* Gal and Lac repressors; Weickert & Adhya, 1992; Dodd & Egan, 1990) have hydrophobic residues at these two positions, followed by a conserved threonine residue (Thr81 in TrpR). For Gal and Lac repressors, these two residues are critical determinants of DNA-binding specificity. The specificity of the Gal repressor can be commuted to that of the Lac repressor by interchanging this pair of residues between the two proteins (Lehming et al., 1990).

Residues Ile79 and Ala80 play important roles in determining the DNA-binding specificity of Trp repressor (Figure 1). Among 38 mutant Trp repressors with single amino acid changes of these residues, 11 have extended recognition specificities, and bind a subset of mutant operators better than wild-type repressor *in vivo*. One of the 38 mutant proteins, Lys79 repressor, has a "new", or "altered", DNA-binding specificity. Lys79 repressor shows reduced affinities for the natural and consensus *trp* operators, and increased affinity for a mutant operator *in vivo*. Repressors with chemical adducts that can catalyze the site-specific cleavage of DNA were constructed from the wild-type Ile79 and mutant Lys79 repressors. The altered specificity of a nuclease derived from the mutant Lys79 repressor is revealed by its preference for binding and cleaving a mutant operator *in vitro*.

EXPERIMENTAL PROCEDURES

Bacteria, Phage, and Plasmids. Bacterial strains are derivatives of *Escherichia coli* K12 and *Salmonella typhimurium* LT2. *E. coli* strain X90/F′*lacI*^{Q} [Δ(*proAB-lac*) *recA argE-am thi*] carries an F′ episome that produces high levels

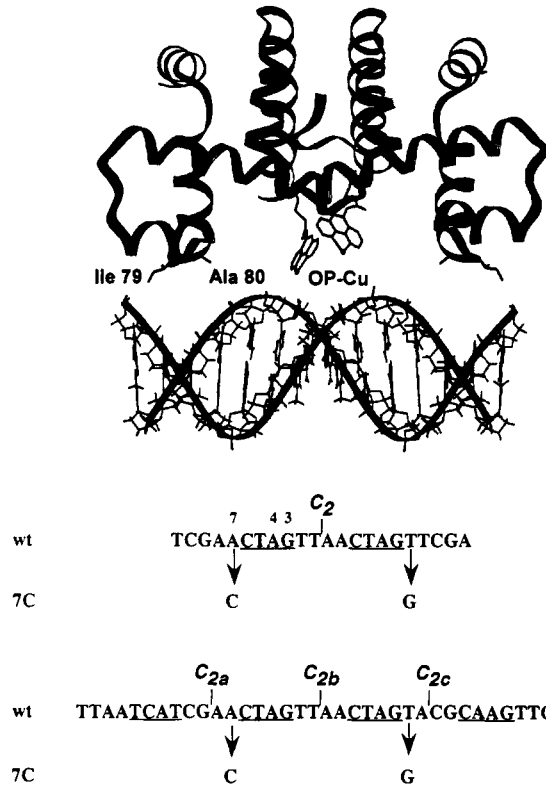

FIGURE 1: (Top) Ribbon diagram of the TrpR dimer structure, showing the Ile79 and Ala80 side chains and the 1,10-phenanthroline–copper adduct with Cys49 (OP-Cu) aligned with the consensus operator, courtesy of Ralf Landgraf. The consensus, minimal *trp* operator sequence carried by a challenge phage (center) corresponds to the central of three tandem binding sites for Trp holorepressor in the natural *trp* operator with highest affinity (bottom). The symmetric "7C" change inactivates the minimal, consensus *trp* operator, as well as the central of three sites in the natural *trp* operator.

of Lac repressor (Amann et al., 1983), and was used as the transformation recipient for mutant plasmids. CG103/RSF2001 [Δ(*lac-pro*) *ΔtrpR-505 thi-1 recA-56 srl*::*Tn10* λCLG145] carries an integrated, specialized transducing λ prophage with a fusion of the *trp* promoter/operator region and start of *trpE* to *lacZ*, and was used as the host for β-galactosidase assays (Arvidson et al., 1991). *S. typhimurium* strain MS1868/F′*lacI*^{Q}, the host for challenge phage infections, and challenge phage derivatives of P22 *Kn9 O-ref2 arc-H1605*(*am*) with the reference-type and symmetric, mutant *trp* operators have been described (Benson et al., 1986; Bass et al., 1987). Plasmid pPY2000, an ampicillin-resistant derivative of pBR322, has a phage M13 origin of replication, and expresses wild-type Trp aporepressor from the *lacUV5* promoter. Plasmid pPY1999 is an otherwise isogenic *trpR*$^{-}$ derivative of pPY2000 missing the 404 bp *Bam*HI (*trpR*) fragment (Bass et al., 1987).

Additional plasmids with codon 79 and codon 80 changes were made by cassette mutagenesis, using single-stranded oligonucleotides to repair the *trpR* gene on a double-stranded plasmid template (Wells et al., 1985), or mismatched-primer mutagenesis (Kunkel, 1985) as described (Pfau et al., 1994). Single-stranded oligonucleotides used for cassette mutagenesis were the following: CGCGTAATCGTCGCCABGCCTGCGCCAAGCT (Leu79, Met79, Val79), CGCGTAATCGTCGCSYGGCCTGCGCCAAGCT (Thr79, Ser79), CGCGTAATCGTCGCWTKGCCTGCGCCAAGCT (His79, Gln79, Asn79, Lys79), CGCGTAATCGTSAWGATGCCTGCGCCAAGCT (Phe80, Leu80, Met80), CGCGTAATCGTACYGATGCCTGCGCCAAGCT (Ser80,

Gly80), and CGCGTAATCGTWTSGATGCCTGCGCCAAGCT His80, Gln80, Glu80, Asp80). Several changes were not obtained with the cassettes, and were made by mutagenesis using mismatch primers with single-stranded plasmid pPY2000 DNA as template (Arvidson et al., 1991); mismatch primers were GGCGCAGGCCAGGCGACGATT (Gln79), GGCGCAGGCTGCGCGACGATT (Cys79), GCAGGCATCGRGACGATTACG (Glu79, Gly79), GCAGGCATCATCACGATTACG (Ile79), and GCAGGCATCTACACGATTACG (Tyr79). The *trpR* gene on each plasmid was sequenced by the method of Sanger et al. (1973). Templates for sequencing were single-stranded plasmid DNAs prepared after infection of an *E. coli* host carrying these plasmids with wild-type phage M13 (Zagursky & Berman, 1984). Plasmids were transferred between bacterial hosts by electroporation (Pfau & Youderian, 1990). Plasmids introduced into the *S. typhimurium* host used for challenge phage assays were transferred into *E. coli* strain X90/F′*lacI*^{Q} and resequenced, to confirm their genotypes.

Plasmids used as sources of purified TrpR protein for binding experiments *in vitro* are derivatives of pBR327 (Soberon et al., 1980) carrying the 404 bp *Bam*HI *trpR* fragment from plasmid pRPG47 (Arvidson et al., 1986) cloned into the unique *Bam*HI site. A derivative of pBR327 encoding Cys49 repressor was constructed by subcloning the *Bam*HI *trpR* fragment from pRPG47 Cys49 (Sutton et al., 1993) into pBR327, and a derivative of this plasmid with the additional Lys79 change was constructed by site-directed mutagenesis using the polymerase chain reaction (PCR). The internal primer, GCAGGCAAGGCGACGATTACGCGT, was used in combination with primers flanking the cloned *trpR* gene on template plasmid pRPG47 Cys49 (GGCCCTTTCGTCTTCAAGAATTC and AGGAATGGTGCATGCAAGGAGATG), to amplify a DNA fragment with the desired mutation (Landt et al., 1990). This fragment was cleaved with *Eco*RI and *Sph*I, and the mutant *trpR* gene was subcloned into the parental plasmid.

To construct a derivative of pBR327 with the natural *trp* operator sequence, the oligonucleotides AATTCGGGATTAATCATCGAACTAGTTAACTAGTACGCAAGTTCACGGGG and GATCCCCCGTGAACTTGCGTACTAGTTAACTAGTTCGATGATTAATCCCG corresponding to the wild-type *trpEDCBA* operator were annealed and subcloned into vector cleaved with *Eco*RI and *Bam*HI. To construct an otherwise isogenic plasmid with the mutant 7C operator sequence, the wild-type *trp* operator was excised from plasmid pBR327 with *Eco*RI and *Bam*HI, and subcloned into pUC19 (Yanisch-Perron et al., 1985). Two-step PCR mutagenesis (Landt et al., 1990) using the internal primer, GCGTCCTAGTTAACTAGGTCGA, external primers derived from the pUC sequences (CCCAGTCACGACGTTGTAAAACG and CCAAGCTTGCATGCCTGCAGGTC), and pUC19 template DNA was used to generate a product, which was cleaved with *Eco*RI and *Bam*HI, and recloned into pBR327. Enzymes used in plasmid constructions were from Bethesda Research Laboratories, New England Biolabs, or Stratagene.

Measurement of Steady-State Levels of Mutant Aporepressors in Vivo. Antibody ("Western") assays of the steady-state levels of Trp repressor production were done as described by Arvidson et al. (1993). *E. coli* CG103/RSF2001 with each mutant derivative of pPY2000 was grown in typrophan "drop-out" medium (Bass et al., 1987) with 50 μg/mL proline, 100 μg/mL thiamine, and 400 μg/mL carbenicillin to a density of 2 × 10^{8}/mL. Proteins in cell lysates were separated by SDS (sodium dodecyl sulfate)–polyacrylamide gel electrophoresis, using a gel system designed for the resolution of low molecular weight proteins (Schagger & von Jagow, 1987), and were transferred to nitrocellulose electrophoretically (Tsang et al., 1983). Nitrocellulose-bound aporepressor was detected by sesquential incubation with a primary rabbit anti-aporepressor antibody (Gunsalus et al., 1986), and a secondary goat anti-rabbit antibody conjugated with alkaline phosphatase, using the Picoblue immunoscreening kit (Stratagene). Steady-state levels of mutant proteins were quantitated by densitometric analysis of photographs of stained gels; band intensities were normalized to the intensity of the higher molecular weight bands which cross-react with the polyclonal antiserum. Control experiments show that the relative intensities of these higher molecular weight bands do not change in response to the addition of tryptophan to the growth medium.

Assays of β-Galactosidase Activity. Kinetic assays of β-galactosidase activity were conducted as described by Arvidson et al. (1991b). Cultures of each CG103/RSF2001 strain with a pPY2000 derivative were grown in tryptophan drop-out medium (Bass et al., 1987) with or without added tryptophan in the wells of microtiter plates at 37 °C, and lysed from without with a high multiplicity of infection of phage T4 in β-galactosidase assay buffer. Liberation of *o*-nitrophenol from the chromogenic substrate *o*-nitrophenyl β-D-galactoside was monitored as A_{414} over time by a computer up-linked microtiter plate spectrophotometer. Activities were calculated using the equation:

$$\text{activity} = 1000bA_{595}^{-1}f^{-1}$$

where b is the slope of the linear least-squares fit to the plot of A_{414} *vs* time (min) and f (0.2 in our assays) is the fraction of cells added to the total volume of lysate. Averages of values for four to six independent determinations varied less than 25%; each determination was from measurements made at 10 or more times after the start of each reaction over a time course of 2 h, using cells sampled at an initial exponential density of $A_{595} = 0.10 \pm 0.02$.

Challenge Phage Assays. Challenge phages are derivatives of *S. typhimurium* phage P22 that place the lysogenic development of P22 under the control of a specific DNA-binding protein. When the reporter gene in the challenge phage assay (*ant*, encoding P22 antirepressor) is expressed, P22 infection is channeled into the lytic developmental pathway. If a host-encoded, specific DNA-binding protein can occupy the *trp* operator on a challenge phage, the phage will lysogenize its infected host. Challenge phages carry a gene encoding neomycin-phosphoribosyl transferase; the frequency of lysogeny of a challenge phage is scored as the frequency of kanamycin-resistant survivors of a *Salmonella* host infected with a high multiplicity of phage, and is a measure of the ability of a host-encoded repressor to bind the phage-borne operator (Benson et al., 1986).

The *Salmonella* host we use for challenge phage assays carries two plasmids: pPY2000, which produces TrpR protein from the *lacUV5* promoter, and the F′*lacI*^{Q} episome. To measure repression as a function of the amount of plasmid-encoded TrpR protein required for the efficient lysogenization of the challenge phage, overnight cultures of MS1868/F′*lacI*^{Q} carrying plasmid pPY2000 (Bass et al., 1987) or each mutant plasmid were diluted 100-fold into LB medium with 50 μg/mL ampicillin and 10 μM IPTG, and grown to a density of 4 × 10^{8}/mL at 37 °C. Each culture was infected with an equal volume of challenge phage P22 *Kn9 O-ref2 arc-*

H1605(am), to give a multiplicity of 25 phage/cell. After adsorption of the phage for 15 min at 25 °C, serial 5-fold dilutions of infected cells were made in the wells of microtiter plates, and 4 μL of each serial dilution was spotted on green tryptophan drop-out plates with the same concentrations of IPTG, ampicillin (100 μg/mL), kanamycin (25 μg/mL), and tryptophan (100 μg/mL). To measure repression as a function of the amount of tryptophan (corepressor) required for survival after infection with challenge phage, overnight cultures of MS1868/F′*lacI*^{Q} carrying plasmid pPY2000 derivatives were diluted 100-fold into LB medium with 50 μg/mL ampicillin and 10 μM IPTG, and grown to a density of 4×10^8/mL at 37 °C. Each culture was infected with P22 *Kn9 O-ref2 arc-H1605(am)* at a multiplicity of 25 phage/cell. After adsorption of the phage for 15 min at 25 °C, infected cells were diluted, and 4 μL of each serial dilution was spotted on green tryptophan drop-out plates with 10^{-5} M IPTG, ampicillin (100 μg/mL), kanamycin (25 μg/mL), and tryptophan at various concentrations.

Numbers of kanamycin-resistant lysogens were scored after incubation at 37 °C for 48 h. Frequencies of lysogeny were calculated as the titers of kanamycin-resistant survivors divided by the titers of infected 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.

Tests for Altered DNA-Binding Specificities in Vivo. Cultures of MS1868/F′*lacI*^{Q} carrying each mutant plasmid were grown to a density of 2.5×10^8/mL at 37 °C IPTG was added to 10^{-5} M, and cells were grown an additional 30 min at 37 °C. Cultures were infected with each of 28 different challenge phages derived from P22 *Kn9 O-ref2 arc-H1605(am)* that carry symmetric changes in the consensus *trp* operator (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 IPTG (10 μM), ampicillin (100 μg/mL), kanamycin (25 μg/mL), and tryptophan (40 μg/mL).

Electrophoretic Assay of Repressor Binding in Vitro. E. coli LE392 [F$^-$ *hsdR514* (R$^-$M$^-$) *supE44 supE58 lacY1 galK2 galT22 metB1 trpR55*; Sambrook et al., 1989] host cells with plasmids pBR327 *trpR*-Cys49 and pBR327 *trpR*-Cys49 Lys79 were diluted 1:100 from overnight cultures, and grown to a density of about 2×10^8/mL (A_{600} = 0.4) in 200 mL of LB medium at 37 °C. IPTG was added to 1 mM, and cells were incubated at 37 °C for 45 min, concentrated by centrifugation, resuspended in 1 mL of buffer containing 25 mM Tris, pH 7.5, 10 mM EDTA, and 50 mM glucose, and sonicated. Cell debris was removed by centrifugation, and the supernatant was supplemented to 0.5% streptomycin sulfate, incubated at 85 °C for 10 min and then at 4 °C for 10 min, and recentrifuged. The supernatant was supplemented to 5 mM β-mercaptoethanol and then stored at −70 °C. Modification reactions were carried out by combining 100 μL of supernatant, 800 μL of 10 mM potassium phosphate buffer (pH 7.8) with 300 mM KCl, and 100 μL of 40 mM 5-(iodoacetamido)-1,10 phenanthroline, and reactions were incubated at 4 °C for 16–24 h. Aliquots of the derivatized protein were supplemented to 10% glycerol, and stored at −70 °C.

To generate end-labeled operator fragments used in the binding assay, pBR327 carrying the wild-type or 7C *trp* operators was cleaved with *Eco*RI, labeled with polynucleotide kinase and [γ-^{32}P]ATP (3500 Ci mmol^{-1}), cleaved with *Bam*HI, and purified by electrophoresis through and elution from a 6% (nondenaturing) polyacrylamide gel.

Protein–DNA complexes were resolved on 10% polyacrylamide gels [75:1 acrylamide:bis(acrylamide)] with 20 mM sodium phosphate buffer (pH 6.0) containing 0.1 mM L-tryptophan, as described (Carey, 1988). Binding reactions were carried out for 15 min at 37 °C in a total volume of 20 μL, and included 100 mM Tris-HCl (pH 7.5), 200 mM KCl, 6 mM $MgCl_2$, 11 mM L-tryptophan, 0.1 mM poly(dI/dC), and 2×10^5 cpm (ca. 0.2 pmol) of labeled operator fragment.

Copper(I)–Phenanthroline Cleavage of the trp Operator. Acrylamide slices containing protein–DNA complexes were incubated in 20 mM sodium phosphate buffer with 0.1 mM L-tryptophan, 3 mM H_2O_2, 3 mM mercaptopropionic acid, and 50 μM $CuSO_4$ for 15 min. Reactions were quenched by the addition of an equal volume of 2.8 mM 2,9-dimethyl-1,10 phenanthroline, and the products were analyzed on sequencing gels as described (Sutton et al., 1993).

RESULTS

To explore the contribution of residues Ile79 and Ala80 to Trp repressor function, plasmids expressing Trp repressors with each of the mutant, single amino acid changes at residues Ile79 and Ala80 were constructed by site-directed mutagenesis of the *trpR* gene, using a combination of strategies (Experimental Procedures). Because a subset of the characterized single amino acid changes in Trp repressor results in a reduced steady-state level of protein produced *in vivo* (Arvidson et al., 1991a; Chapman et al., 1994), we measured the steady-state levels for each mutant TrpR protein relative to the wild-type level *in vivo*. Each mutant plasmid was introduced into the same *E. coli* host, and the amount of repressor produced from each recombinant strain was measured with rabbit anti-TrpR antiserum. Among the 38 mutant proteins with Ile79 or Ala80 changes, 35 were found to be expressed at steady-state levels within 2-fold of that of the wild-type repressor (Figure 2). Two mutant repressors, Arg79 and Cys80, are made at significantly higher steady-state levels, and only Gly80 repressor is made at a significantly lower level, at about 40% of the wild-type level.

To compare the activities of mutant and wild-type repressors *in vivo*, we assayed the ability of each repressor to turn down the expression of β-galactosidase activity from a single-copy *trpE-lacZ* fusion operon in *E. coli* (Figure 3). As shown in Figure 4, the *trpR*$^-$ *E. coli* host with a plasmid that does not carry *trpR* produces about 1400 units of β-galactosidase activity, with or without tryptophan added to the culture medium. An otherwise isogenic host with a *trpR*$^+$ plasmid produces 270 units of β-galactosidase in media without added tryptophan, and 30 units in media with tryptophan. Repression of the *trpE-lacZ* fusion operon by wild-type TrpR protein is about 50-fold with added tryptophan.

Few single amino acid changes at residues Ile79 and Ala80 abolish repressor function in this assay *in vivo*, and a wider variety of changes is tolerated without loss of function at Ile79 than at Ala80. Changes to Pro or the acidic amino acids Asp (D) and Glu (E) abolish repressor activity at both residue positions; changes of Ala80 to the larger, hydrophobic residues Phe (F) and Trp (W) also abolish activity. The majority of mutant repressors retains at least partial activity. A total of 8 of 19 mutants with Ile79 changes [Ala, Cys, Ser, Met, Val, Gly, Gln (Q), and Leu] and 2 of 19 mutants with Ala80 changes (Leu and Gly) have activities close to that of wild-type repressor.

These β-galactosidase assays yield measurements of activity which reflect the rate of transcription initiation from the

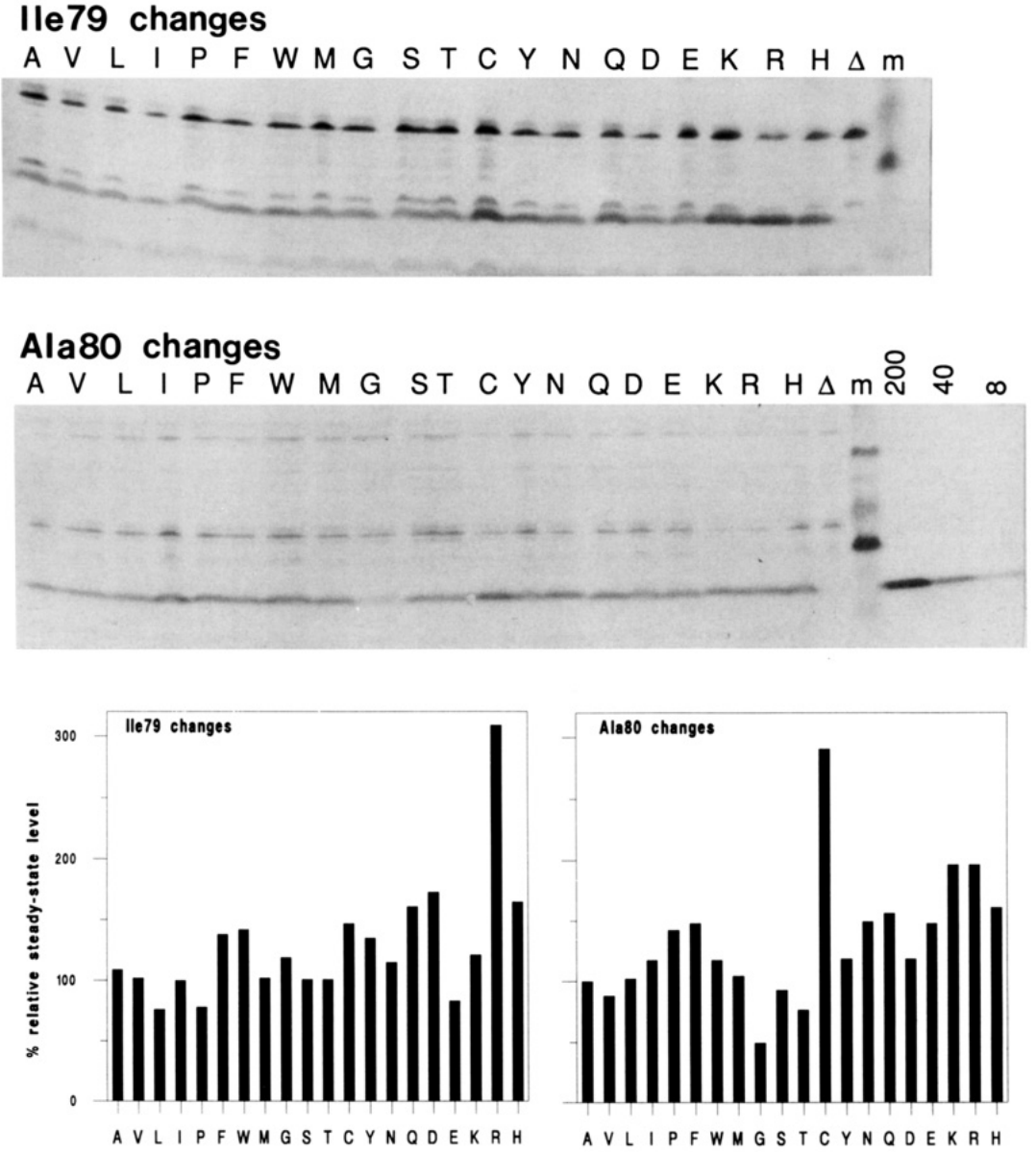

FIGURE 2: Mutant aporepressors with Ile79 and Ala80 changes are made at similar steady-state levels *in vivo*. (Top) Proteins present in *E. coli* host CG103/RSF2001 with wild-type and mutant derivatives of plasmid pPY2000 were resolved by SDS–polyacrylamide gel electrophoresis, and TrpR protein was identified immunologically (Arvidson et al., 1991a, 1993). "Δ" indicates the proteins present in cells without *trpR*. Numbered lanes were loaded with purified wild-type TrpR protein; numbers indicate the nanograms loaded in each lane. Wild-type and mutant proteins present in cell extracts are indicated by the single-letter amino acid code. "m" designates lanes loaded with prestained molecular weight standards (Bio-Rad Laboratories catalog no. 161-0305). Under these conditions, we estimate that (wild-type) plasmid pPY2000 produces about 8000 molecules of TrpR protein/cell, or about 25–40 times the wild-type complement in *E. coli* (Gunsalus et al., 1986). Only the Gly80 repressor is made at steady-state levels significantly lower than wild-type TrpR in *E. coli*. (Bottom) Bar-graph representation of the results of quantitation of the steady-state levels of mutant proteins (wild type = 100%).

natural *E. coli trp* promoter–operator complex controlling *lacZ* expression *in vivo*. To characterize these mutant repressors further, we assayed their activities using the challenge phage assay for DNA binding *in vivo* (Benson et al., 1986; Figure 3). Challenge phage assays provide a more discriminating assessment of the activities of mutant Trp aporepressors *in vivo*, because they are more sensitive to subtle changes in both the amount of aporepressor and the amount of the corepressor, tryptophan, present in host cells (Bass et al., 1987).

Challenge phages are derivatives of temperate *Salmonella* phage P22 *Kn9 arc-H1605(am)* which have a cloned, minimal *trp* operator controlling *ant* (antirepressor) transcription (Figure 1). Methylation protection studies show that this operator has a single repressor dimer-binding site, centered six base pairs (bp) downstream of the *ant* transcription startpoint (Kumamoto et al., 1987; Kumamoto, 1989). *S. typhimurium* cells which express a mutant Trp repressor survive infection with a challenge phage if and only if the mutant repressor binds the phage-borne operator; the state of occupancy of the phage-borne operator determines the fate of the infected cell. If repression occurs *in vivo*, an infecting challenge phage will develop lysogenically, integrate as prophage, and confer kanamycin resistance upon its surviving host cell. If repression fails, *ant* expression leads to lytic development of the phage, and the infected host cell dies. A challenge phage carrying the consensus, minimal *trp* operator lysogenizes a host that produces wild-type repressor under two conditions: a sufficient amount of wild-type TrpR protein must be produced by the host, and host cells must be grown in media supplemented with tryptophan.

The *Salmonella* host strain used in this assay is designed to permit the controlled expression of Trp repressor. This strain, MS1868/F′*lacI*^{Q}, carries an F factor which produces

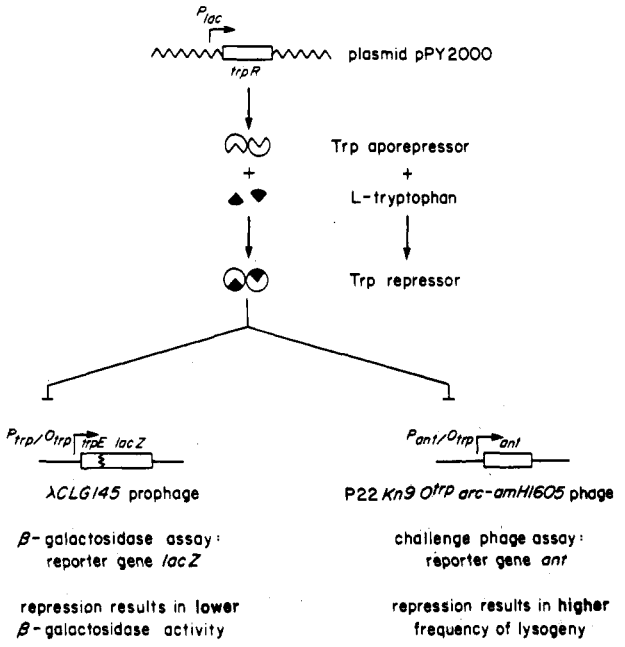

FIGURE 3: Assays for Trp repressor activity *in vivo*. Trp aporepressor produced by plasmid pPY2000 (or its mutant derivatives) binds corepressor to form the active Trp repressor (holorepressor) complex. (Left) Trp holorepressor binds the natural *trp* operator, to repress transcription of a single-copy *trpE/lacZ* fusion operon, measured as the inhibition of β-galactosidase synthesis. Inactive mutant repressors cannot repress transcription of the *lacZ* fusion gene; in their presence, high levels of enzyme are produced. (Right) Infection with a *trp* challenge phage (carrying a minimal operator) follows one of two developmental pathways, depending on the expression of P22 antirepressor. Binding of an active Trp repressor to an operator represses *ant* transcription, permitting lysogenic development and host survival (what is measured). Expression of antirepressor during P22 infection triggers lytic development and the programmed death of the infected host. In this assay, the formation of active Trp holorepressor complex requires binding of tryptophan, and depends on the affinity of aporepressor for tryptophan, as well as other linked equilibria. Experimentally, we can drive the conversion of aporepressor to holorepressor by adding an excess of tryptophan to the medium; conversely, because tryptophan is an essential amino acid that is synthesized by the living cell, some holorepressor will be present *in vivo*, even when tryptophan is not added to the medium.

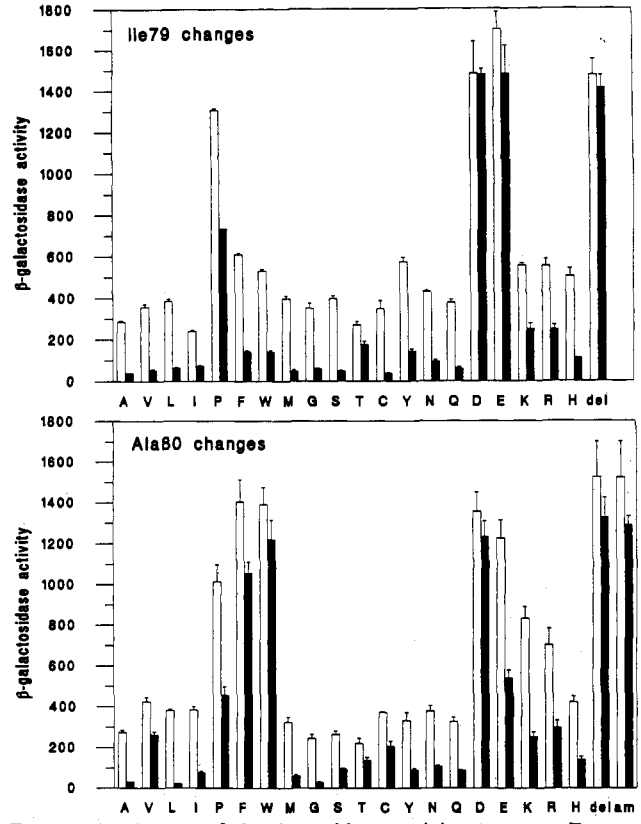

FIGURE 4: Assays of β-galactosidase activity *in vivo*. Enzyme activities produced from a single-copy *trpE/lacZ* fusion operon regulated by each of the mutant Trp repressors with Ile79 and Ala80 changes are shown in histograms, as described under Experimental Procedures. Error bars represent the standard deviations of values determined in triplicate from assays of cells grown without (open bars) or with (filled bars) added tryptophan. Because regulation of tryptophan biosynthesis is homeostatically controlled by Trp holorepressor activity, mutant repressors which are less active than the wild type will result in higher β-galactosidase activities, and mutant repressors which are more active than the wild type will result in lower β-galactosidase activities in this assay under conditions of tryptophan-limited growth [see, for examples, Kelley and Yanofsky (1985) and Klig et al. (1988)].

high, constitutive levels of Lac repressor protein. The plasmids we have constructed produce mutant Trp repressors from the *lacUV5* promoter, regulated by the *lac* operator. In the absence of an inducer of Lac repressor such as IPTG (isopropyl β-D-galactoside), expression of Trp repressor from these plasmids is low in the *Salmonella* host, because F-encoded Lac repressor turns down transcription of *trpR* from the *lacUV5* promoter. With added IPTG, *trpR* expression is induced in a dose-dependent manner. A plot of the logarithm of the titer of cells which survive challenge phage infection *vs* added IPTG concentration gives a sigmoidal dose–response curve, indicating that an induced level of Trp repressor is required for efficient repression and survival in this assay (Figures 5 and 6, left panels).

Previous results show that challenge phage assays done with an excess of exogenous tryptophan and varying amounts of Trp aporepressor allow us to rank the relative DNA-binding affinities of mutant *holo*repressors *in vivo* (Arvidson et al., 1993; Shapiro et al., 1993). This is the same ranking determined by direct measurements of affinity *in vitro* (Hurlburt & Yanofsky, 1990). As shown in the left panels of Figures 5 and 6, 10 mutant holorepressors with Ile79 or Ala80 changes have activities similar to that of wild-type repressor. These include nine mutant repressors with Ile79 changes (to Ala, Cys, His, Gln (Q), Gly, Met, Ser, Thr, and Val) and one mutant repressor with an Ala80 change (Gly). Most repressor mutants have slightly lower activities than the wild-type protein, and the subset of mutant repressors which are inactive in β-galactosidase assays is a proper subset of the mutant repressors inactive in the challenge phage assay. The order of activities measured for the binding of mutant repressors to a minimal, consensus *trp* operator using the challenge phage assays is remarkably similar to that obtained for the binding of mutant repressors to the compound, wild-type *trp* operator using the β-galactosidase assay (Figures 3, 5, and 6). For example, the two most active mutant repressors with Ala80 changes, Gly80 and Leu80, in the β-galactosidase assay (Figure 3, bottom) also show the highest activities in the challenge phage assays of holorepressor and aporepressor activity (Figure 6, top four panels), and the five least active mutant repressors with Ala80 changes, Pro80, Phe80, Trp80, Asp80, and Glu80, are inactive in the challenge phage assays.

Seven Mutant Trp Repressors with Ile79 or Ala80 Changes Are Super-Aporepressors. The efficient lysogenization of a *Salmonella* host by a challenge phage with a minimal *trp* operator depends not only on a high steady-state level of Trp aporepressor but also on the presence of exogenous tryptophan, to convert aporepressor into holorepressor. As shown in Figures 5 and 6 (left panels), our control host that produces wild-type aporepressor cannot support lysogeny in the absence

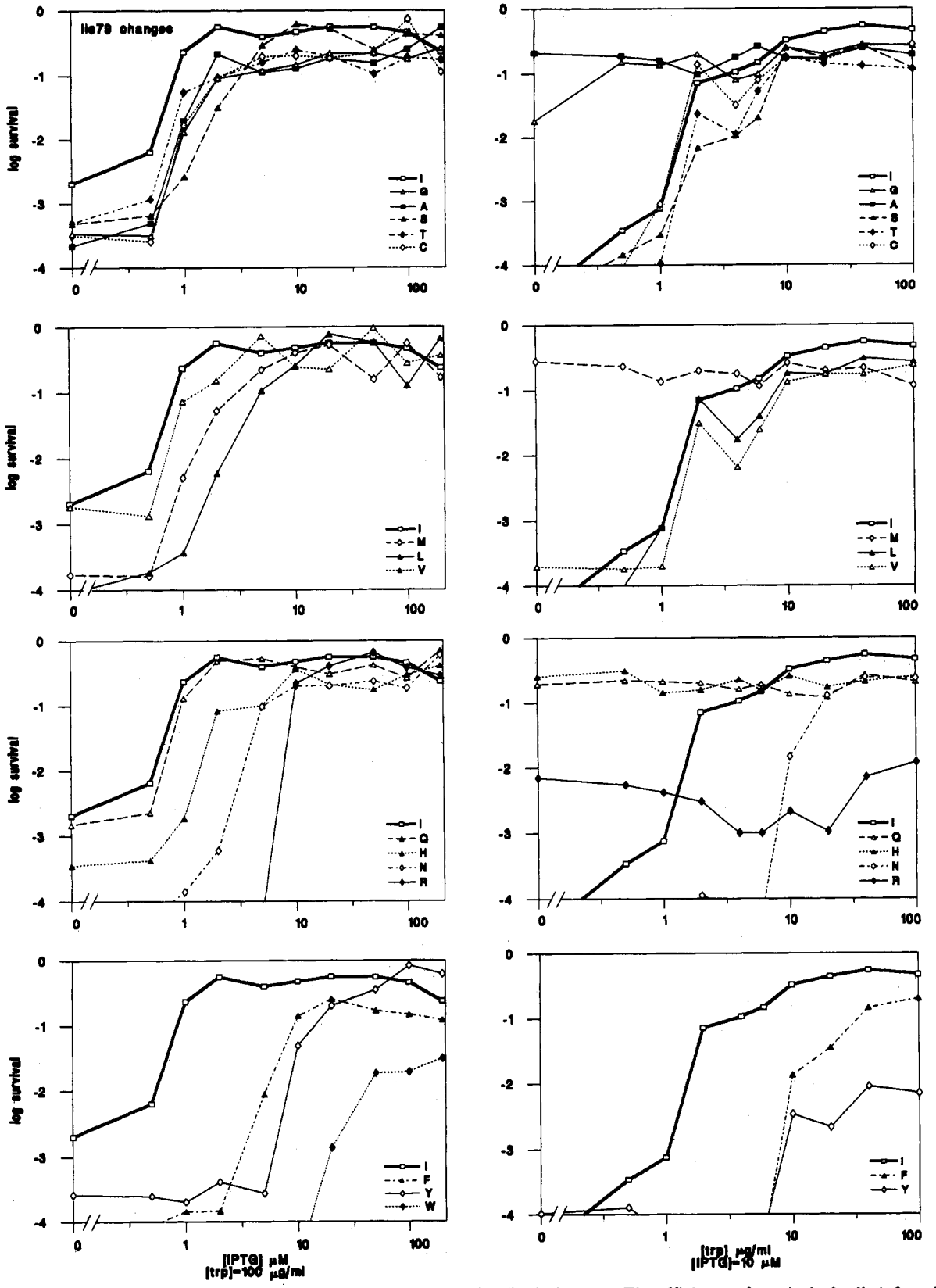

FIGURE 5: Challenge phage assays *in vivo* of mutant Trp repressors with Ile79 changes. The efficiency of survival of cells infected with a challenge phage is a measure of how well the phage-borne, minimal *trp* operator is occupied by plasmid-encoded TrpR protein. For the results shown in the left panels, L-trptophan is present in excess, and the steady-state level of TrpR protein is varied, by the addition of different concentrations of IPTG (plotted on a logarithmic scale). Under these conditions, TrpR protein is expected to exist primarily as holorepressor, allowing us to rank the relative binding affinities of mutant holorepressors (Arvidson et al., 1993; Shapiro et al., 1993). In the right panels, results are shown for infections in which the concentration of inducer (10 μM IPTG) and thus the steady-state level of TrpR expression is held constant, while the concentration of the corepressor, tryptophan, is varied. Values for the wild-type repressor are connected by boldface lines.

of tryptophan, and survives infection if sufficient corepressor is added to the growth medium. The analysis of mutant repressors with Ile79 and Ala80 changes shows that six mutant proteins with Ile79 changes [Gly, Ala, Met, Gln (Q), His, and

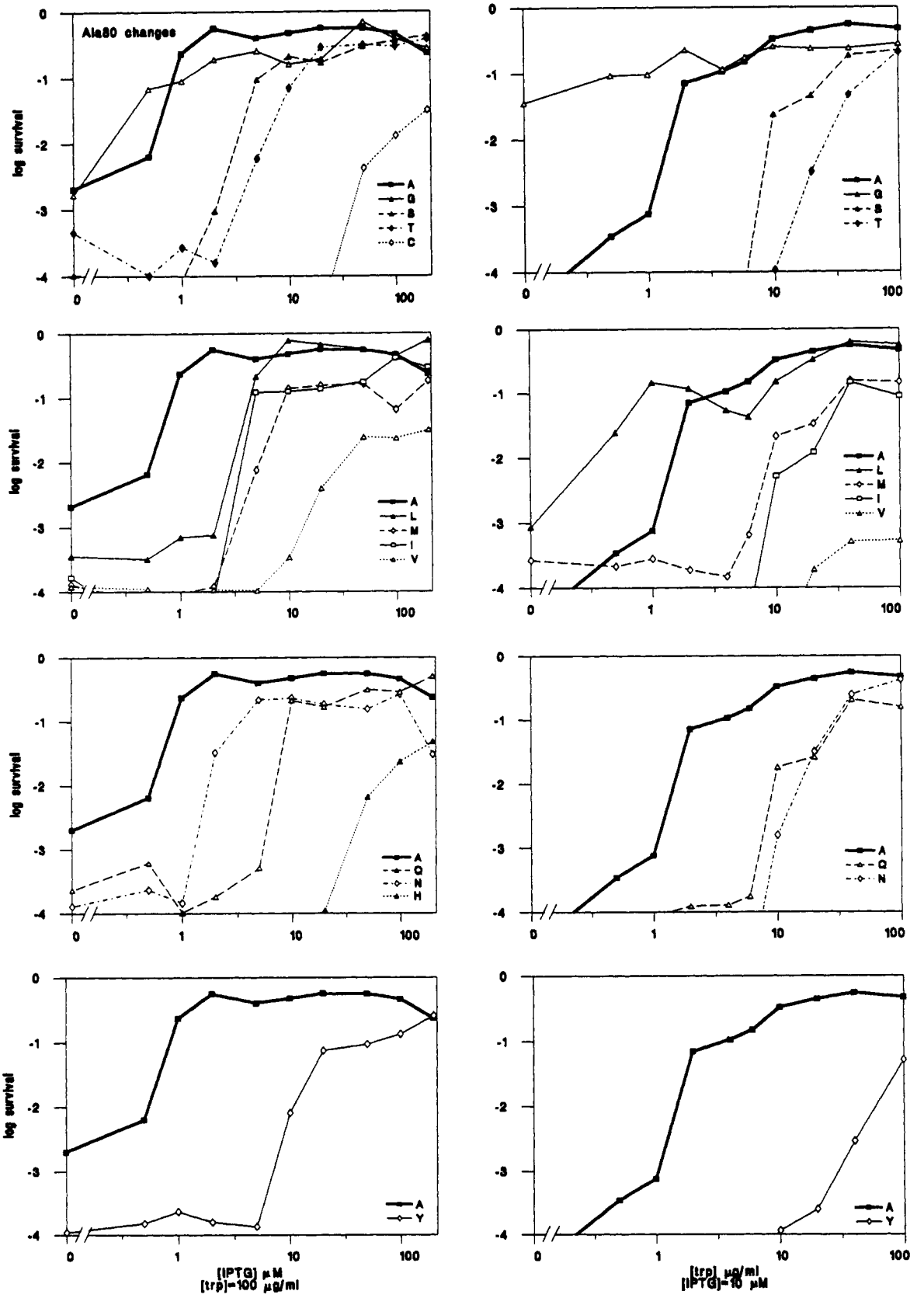

FIGURE 6: Challenge phage assays *in vivo* of mutant Trp repressors with Ala80 changes. Legend as in Figure 5.

Arg (R)] and two mutant proteins with Ala80 changes (Gly and Leu) require less added tryptophan for activation than wild-type repressor. These eight mutant proteins show a gain in function and are super-*apo*repressors. All of the super-aporepressors have activities similar to that of the wild-type protein as holorepressors (Figures 5 and 6, right panels).

Twelve Mutant Trp Repressors with Ile79 or Ala80 Changes Have New DNA-Binding Specificities in Vivo. To test whether mutant Trp repressors have different specificities of target recognition than wild-type repressor, we used the challenge phage assay to measure the ability of wild-type and mutant proteins to bind a set of 27 different mutant-binding

Table 1: Twelve Mutant Trp Repressors with Ile79 and Ala80 Changes Have New or Extended Binding Specificities[a]

	repressor															
operator	**Ile79**	Lys79	Arg79	Gly79	Ser79	His79	Phe79	**Ala80**	Gly80	Ser80	Thr80	Cys80	Ile80	Leu80	Met80	Tyr80
wt	10	–	5	8	20	20	10	10	3	10	9	0.3	2	20	20	0.02
7G:C	30	–	–	20	20	0.002	–	30	10	0.2	0.02	–	–	0.03	–	–
7T:A	30	–	–	0.02	0.002	0.5	–	30	8	0.03	–	–	–	0.01	0.004	–
7C:G	0.3	**2**	**20**	**10**	**20**	0.6	–	0.3	**2**	0.004	0.01	–	–	0.4	0.2	0.008
6G:C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6A:T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6T:A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5G:C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5A:T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5C:G	3	–	0.4	10	**20**	9	10	3	6	**20**	8	0.7	–	1.0	**20**	0.1
4G:C	–	–	–	–	–	–	–	–	–	**0.4**	**0.02**	**0.009**	–	–	**8**	**0.008**
4T:A	–	–	–	–	–	–	–	–	–	**0.003**	**0.001**	–	**0.05**	**0.3**	**0.2**	**0.004**
4C:G	20	–	0.003	20	0.4	50	0.3	20	0.02	0.04	0.004	–	–	0.1	0.005	10
3A:T	–	–	–	**0.007**	–	–	–	–	**0.04**	**0.001**	–	–	–	**1.0**	**0.002**	**1**
3T:A	30	–	–	0.2	0.01	3	–	30	2	0.03	0.08	–	0.01	30	0.2	0.6
3C:G	0.8	–	0.006	0.6	0.04	3	0.02	0.8	0.04	0.02	0.06	–	0.02	0.7	0.03	4
2G:C	30	–	–	0.05	–	0.01	–	30	3	0.03	–	–	0.01	20	0.5	0.1
2A:T	0.7	–	–	0.02	–	0.006	–	0.7	0.7	0.003	–	–	0.02	0.4	0.02	0.004
2C:G	30	–	0.02	40	8	40	–	30	10	1	0.04	–	0.02	20	2	0.004
1C:G	10	–	–	20	30	4	0.004	10	10	10	10	0.02	0.6	40	20	0.2

[a] Symmetric operators carried by challenge phages are designated by operator position and variant base pair (see Figure 1, center). Operators are numbered outward from the C_2 dyad on the top strand of the left, consensus (Bass et al., 1987) operator half-site. The relative affinity of each holorepressor for each operator is given as the percentage of repressor-producing cells which survive infection with a challenge phage carrying that operator. Results indicating novel interactions are indicated in boldface and underlined. Data represent the average of at least three independent determinations; "–" indicates values $<10^{-5}$. Data shown for the Lys79, Arg79, Phe79, Thr80, and Cys80 mutant repressors are consistent with previous results (Bass et al., 1988).

sites (Table 1; Bass et al., 1987, 1988). In this experiment, each mutant-binding site is carried on a different challenge phage, and has a symmetrically related pair of base pair changes in the minimal consensus *trp* operator. Initially, we screened the ability of each of 6 different challenge phages with operators insensitive to wild-type repressor binding to lysogenize *Salmonella* hosts which produce each of the 38 mutant repressors, and found that 12 mutant repressors could bind 1 or more of the 6 defective mutant operators *in vivo*. The interaction of each of these 12 mutant repressors with each of the 27 *trp* operators was examined in detail. Detailed measurements were also done with several mutant repressors which resemble the wild-type repressor in phenotype, for the sake of comparison.

As shown in Table 1, four mutant repressors with Ile79 changes (Arg, Gly, Lys, and Ser) have different binding specificities. All four of these mutant repressors bind the 7C:G operator better than the wild-type repressor. Among these mutant repressors, Lys79 has an *altered* (or "*new*") binding specificity; a host expressing this mutant protein does not survive infection with a challenge phage carrying the consensus (7A:T) operator. In contrast, Arg79, Gly79, and Ser79 repressors have *extended* specificities of binding. These mutants bind the mutant 7C:G operator better than wild-type, and retain the ability to bind the consensus 7A:T operator.

Eight changes at Ala80 that result in extended binding specificities suppress changes in a different region of the operator, at base pairs 4 and 3 (Figure 1). These are Cys80, Met80, Ser80, Thr80, and Tyr80, which bind operator 4G:C, Ile80, Leu80, Met80, Ser80, Thr80, and Tyr80, which bind operator 4T:A; and, Gly80, Leu80, Met80, Ser80, and Tyr80, which bind operator 3A:T. Hosts producing each of these mutant repressors survive infection with a challenge phage carrying a mutant operator with efficiencies more than 5-fold higher than the efficiency of survival of otherwise isogenic cells producing wild-type repressor. All of these mutant repressors retain the ability to bind the wild-type operator.

Lys79 Repressor Has an Altered Binding Specificity in Vitro. To study the altered binding specificity of the mutant Lys79 repressor *in vitro*, we constructed a plasmid which produces the double-mutant repressor with Ile79→Lys and Glu49→Cys changes. In both the co-crystal and NMR structures of the holorepressor–operator complexes, pairs of Glu49 side chains in the holorepressor dimer point toward the minor groove near the C_2 dyad axis of the *trp* operator, and result in electrostatically unfavorable interactions. (Both the Glu49 side chains and operator phosphates have potential negative charges.) Thus, the change of Glu49→Lys results in dramatic super-holorepressor and super-aporepressor phenotypes (Kelley & Yanofsky, 1985; Klig & Yanofsky, 1988; Hurlburt & Yanofsky, 1990; Shapiro et al., 1993). The change of Glu49→Cys improves repressor binding slightly, and introduces a sole cysteine residue into each protomer of the repressor dimer. Challenge phage assays of the activities of the single-mutant Cys49 and double-mutant Cys49 Lys79 repressors show that these repressors retain the wild-type and altered recognition specificities of their wild-type and Lys79 parents, respectively (data not shown).

The thiol group of this cysteine may be used as a site for the specific chemical modification of TrpR protein (Sutton et al., 1993). Cys49 and Cys49 Lys79 repressors were alkylated with 5-(iodoacetamido)-1,10-phenanthroline, to attach the 1,10-phenanthroline moiety to the sulfur atom of Cys49 (Sutton et al., 1993). The derivatized proteins, Cys49-OP and Lys79 Cys49-OP, were assayed for their ability to bind a 50 bp fragment of DNA including the natural *trp* operator, which is composed of three tandem binding sites for Trp holorepressor dimers (Kumamoto et al., 1987; Sutton et al., 1993). We also tested their ability to bind a mutant, 50 bp operator with symmetric 7A:T→C:G changes affecting the strongest, central dimer-binding site (Figure 1).

Figure 7 shows the results of an experiment in which the binding of Cys49-OP and Lys79 Cys49-OP proteins to the wild-type and mutant 7C:G *trp* operators was assayed using gel electrophoresis. Each repressor was incubated with end-labeled operator fragment, and protein–DNA complexes were separated from free operator fragment on the basis of their difference in electrophoretic mobility (Carey, 1988). As shown

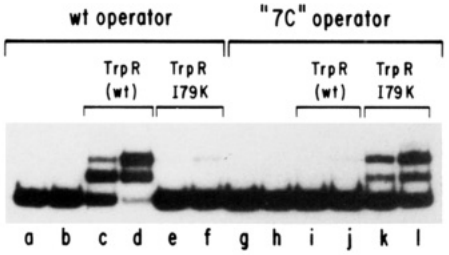

FIGURE 7: Gel retardation of E49C-OP (wt) and 179K E49C-OP mutant Trp repressors bound to the natural (wt) and mutant ("7C") *trp* operators was performed as described by Carey (1988). All lanes contain a restriction fragment with the 5′-labeled *trpEDCBA* operator. Lanes: (a, b) labeled wild-type operator alone; (c, d) complexes of 0.2 and 0.6 nM wild-type TrpR E49C-OP protein with wild-type operator, respectively; (e, f) complexes of 0.2 and 0.6 nM mutant 179K protein with wild-type operator, respectively; (g, h) labeled mutant ("7C") operator alone; (i, j) complexes of 0.2 and 0.6 nM wild-type protein with mutant operator; (k, l) complexes of 0.2 and 0.6 nM mutant protein with mutant operator. In both cases (wild-type + wild-type and mutant + mutant), two protein–DNA complexes are formed. The higher (more slowly migrating) complex has 2:1 repressor dimer/operator fragment stoichiometry, whereas the complex with intermediate mobility has 1:1 stoichiometry (Carey et al., 1991).

in Figure 7, Cys49-OP protein slows the migration of the wild-type *trp* operator fragment (lowest band), indicating that protein–DNA complexes are formed under these conditions (lanes c and d). Two bound species are observed. These correspond to a more rapidly migrating complex with 1:1 holorepressor dimer:operator stoichiometry (middle band) and a slower complex with 2:1 stoichiometry (highest band) (Carey et al., 1991; Haran et al., 1992). Similar concentrations of the Lys79 Cys49-OP protein slow the migration of the mutant 7C *trp* operator fragment (lanes k and l). Conversely, the combination of Cys49-OP protein with the 7C operator and Lys79 Cys49-OP protein with the wild-type operator do not form complexes efficiently (lanes e and f, i and j). These results show that the modified Lys79 protein has an altered DNA-binding specificity *in vitro*.

To identify the sites within these operator fragments to which Trp repressor is bound, each complex was activated by the addition of Cu^{2+} ion, a thiol reductant, and hydrogen peroxide, to catalyze the site-specific scission of DNA (Chen & Sigman, 1987). 1,10-Phenanthroline–cuprous complex with hydrogen peroxide attacks the 3′ C1-position of ribose in the minor groove of DNA (Goyne & Sigman, 1987; Kubawara et al., 1986; Sigman et al., 1979). When 5-acetamido-1,10-phenanthroline is attached to Cys49 repressor, the derivatized protein cleaves riboses predicted to be adjacent to residue 49 in the minor groove at the C_2 dyad of the *trp* operator (Sutton et al., 1993).

As shown in Figures 8 and 9, the Cys49-OP nuclease cleaves the natural *trp* operator preferentially at a pocket within the central dimer-binding site of the operator in the 1:1 complex, and at two main pockets in the 2:1 complex, the central site and a downstream site. Much weaker cleavage is also observed at a third (upstream) site for the 2:1 complex [see also Sutton et al. (1993)]. These results are simply consistent with the results of both methylation protection experiments (Kumamoto et al., 1987) and mutational analysis of the operator (Elledge & Davis 1989; Arvidson, 1989), which also suggest that the *trp* operator is composed of three tandem dimer-binding sites. The nuclease derived from wild-type repressor clearly prefers to bind two of these three tandem sites.

Cleavage of the 7C operator by the Cys49-OP Lys79 repressor results in a slightly different pattern than that

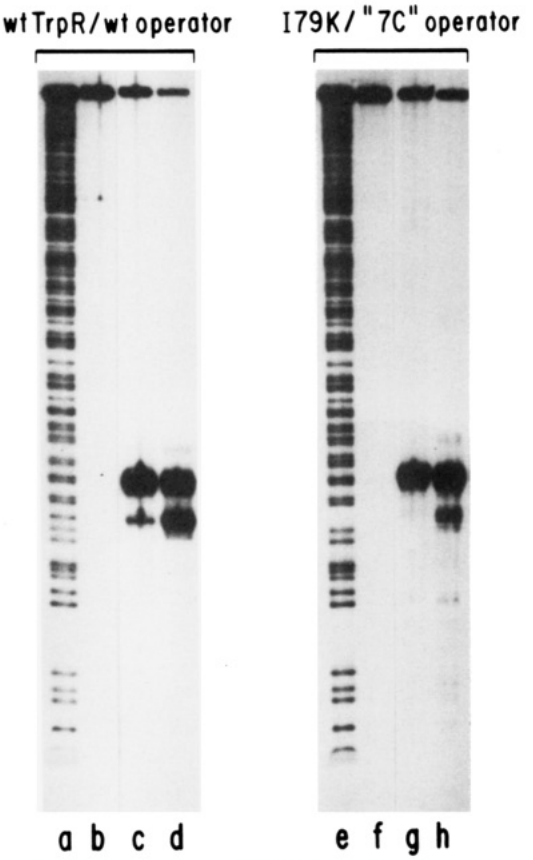

FIGURE 8: Scission patterns of 1,10-phenanthroline–copper endonucleases within the wild-type and mutant *trp* operators. All scission chemistry was carried out within the gel matrix. Lanes: (a) Maxam–Gilbert G+A chemical sequencing lane of the wild-type operator; (b) wild-type operator; (c) wild-type operator + wild-type endonuclease, 1:1 complex; (d) wild-type operator + wild-type endonuclease, 2:1 complex; (e) Maxam–Gilbert G+A chemical sequencing lane of the mutant operator; (f) mutant operator; (g) mutant operator + mutant endonuclease, 1:1 complex; (h) mutant operator + mutant endonuclease, 2:1 complex. Note that the efficiency of cleavage is high for all four complexes; uncleaved operator fragment (top of gel) accounts for less than 20% of the total label in lanes c, d, g, and h.

FIGURE 9: Sites of cleavage of 1,10-phenanthroline–copper endonucleases within the wild-type and mutant *trp* operators. Arrows indicate the relative efficiencies of endonucleolytic cleavage of the template strand of *trp* operators observed for the 2:1 complexes formed between (top) Cys49-OP nuclease and the wild-type operator, and (bottom) Lys79 Cys49-OP nuclease and the mutant 7C:G operator.

observed for the wild-type complex. In the mutant 2:1 complex, scission of the 7C operator is observed at the same two main pockets as in the wild-type complex, but the efficiency of cleavage is lower at the second (downstream) pocket than in the wild-type complex. This result confirms the altered binding specificity of the mutant protein, because this second site in the mutant operator does not have 7A:T→C:G changes

(Figure 9). The decreased efficiency of scission at the second pocket in the 2:1 complex could be due to a new interaction of the mutant repressor with the mutant operator at or near base pair 7 of the primary dimer-binding site which inhibits cleavage at the second binding site, a poorer or less intimate interaction between the mutant repressor and this second site, or a combination of these factors.

DISCUSSION

The product of the *E. coli trpR* gene, Trp repressor, is a small, stable protein that is activated allosterically to bind specific DNA sequences. Trp repressor regulates gene expression in response to a physiological signal, the availability of intracellular L-tryptophan, and modulates the expression of a variety of genes involved in the transport and biosynthesis of this essential amino acid.

In this report, we have described the phenotypes of 38 different mutant Trp repressors with single amino acid changes of residues Ile79 and Ala80. All but one of these mutant proteins are made in steady-state levels similar to that of the wild-type; thus, their activities *in vivo* may be compared with that of wild-type repressor directly. Many of these mutant repressors have complex phenotypes *in vivo*, and differ from the wild type in their interactions with tryptophan, with operator DNA, with nonspecific DNA, or with combinations of these ligands.

Single amino acid changes of residues Ile79 and Ala80 of Trp repressor result in one of *three* different phenotypic consequences: a loss of function (for example, changes to Pro), a preservation of function (changes to Val), or a gain of function (changes to Gly). Eight mutant repressors with Ile79 and Ala80 changes (Gly79, Ala79, Met79, Gln79, His79, Arg79, Gly80, and Leu80) exhibit a gain in function *in vivo*. When tryptophan is limiting, TrpR protein is expected to exist predominantly as aporepressor, not holorepressor, in the challenge phage assay. These eight proteins are more active than wild-type TrpR under these conditions, and thus are super-*apo*repressors. However, we do not wish to imply that mutant aporepressor species can affect repression under these conditions. Rather, these aporepressors likely require less tryptophan for activation or have lower affinities for nonspecific DNA (Arvidson et al., 1993).

In contrast, single amino acid changes in many other proteins lead to only *two* possible phenotypes, either to the loss or retention of function. For example, most single amino acid changes in β-lactamase or hexokinase affect function little, whereas a small subset of changes inactivate these enzymes (Ma et al., 1989; Palzkill & Botstein, 1992). These enzymes have rapid turnover rates limited primarily by the kinetics of substrate diffusion. In contrast, optimal Trp repressor function does not correlate with maximized ligand binding. This is because Trp repressor must be able to associate and dissociate with its ligands at rates that are responsive to sudden changes in cellular physiology, rates that determine modest (millimolar to nanomolar) affinities for these ligands.

Because many changes in TrpR protein result in a gain of function, we cannot describe the relationships between the structure and function of this protein in the same way we describe the structure and function of enzymes. In particular, residues Ile79 and Ala80 of Trp repressor have functional roles that should not be categorized simply in terms of their "information content", or fraction of possible amino acid changes which do not impair function (Bowie et al., 1990). Ile79 and Ala80 are neither essential nor dispensable for function; rather, they play a third kind of role in function, that of balancing the interactions of TrpR protein with two different ligands. This balance is illustrated best by the observation that changes of Ile79 to amino acid residues with both shorter and longer aliphatic, hydrophobic side chains result in a super-*apo*repressor phenotype, but not in a super-*holo*repressor phenotype (Figure 5).

The observation that 12 of the 38 mutant Trp repressors with Ile79 and Ala80 changes have different DNA-binding specificities shows that the interaction of this protein with its DNA ligand is extraordinarily plastic. Four mutant repressors with Ile79 changes (Arg79, Gly79, Lys79, and Ser79) alter the interaction of repressor with base pair 7 of the *trp* operator, and nine mutant repressors with Ala80 changes (Cys80, Gly80, Ile80, Leu80, Met80, Ser80, Thr80, and Tyr80) alter the interaction of repressor with base pairs 4 and 3 of the operator. If we assume that these changes affect direct protein–DNA contacts, then we would predict that the side chains of Ile79 and Ala80 would contact the operator at or near these base pairs. Consistent with this idea, in the solution structures of the holorepressor–operator complex, contact NOE's are observed between side chain protons of Ile79 and protons of thymines 8 and −4, and between Ala80 and thymine −4 (Zhang et al., 1994).

How do changes of Ile79 and Ala80 result in new DNA-binding specificities? Lys79 repressor may make a new protein–DNA contact at or near base pair 7 of the operator. Its long, basic side chain may interact directly with this base pair or with an adjacent operator phosphate. Indeed, similar new interactions may be made by Arg79 repressor, which binds both the consensus and mutant 7C operators *in vivo*. Two mutant repressors, Gly79 and Ser79, alter the interaction at two different nonadjacent base pairs (7 and 3, and 7 and 5, respectively; Table 1). Because these changes result in the suppression of operator changes at several different sites, they may act indirectly, perhaps by making the local structure of the "recognition" α-helix more flexible, and thereby allowing several new, alternative combinations of protein–DNA contacts.

Changes of either Ile79 or Ala80 to Gly increase aporepressor activity. This result supports the idea that the Ile79 and Ala80 side chains are important for operator recognition, because (1) increasing the flexibility of the start of α-helix E can improve the association of TrpR protein with tryptophan, operator DNA, or both, and (2) changes of these residues to glycine incur a cost in the specificity of binding. The extended specificity phenotypes of the Gly79 and Gly80 repressors suggest that these mutant proteins have a decreased ability to distinguish between the set of natural operators bound by wild-type repressor and competing (nonspecific) DNA sequences.

Comparisons of the DNA-binding specificities of mutant repressors with Ala80 changes suggest that several repressors with Ala80 changes create new, direct operator contacts. For example, Tyr80 repressor has an extended specificity of binding, and recognizes all of the mutant operators with changes at base pairs 4 and 3 which are not recognized by the wild-type repressor (4G, 4T, and 3A). In contrast, Phe80 repressor is inactive. This result suggests that the hydroxyl group of tyrosine, which is absent from phenylalanine, may make new hydrogen bonds with functional groups of the nucleotide bases at base pairs 4 and 3. Similarly, although Val80 repressor has a recognition specificity which resembles the wild type (Ala80), both Ser80 and Thr80 repressors have extended specificities of recognition, and bind mutant operators with base pair 4 changes that wild-type repressor cannot.

Again, this result suggests that the Ser80 and Thr80 hydroxyl groups can make new, direct contacts with functional groups of this operator base pair. Ser80 repressor can bind the same mutant operators recognized by Thr80 repressor, as well as operator 3A:T. Perhaps the threonine methyl group, absent from serine, sterically occludes the productive interaction of Thr80 repressor with operator 3A:T. Models of the particular interactions between mutant repressors and operators based on these genetic results await biochemical corroboration.

The fact that nearly one-third of the possible amino acid changes of Ile79 and Ala80 result in new binding specificities and the likelihood that a subset of mutant repressors with Ala80 changes makes new operator contacts add to a growing list of evidence which argues that Trp repressor can make direct operator contacts. These results suggest that the solution structures of the Trp holorepressor–operator complex, which reveal an extensive network of direct interactions between protein and DNA species (Zhang et al., 1994), are a more accurate reflection of the structure of this complex than the co-crystal structures, which show that the majority of repressor–operator interactions are water-mediated (Otwinowski et al., 1988; Lawson & Carey, 1993). Trp repressor is an extremely dynamic molecule in solution (Arrowsmith et al., 1991a,b; Czaplicki et al., 1991; Zhao et al., 1993; Zhang et al., 1994), and, like insulin, no single crystal may provide an accurate representation of its structure, or of its interactions with ligands (Hua et al., 1991).

One mutant repressor stands out among the 12 mutant repressors with changed binding specificities. The mutant Lys79 repressor has a classical altered specificity phenotype both *in vivo* and *in vitro* (Youderian et al., 1983). It binds the wild-type operator less well than wild-type repressor yet binds the mutant 7C:G operator better than wild-type repressor. This switch in DNA-binding specificity is evidenced *in vitro* in two different ways. First, Lys79 repressor binds a mutant *trp* operator under conditions where wild-type repressor can bind the wild-type *trp* operator, but where neither wild-type repressor binds the mutant operator nor Lys79 repressor binds the wild-type operator (Figure 7). Second, a phenanthroline–copper derivative of Lys79 repressor shows a marked preference for cleavage of the central binding site within this mutant operator (Figure 8).

Copper(I)–phenanthroline derivatives of Trp repressor accomplish double-stranded cleavage of their target sites efficiently (Figure 8). We have demonstrated that such nucleases can be constructed with mutant, altered-specificity Trp repressors and that these modified proteins exhibit different target specificities. It should be possible to construct a generation of nucleases which cleave a variety of rare recognition sequences derived from mutant Trp repressors.

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