Effects of Mutations in the L-Tryptophan Binding Pocket of the trp RNA-binding Attenuation Protein of Bacillus subtilis

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The Bacillus subtilis tryptophan biosynthetic genes are regulated by the trp RNA-binding attenuation protein (TRAP). Cooperative binding of l-tryptophan activates TRAP so that it can bind to RNA. The crystal structure revealed that l-tryptophan forms nine hydrogen bonds with various amino acid residues of TRAP. We performed site-directed mutagenesis to determine the importance of several of these hydrogen bonds in TRAP activation. We tested both alanine substitutions as well as substitutions more closely related to the natural amino acid at appropriate positions. Tryptophan binding mutations were identified in vivo having unchanged, reduced, or completely eliminated repression activity. Several of the in vivo defective TRAP mutants exhibited reduced affinity for tryptophan in vitro but did not interfere with RNA binding at saturating tryptophan concentrations. However, a 10-fold decrease in TRAP affinity for tryptophan led to an almost complete reduction in trp operon regulation in vivo and reduced affinity of TRAP for trp leader RNA in vitro. TRAP's stem loop results in a substantial reduction in trp operon regulation in vivo and reduced affinity of TRAP for trp leader RNA in vitro. TRAP's stem loop RNA interaction reduces the number of (G/U)AG repeats required for stable TRAP-trp leader RNA association. Thus it appears that the 5'-structure increases the rate of TRAP-trp leader RNA association, promoting the likelihood that tryptophan-activated TRAP binds to the nascent trp leader transcript before the antiterminator can form.

Cooperative binding of l-tryptophan between every adjacent TRAP subunit activates the 11-subunit TRAP complex. The crystal structure of TRAP complexed with tryptophan revealed that tryptophan forms nine hydrogen bonds with various amino acid residues of TRAP (Fig. 2). We performed site-directed mutagenesis to determine the relative importance of several of these hydrogen bonds in TRAP activation. We tested both alanine-substituted mutants as well as substitutions more closely related to the natural amino acid at appropriate positions. Several of these substitutions were expected to disrupt hydrogen bond formation between TRAP and tryptophan, whereas others were not expected to disrupt TRAP function. The regulatory activity of each mutant TRAP was tested in vivo, whereas the tryptophan binding and RNA binding activities of each mutant protein were tested in vitro. Our results indicate that one hydrogen bond between TRAP and tryptophan that was predicted by the crystal structure is dispensable for TRAP activity, whereas two other hydrogen bonds appear to be essential for TRAP function. Another mutant protein exhibited tryptophan-independent RNA binding activity. We also found that trp leader RNA increases the affinity of TRAP for tryptophan.

Expression of the Bacillus subtilis tryptophan biosynthetic (trp) genes is negatively regulated in response to tryptophan by TRAP,1 the trp RNA-binding attenuation protein, which is the product of the mtrB gene (reviewed in 1). TRAP-mediated regulation of the trp genes includes a transcription attenuation and two translation control mechanisms. The untranslatable trp operon leader transcript contains inverted repeats that allow the mRNA to form three RNA secondary structures. Two of these structures, the antiterminator and terminator, overlap by four nucleotides and therefore are mutually exclusive (Fig. 1) (2-5). The TRAP binding target in the trp leader transcript consists of 11 (G/U)AG repeats, six of which are present within the antiterminator (6). When activated by tryptophan, TRAP binds to the 11 triplet repeats, which prevents formation of the antiterminator structure. This allows formation of the overlapping terminator, which promotes transcription termination upstream of the trp structural genes. In the absence of TRAP binding, the antiterminator structure forms, which results in transcription of the entire operon (7, 8). An additional RNA secondary structure forms at the extreme 5'-end of the B. subtilis trp leader transcript. Disruption of the 5'-stem loop results in a substantial reduction in trp operon regulation in vivo and reduced affinity of TRAP for trp leader RNA in vitro (9). TRAP's 5'-stem loop RNA interaction reduces the number of (G/U)AG repeats required for stable TRAP-trp leader RNA association. Thus it appears that the 5'-structure increases the rate of TRAP-trp leader RNA association, promoting the likelihood that tryptophan-activated TRAP binds to the nascent trp leader transcript before the antiterminator can form.2

Cooperative binding of l-tryptophan between every adjacent TRAP subunit activates the 11-subunit TRAP complex. The crystal structure of TRAP complexed with tryptophan revealed that tryptophan forms nine hydrogen bonds with various amino acid residues of TRAP (Fig. 2) (10). We performed site-directed mutagenesis to determine the relative importance of several of these hydrogen bonds in TRAP activation. We tested both alanine-substituted mutants as well as substitutions more closely related to the natural amino acid at appropriate positions. Several of these substitutions were expected to disrupt hydrogen bond formation between TRAP and tryptophan, whereas others were not expected to disrupt TRAP function. The regulatory activity of each mutant TRAP was tested in vivo, whereas the tryptophan binding and RNA binding activities of each mutant protein were tested in vitro. Our results indicate that one hydrogen bond between TRAP and tryptophan that was predicted by the crystal structure is dispensable for TRAP activity, whereas two other hydrogen bonds appear to be essential for TRAP function. We also determined that the trp leader RNA increases the affinity of TRAP for tryptophan, presumably by stabilizing the interaction of TRAP with tryptophan.

EXPERIMENTAL PROCEDURES

Plasmids and Bacterial Strains—The B. subtilis strains used in this study are described in Table 1. The plasmid pTZ18mtrAB was described (5). Site-directed mutagenesis of mtrB using pTZ18mtrAB, mutagenic DNA oligonucleotides, and the MORPH mutagenesis kit was carried out according to the manufacturer's instructions (5 Prime-3 Prime, Inc.). Escherichia coli strain DH5α was used for plasmid constructions, whereas E. coli strain K38/pGP1-2 (11) was used to overproduce wild type and mutant TRAP proteins. The 1.1-kilobase EcoRI-HindIII fragment containing the wild type or mutant mtrB alleles were subcloned into pMK3 (12). These plasmids were used to transform the TRAP-deficient B. subtilis strain CYBS306 (13). The resulting strains were used to examine the in vivo TRAP activities of wild type and mutant TRAP proteins.

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1 The abbreviations used are: TRAP, trp RNA-binding attenuation protein; WT, wild type.
2 H. Du, A.V. Yakhnin, S. Dharmaraj, and P. Babitzke, unpublished results.
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**FIG. 1. Model of the trpEDCBA operon transcription attenuation mechanism.** A, under tryptophan-limiting conditions TRAP is not activated. During transcription the 5'-stem loop and antiterminator structures form. Antiterminator formation prevents formation of the terminator, resulting in transcription readthrough into the trp structural genes. B, under excess tryptophan conditions TRAP is activated. During transcription TRAP interacts with the 5'-stem loop and with the (G/U)AG repeats as soon as they are synthesized, thereby wrapping the RNA around the periphery of the TRAP complex. TRAP binding prevents formation of the antiterminator, which allows formation of the terminator, and hence termination of transcription before RNA polymerase can reach the trp operon structural genes. The (G/U)AG repeats are indicated in boldface type. The four nucleotides that the antiterminator and terminator structures have in common (ACCC) are indicated by the outlining type.

**FIG. 2. Hydrogen bonds formed between wild type TRAP and L-tryptophan.** The amino group of L-tryptophan is hydrogen-bonded to the main chain carbonyls of residues Thr-25, Gly-27, and Asp-29, as well as to the side chain hydroxyl of Thr-30. One carboxyl oxygen of L-tryptophan forms hydrogen bonds with the main chain amide of Gly-27, the side chain hydroxyl of Ser-53, and a water molecule. The other carboxyl oxygen forms a hydrogen bond with the side chain hydroxyl groups of both Thr-49 and Thr-52. Finally, the secondary amine present in the indole ring of L-tryptophan is hydrogen-bonded to the main chain carbonyl of Gln-47 (10). The amino acid residues that were mutagenized in this study are shown in boldface type.

β-Galactosidase Assay—The effect of single amino acid changes on the expression of a trpE/-lacZ translational fusion was measured using β-galactosidase assays as described previously (9). Cells were cultured in minimal medium containing 0.2% acid-hydrolyzed casein and 0.2% glucose in the presence or absence of L-tryptophan (50 μg/ml). The culture medium was supplemented with tetracycline (12.5 μg/ml), chloramphenicol (5 μg/ml), and kanamycin (5 μg/ml). The β-galactosidase activity is reported in Miller units (14).

TRAP Purification—Wild type and mutant mtrB genes were overexpressed using the T7 RNA polymerase system described by Tabor and Richardson (11). Proteins were purified by a published procedure (8) or by a simplified protocol. Cells (2.5 g of wet weight) were suspended in 15 ml of 40 mM sodium phosphate (pH 7.0) and 1 mM EDTA. 150 μl of isomyl alcohol was added to prevent foaming during cell lysis. Cells were lysed by continuous sonication for 10 min (50% pulses) in an ice-water bath. Cell debris was removed by ultracentrifugation (100,000 × g) for 1 h at 4 °C. The supernatant was then heated for 10 min in boiling water. All TRAP mutants remained soluble during this step. Denatured proteins were pelleted by centrifugation (20,000 × g) for 30 min at 4 °C. The supernatant was loaded at room temperature onto a 3-ml P11 phosphocellulose (Whatman) column equilibrated with buffer A (12 mM sodium phosphate (pH 7.2), 50 mM sodium chloride, and 0.1 mM EDTA). The column was washed with 7.5 ml of buffer A and then with 10 ml of buffer A containing 0.5 M sodium chloride. TRAP was eluted with 7.5 ml of buffer A containing 1 M sodium chloride. Protein was concentrated using a Centricon 30 ultrafiltration unit (Millipore). The retentate was diluted 3-fold in buffer A and concentrated as described above to a final volume of 1.2 ml. The pellet formed during this step was removed by centrifugation for 15 min (12,000 × g). β-Mercaptoethanol (0.1% final concentration) was added to all buffers for the SS3C mutant protein purification. TRAP purity was assessed by electrophoresis through 16% sodium dodecyl sulfate-polyacrylamide gels. Protein concentrations were determined by the BCA assay (Pierce). Wild type and mutant TRAP proteins purified according to this procedure were as active as those purified by a previous method (8).

Equilibrium Dialysis—The affinities of wild type and mutant TRAP proteins were determined by equilibrium dialysis using [3H]-L-tryptophan (NEN Life Science Products) and purified TRAP by modifying a published procedure (10). The buffer used in this analysis consisted of 50 mM Tris-HCl (pH 8.0), 250 mM KCl, and 4 mM MgCl2. [3H]-L-Tryptophan at various concentrations (1–2187 nM) and TRAP (1–15 nM) were injected into opposite sides of 12–14-kDa cut-off membrane in an EMD101 equilibrium dialyzer (Amersham Pharmacia Biotech). After incubation at 4 °C for 16–24 h, duplicate 10-μl aliquots were quantified by liquid scintillation counting. The data were fit to the Hill equation to describe the cooperative multisite binding of tryptophan to TRAP (see below).

Gel Mobility Shift Assay—The RNA used in the gel mobility shift experiments was synthesized in vitro using the Ambion MEGAscript kit and plasmid pPB77 linearized with BstBI as template (6). This transcript contained nucleotides 1 to +111 of the B. subtilis trp leader. The RNA was gel-purified (15) and subsequently dephosphorylated with calf intestinal phosphatase and 5′-phosphate 5′-exonuclease. The RNA was labeled with 32P-ATP.

**Binding reaction mixtures (8 μl) contained 50 mM Tris-acetate (pH 8.0), 4 mM magnesium acetate, 5 mM dithiothreitol, 10% (v/v) glycerol, 0.2 mg/ml E. coli tRNA, 0.1 mg/ml xylene cyanol and 400 μM/ml RNAsin (Promega). 0.2 mM 5′-End-labeled trp leader RNA and 1.2 mM L-tryptophan were used for TRAP dilution experiments. 0.5 mM 5′-End-labeled trp leader RNA and 320 nM TRAP were used for l-tryptophan dilution experiments. Reaction mixtures were incubated at room temperature for 20 min. The samples were then fractionated on 12–14-kDa cut-off membrane in an EMD101 equilibrium dialyzer (Amersham Pharmacia Biotech). After incubation at 4 °C for 16–24 h, duplicate 10-μl aliquots were quantified by liquid scintillation counting. The data were fit to the Hill equation to describe the cooperative multisite binding of tryptophan to TRAP (see below).

**RNA affinity** = a × [TRAP]/[Kd + [TRAP]] (Eq. 1)

where a is the saturation level of the fraction RNA bound ([RNAa]); Kd is the concentration of free protein, [TRAP], at which the RNA reaches 50% saturation; RNA is the fraction of RNA bound between 0 and 1; [TRAP], is the concentration of free protein, TRAP, and was assumed to be the concentration of total TRAP added because the total TRAP concentration was at least in 12-fold molar excess over RNA. The tryptophan and RNA binding data were also fit to the cooperativity equations,

TRP = a × [([TRP]/[S0.5])1/(1 + ([TRP]/[S0.5]))] (Eq. 2)

RNA = a × ([TRP]/[S0.5])1/(1 + ([TRP]/[S0.5])) (Eq. 3)
In Vivo Regulation of Wild Type and Mutant TRAP Proteins—The x-ray crystal structure of TRAP in the presence of bound tryptophan indicates that tryptophan forms nine hydrogen bonds with the RNA. Note that structural information of non-tryptophan-activated TRAP permitted us to describe the cooperativity of binding (16).

Filter Binding Assays—The binding of [\textsuperscript{\textit{H}}]-tryptophan to wild type and mutant TRAP proteins in the presence or absence of RNA was examined by modifying a published filter binding assay (17). Reaction mixtures (0.1 ml) containing various concentrations of [\textsuperscript{\textit{H}}]-tryptophan were incubated for 20 min at 37 °C with 0.2 μM TRAP, 1 mM dithiothreitol, and 5 units RNasin in buffer containing 50 mM Tris-HCl (pH 8.0), 250 mM KCl, and 4 mM MgCl\textsubscript{2}. In some assays, gel-purified RNA (see gel mobility shift assay) was added to binding assays at a 10-fold excess (2 μM) over the TRAP concentration. Mixtures were filtered, the filters were washed four times with 0.1 ml of the same buffer, and the retained tryptophan was quantified in a scintillation counter.

RESULTS

In Vivo Regulation of Wild Type and Mutant TRAP Proteins—The x-ray crystal structure of TRAP in the presence of bound tryptophan indicates that tryptophan forms nine hydrogen bonds with various amino acid residues of TRAP (Fig. 2) (10). Based on this structural information, point mutations were introduced into mtrB by site-directed mutagenesis to alter amino acid residues predicted to be involved in tryptophan binding. These TRAP mutants were also isolated to decipher which of the hydrogen bonds were required for the presumed conformational changes that allow TRAP interaction with RNA. Note that structural information of non-tryptophan-activated TRAP is unavailable. We tested both alanine-substituted mutants as well as substitutions more closely related to the natural amino acid at appropriate positions.

A trpE\textsuperscript{−}lacZ translational fusion was used to assess the level of trp operon expression in B. subtilis strains containing wild type or mutant TRAP proteins (Table I). Each of these strains contained the entire trp operon at its natural chromosomal location, a single copy trpE\textsuperscript{−}lacZ translational fusion under the control of the wild type trp promoter and leader integrated into amyE and a defective chromosomal copy of mtrB (13). This genetic background was used to measure the relative levels of trp operon expression from the trpE\textsuperscript{−}lacZ translational fusion in strains containing wild type or mutant plasmid-borne mtrB alleles. β-Galactosidase activity in the positive control strain bearing wild type (WT) mtrB was reduced 700-fold when cultures were grown in the presence of tryptophan compared with cultures grown in its absence (Table I). In the negative control strain (no TRAP) β-galactosidase activity was similar when cultures were grown in the presence or absence of tryptophan (Table II). Of the eleven strains containing mutant TRAP, two exhibited wild type regulation (Q47E, S53A), four showed intermediate regulation (Q47A, Q47M, T52A, and S53C), and five were unable to regulate expression in response to tryptophan (T30A, T30V, T49A, T49V, and T52V) (Table II). Thus, nine of the eleven mtrB mutations severely affected the ability of the resulting TRAP complex to regulate trp operon expression in vivo.

Defects of the Mutant TRAP Proteins Determined in Vitro—To determine if the in vivo defects caused by the mtrB mutations were due to altered tryptophan binding, equilibrium dialysis experiments were performed in vitro with purified wild type or mutant TRAP proteins. The S\textsubscript{50} value determined for wild type TRAP was 6.6 μM (Table III). The Q47E and S53A mutant TRAP proteins bound RNA with similar affinities as wild type TRAP, consistent with the in vivo expression study (Tables II and III). We did not observe detectable tryptophan binding with the remaining TRAP mutants at a 1-μM protein concentration; however, tryptophan binding with reduced affinity was observed for the Q47A, Q47M, T52A, and T52V mutant proteins at a concentration of 15 μM (Table III). Note that three of these four substitutions (Q47A, Q47M, T52A) retained partial regulatory activity in vivo. Also note that whereas equilibrium dialysis did not detect tryptophan binding to S53C in vitro (Table III), this mutant protein retained partial regulatory activity in vivo. Thus with the exception of T52V and S53C, the affinity of the mutant proteins for tryptophan determined in vitro correlated with their regulatory activity in vivo.

To determine if the mutant TRAP proteins could bind to trp leader RNA we performed gel mobility shift assays using a saturating tryptophan concentration (1.2 mM). We found that the T49A substitution led to the complete loss of RNA binding activity. All of the remaining proteins, as well as wild type TRAP, bound leader RNA in a cooperative manner (positive n value) (Fig. 3 and Table III). Of these mutant proteins, only T30A, T30V, T49V, and S53C exhibited a reduced affinity for RNA (at least 3-fold). However, we did observe a 2- to 3-fold increase in the affinity for RNA with the S53A mutant protein. The affinities of the remaining mutant proteins were comparable to that of wild type TRAP (Fig. 3 and Table III). With the exception of T30V, RNA binding was dependent on tryptophan (Fig. 4 and Table III).

Because wild type TRAP can only bind RNA tightly when

| Strains | Genotypea | Plasmid-encoded mtrB allele |
|---------|-----------|---------------------------|
| CYBS306<sup>b</sup> | mtrB<sup>H</sup>Tc amyE<sup>−</sup>-[trpP (−412 to +203)]<sup>c</sup> | None |
| PLEBS151 | CYBS306/pMK3 (Km<sup>′</sup>) | None |
| PLEBS152 | CYBS306/pRT10 (Km<sup>′</sup>) | WT |
| PLEBS162 | CYBS306/pJJT7 (Km<sup>′</sup>) | T50A |
| PLEBS155 | CYBS306/pRT13 (Km<sup>′</sup>) | T30V |
| PLEBS161 | CYBS306/pJJT6 (Km<sup>′</sup>) | Q47A |
| PLEBS154 | CYBS306/pRT12 (Km<sup>′</sup>) | Q47E |
| PLEBS153 | CYBS306/pRT11 (Km<sup>′</sup>) | Q47M |
| PLEBS164 | CYBS306/pJJT10 (Km<sup>′</sup>) | T49A |
| PLEBS156 | CYBS306/pJJT1 (Km<sup>′</sup>) | T49V |
| PLEBS157 | CYBS306/pJJT2 (Km<sup>′</sup>) | T52A |
| PLEBS165 | CYBS306/pJJT11 (Km<sup>′</sup>) | T52V |
| PLEBS163 | CYBS306/pJJT9 (Km<sup>′</sup>) | S53A |
| PLEBS161 | CYBS306/pJJT6 (Km<sup>′</sup>) | S53C |

<sup>a</sup> trpP denotes the trp operon promoter; prime indicates truncation of the gene. −412 to +203 denotes the DNA fragment containing the trp promoter and neighboring regions that were incorporated.

<sup>b</sup> Ref. 13.

\[ \text{RNA}_a = a \times \left( \frac{[\text{TRAP}]}{S_{50}} \right)^n / \left( 1 + \frac{[\text{TRAP}]}{S_{50}} \right)^n \]  
(Eq. 4)

where \( a \) is the saturation level of the fraction of bound RNA (\( \text{RNA}_a \)) or tryptophan (\( \text{TRP}_a \)), \( S_{50} \) is the concentration of free protein, \( [\text{TRAP}]_p \) or free tryptophan, \( [\text{TRP}]_p \), at which the \( \text{RNA}_a \) or \( \text{TRP}_a \), respectively, reach 50% saturation; \( \text{RNA}_a \) and \( \text{TRP}_a \) are the fractions of bound RNA or tryptophan, respectively, between 0 and 1; [TRAP], and [Trp], are the concentrations of free TRAP 11-mer or tryptophan, respectively, which were assumed to be the concentration of total TRAP or tryptophan added. The cooperativity coefficient, \( n \), is used to describe the cooperativity of binding (16).

RNA\textsubscript{50} denotes the DNA fragment containing the trp operon expression from the trpE\textsuperscript{−}lacZ translational fusion (10). Based on this structural information, point mutations were introduced into mtrB by site-directed mutagenesis to alter amino acid residues predicted to be involved in tryptophan binding. These TRAP mutants were also isolated to decipher which of the hydrogen bonds were required for the presumed conformational changes that allow TRAP interaction with RNA. Note that structural information of non-tryptophan-activated TRAP is unavailable. We tested both alanine-substituted mutants as well as substitutions more closely related to the natural amino acid at appropriate positions.

A trpE\textsuperscript{−}lacZ translational fusion was used to assess the level of trp operon expression in B. subtilis strains containing wild type or mutant TRAP proteins (Table I). Each of these strains contained the entire trp operon at its natural chromosomal location, a single copy trpE\textsuperscript{−}lacZ translational fusion under the control of the wild type trp promoter and leader integrated into amyE and a defective chromosomal copy of mtrB (13). This genetic background was used to measure the relative levels of trp operon expression from the trpE\textsuperscript{−}lacZ translational fusion in strains containing wild type or mutant plasmid-borne mtrB alleles. β-Galactosidase activity in the positive control strain bearing wild type (WT) mtrB was reduced 700-fold when cultures were grown in the presence of tryptophan compared with
activated by tryptophan, the gel mobility shift assay was also used to examine the affinities of wild type and mutant TRAP for tryptophan indirectly using serial tryptophan dilutions. However this technique could not be used for the T30A, T49A, T49V, T52A, T52V, S53A, and S53C mutants, which had substantially reduced or eliminated RNA binding activity. In contrast to the TRAP dilution experiments, the positions of shifted RNA complexes in the tryptophan dilution experiments were dependent on the tryptophan concentration (Fig. 4). This effect was likely caused by disruption of the TRAP-RNA complex during gel running due to the dissociation of tryptophan. The Q47E mutant protein that exhibited wild type-like regulation in vivo bound tryptophan with a similar affinity as wild type TRAP. Interestingly, the S53A mutant TRAP had a 2.5-fold higher affinity than wild type TRAP for tryptophan (Fig. 4 and Table III). We also found that the T30V mutant protein possessed tryptophan-independent RNA binding activity. All of the remaining mutant proteins had reduced affinities for tryptophan (Table III) that correlated with their reduced ability to regulate trp operon expression in vivo (Table II). With the exception of T52V, all of the tryptophan-dependent mutant TRAP proteins retained the positive cooperativity of tryptophan binding that was previously observed for wild type TRAP (Table III) (10, 16). Note that T52V TRAP exhibited a negative cooperativity for tryptophan binding in this assay in contrast to the positive cooperativity that was demonstrated for this protein by equilibrium dialysis (Table III). Also note that the dissociation constants of tryptophan binding determined by gel mobility shift assays were substantially lower than those determined by equilibrium dialysis (Table III).

**TRAP-trp Leader RNA Association Stabilizes the Interaction of TRAP with Tryptophan**—It was possible that the apparent discrepancy in tryptophan $S_{0.5}$ values measured by equilibrium dialysis and gel retardation techniques may have resulted because the equilibrium dialysis experiments were carried out in the absence of RNA, whereas the gel mobility shift assay measured the affinity of tryptophan indirectly via RNA binding. This suggested that TRAP-trp leader RNA interaction could increase the affinity of TRAP for tryptophan. A filter binding assay was used to test this hypothesis. We found that the affinity of TRAP for tryptophan increased substantially in the presence of trp leader RNA. The relative amount of tryptophan

### Table II

**In vivo regulatory defects of TRAP mutants**

| TRAP mutant | $-\text{Trp}$ | $+\text{Trp}$ | $\beta$-Galactosidase activity $^c$ | $\beta$-Galactosidase ratio $^{d,n}$ |
|-------------|----------------|----------------|------------------------------------|-----------------------------------|
| No TRAP     | 970 ± 140      | 940 ± 130      | 1.0                                |                                   |
| Wild Type   | 70 ± 11        | 0.1 ± 0.01     | 700                                |                                   |
| T30A        | 1150 ± 80      | 1210 ± 120     | 0.9                                |                                   |
| T30V        | 810 ± 40       | 700 ± 50       | 1.1                                |                                   |
| Q47A        | 77 ± 17        | 11             | 11                                 |                                   |
| Q47M        | 61 ± 8         | 0.1 ± 0.02     | 610                                |                                   |
| T49A        | 140 ± 50       | 71 ± 25        | 2.0                                |                                   |
| T49V        | 1440 ± 130     | 1360 ± 90      | 1.1                                |                                   |
| T52A        | 10 ± 0.7       | 5.1            | 5.1                                |                                   |
| T52V        | 1130 ± 230     | 930 ± 130      | 1.2                                |                                   |
| S53A        | 44 ± 12        | 0.1 ± 0.04     | 440                                |                                   |
| S53C        | 148 ± 11       | 69 ± 8         | 2.1                                |                                   |

$^a$ Thr-30, Thr-49, Thr-52, and Ser-53 form side chain hydrogen bonds with l-tryptophan, whereas Gln-47 forms main chain hydrogen bonds with l-tryptophan (see Fig. 2).

$^b$ B. subtilis strains carried a null chromosomal mtrB (TRAP) allele, a trpE$^{-}$-lacZ translational fusion under control of the trp promoter and leader region, and a plasmid carrying the indicated mtrB (TRAP) allele. $\beta$-Galactosidase activity expressed from the trpE$^{-}$-lacZ fusion is given in Miller units. Values are the averages of three independent experiments ± S.D.

### Table III

**In vitro activities of mutant TRAP proteins**

| TRAP mutant | 1 μM TRAP | 15 μM TRAP | Equilibrium dialysis $^b$ | Gel shift analysis $^d$ |
|-------------|-----------|------------|---------------------------|------------------------|
|             | $S_{0.5}$ | $n$        | $S_{0.5}$ | $n$ | $K_s$ | $S_{0.5}$ | $n$ | $S_{0.5}$ | $n$ |
| WT          | 6.6 ± 0.1 | 1.7 ± 0.1  | nd $^e$   | 21 ± 4 | 17 ± 0.4  | 1.8 ± 0.1 | 1 ± 0.1 | 1.3 ± 0.2 |
| T30A        | NB $^f$   | 740 ± 140  | 340 ± 10 | 1.6 ± 0.1 | nd |
| T30V        | NB        | 72 ± 16    | 53 ± 1   | 2.0 ± 0.1 | nd |
| Q47A        | 71 ± 2    | 1.8 ± 0.1  | 25 ± 3   | 22 ± 1   | 1.3 ± 0.1 | 27 ± 0.5 | 2.1 ± 0.1 |
| Q47M        | 6.4 ± 2   | 2.3 ± 0.1  | 30 ± 3   | 26 ± 1   | 1.4 ± 0.1 | 2 ± 0.1 | 1.3 ± 0.1 |
| T49A        | NB        | 29 ± 4    | 25 ± 2   | 1.4 ± 0.1 | 50 ± 1 | 2.2 ± 0.1 |
| T49V        | NB        | 220 ± 6   | 1.7 ± 0.1 | 1.7 ± 0.1 | nd |
| T52A        | 385 ± 69  | 1.2       | 11 ± 2   | 10 ± 1   | 1.5 ± 0.1 | 18 ± 0.4 | 1.9 ± 0.1 |
| T52V        | 18 ± 1    | 1.7 ± 0.1  | 18 ± 2   | 17 ± 1   | 1.4 ± 0.1 | 11 ± 0.8 | 0.8 ± 0.04 |
| S53A        | 4.3 ± 0.7 | 2.1 ± 0.5  | 7 ± 1    | 7 ± 0.1   | 1.6 ± 0.1 | 0.4 ± 0.01 | 2.6 ± 0.1 |
| S53C        | 135 ± 35  | 92 ± 4     | 2.4 ± 0.2 | 72 ± 5   | 1.3 ± 0.1 |

$^a$ The levels of the fraction of bound l-tryptophan were fit to a cooperative binding equation to give binding constants ($S_{0.5}$).

$^b$ RNA gel mobility shifts were performed as a function of TRAP concentration ([TRAP]) or l-tryptophan concentration ([L-tryptophan]). Data for the [TRAP] assay was fit to both simple ($S_0.5$) and cooperative ($S_0.5$ and $S_0.5$) binding equations, whereas the [L-tryptophan] assay was only fit to a cooperative binding equation. Values are averages of at least two independent experiments ± S.E.

$^c$ Thr-30, Thr-49, Thr-52, and Ser-53 form side chain hydrogen bonds with l-tryptophan, whereas Gln-47 forms main chain hydrogen bonds with l-tryptophan (see Fig. 2).

$^d$ $n$, cooperativity (Hill) coefficient.

$^e$ nd, not determined.

$^f$ NB, no binding detected.
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DISCUSSION

Through the use of site-directed mutagenesis several mutant forms of TRAP were generated, each containing a different single amino acid substitution in the tryptophan binding pocket. The TRAP mutants were used to decipher the relative importance of various hydrogen bonds that form between TRAP and tryptophan. A trpE'-'lacZ translational fusion reporter construct was used to assess the amount of trp operon expression in B. subtilis strains containing wild type or mutant TRAP. Several classes of tryptophan binding mutations were identified in vivo having unchanged (Q47E and S53A), reduced (Q47A, Q47M, T52A, and S53C), or completely eliminated (T30A, T30V, T49A, T49V, and T52V) repression activity (Table II). The RNA binding activity of mutant TRAP proteins was examined in vitro using a gel mobility shift assay with purified wild type or mutant proteins. Three of the five TRAP mutants that were inactive in vivo (T30A, T49A, and T49V) exhibited a more than 10-fold reduction in affinity for trp leader RNA at a saturating tryptophan concentration (Table III). Thus, the functional defects observed in vivo for the remaining TRAP mutants could not be attributed to the inability to bind to trp leader RNA.

The affinity of wild type and mutant TRAP proteins for tryptophan was examined in vitro by equilibrium dialysis, gel mobility shift, and filter binding experiments (Figs. 3–5, Table III). We determined that the wild type and mutant TRAP proteins bound trp leader RNA in a cooperative manner, although the nature of this cooperativity is unknown. Cooperative RNA binding was not a result of the ligand dependence because the tryptophan-independent T30V mutant protein exhibited the same positive cooperativity (Table III). We also found that the affinities of the Q47E and S53A TRAP proteins for tryptophan were similar to wild type TRAP, consistent with the in vivo results where we observed wild type-like regulation (Table II). Several of the mutant proteins (Q47A, Q47M, T52A, and T52V) bound tryptophan with reduced affinity but did not interfere with RNA binding at saturating tryptophan concentrations (Table III), suggesting that the in vivo concentration of...
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tryptophan was too low to activate these mutant proteins (Table II). In contrast, the reduced affinity between tryptophan and other mutant TRAP proteins (T30A, T49A, T49V, and S53C) resulted in a substantial reduction in affinity for trp leader RNA (Table III). We found that a 10-fold decrease in TRAP affinity for tryptophan in vitro led to an almost complete loss of regulation in vivo, whereas increased affinity for tryptophan and RNA by the S53A mutant protein had little or no effect on the in vivo regulatory activity of TRAP. Taken together, our results indicate that the hydrogen bond between Ser-53 and tryptophan is dispensable for TRAP function, whereas the hydrogen bonds formed with Thr-30 and Thr-49 appear to be essential. In addition, we found that the interaction between TRAP and tryptophan was stabilized when bound to RNA (Fig. 5).

Many of the results of this study were not predicted. For instance in wild type TRAP, Gln-47 forms a natural hydrogen bond with tryptophan through a main chain interaction. Thus, substitutions at this position were not expected to affect TRAP function. However we found that only the conservative Q47E replacement did not result in functional defects. In addition, the S53C substitution was expected to maintain the natural hydrogen bond in contrast to the disrupting S53A substitution. However, we found that the hydrogen bond formed by Ser-53 was dispensable for tryptophan binding, because S53A substitution results in a wild type-like protein. The severe effect of the S53C substitution may be due to the more acidic nature of the cysteine sulphydryl group in comparison with the serine hydroxyl group. The partial ionization of the cysteine side chain could be deleterious for the interaction with the negatively charged carboxyl group of tryptophan.

The most surprising finding is that the T30V substitution results in the constitutive conformational change(s) required to allow TRAP interaction with RNA. It is possible that the valine side chain in the T30V mutant protein results in hydrophobic interactions with other amino acid residues normally not involved in binding the indole ring of tryptophan, thereby mimicking the tryptophan-bound conformation of wild type TRAP. This assumption is consistent with the observation that no tryptophan binding could be detected for this mutant by any of the techniques used in this study. Finally, whereas the dependence of tryptophan activation to allow TRAP interaction with RNA (Fig. 5).

The results obtained in vitro are consistent with each other, but there are some apparent discrepancies with the in vivo observations. We would have expected that the tryptophan-independent T30V mutant protein would have resulted in low expression in the presence or absence of tryptophan in vivo. Instead, this mutant strain exhibited high level constitutive expression. This protein was successfully overproduced in E. coli in soluble form and was as stable in vitro as wild type TRAP. Thus, the inability to repress trp operon expression in vivo was unlikely due to incorrect folding. It is possible that the artificially active conformation of this mutant protein caused a relaxation in its specificity in RNA recognition and a corresponding competition by the random cellular RNAs. However, the ability of this mutant protein to bind to a randomly chosen competitor RNA could not be demonstrated in vitro (data not shown).

An apparent discrepancy was also observed for T52V. This mutant protein had affinities for RNA and tryptophan that were similar to the T52A substitution at the same position; however, T52V resulted in the complete loss of in vivo repression, whereas T52A only exhibited reduced repression. Because T52V was the only substitution that resulted in negative cooperativity in tryptophan binding, the loss of cooperativity might be the reason for its severe defect in vivo. The opposite situation was observed for the S53C substitution. S53C retained some in vivo regulatory activity but exhibited greatly reduced affinities for both RNA and tryptophan in vitro. The reducing conditions used for purification and the in vitro assays for this mutant protein make it unlikely that the reduced affinities observed in vivo were due to oxidation. As was mentioned above, the defect of this mutant may be due to the partial ionization of its cysteine sulphydryl group. The pH used in the in vitro experiments (pH 8.0) may be more favorable for cysteine ionization than the pH in vivo.

Three-dimensional structures allow us to determine all protein-ligand contacts but are not capable of establishing the relative importance of these interactions. Our structure-function analysis using site-directed mutagenesis allowed us to obtain a substantial amount of information concerning the activation of TRAP by tryptophan and its role in regulating trp gene expression. Thus our study highlights the importance of testing predictions based on structural information.

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