The Anti-trp RNA-binding Attenuation Protein (Anti-TRAP), AT, Recognizes the Tryptophan-activated RNA Binding Domain of the TRAP Regulatory Protein*

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In *Bacillus subtilis*, the *trp* RNA-binding attenuation protein (TRAP) regulates expression of genes involved in tryptophan metabolism in response to the accumulation of l-tryptophan. Tryptophan-activated TRAP negatively regulates expression by binding to specific mRNA sequences and either promoting transcription termination or blocking translation initiation. Conversely, the accumulation of uncharged tRNA\(^{\text{trp}}\) induces synthesis of an anti-TRAP protein (AT), which forms a complex with TRAP and inhibits its activity. In this report, we investigate the structural features of TRAP required for AT recognition. A collection of TRAP mutant proteins was examined that were known to be partially or completely defective in tryptophan binding and/or RNA binding. Analyses of AT interactions with these proteins were performed using in vitro transcription termination assays and cross-linking experiments. We observed that TRAP mutant proteins that had lost the ability to bind RNA were no longer recognized by AT. Our findings suggest that AT acts by competing with messenger RNA for the RNA binding domain of TRAP. *B. subtilis* AT was also shown to interact with TRAP proteins from *Bacillus halodurans* and *Bacillus stearothermophilus*, implying that the structural elements required for AT recognition are conserved in the TRAP proteins of these species. Analyses of AT interaction with *B. stearothermophilus* TRAP at 60 °C demonstrated that AT is active at this elevated temperature.

The *trp* RNA-binding attenuation protein (TRAP)\(^1\) of *Bacillus subtilis* coordinates regulates expression of the genes of tryptophan metabolism in response to the intracellular level of free l-tryptophan (1). Six of the seven *trp* biosynthetic genes are clustered in the *trpECDFBA* operon, whereas the seventh, *trpG*, is located in the folate operon (2). TRAP also regulates expression of *yhaG* (3), a gene specifying a protein presumably involved in tryptophan transport, and possibly another gene, of unknown function, *ycbK* (4). The mechanisms by which tryptophan-activated TRAP regulates expression of all of these target genes depend on its ability to bind a specific RNA sequence, designated a TRAP binding site. The site is composed of multiple (G/U)AG repeats, generally separated by 2 nucleotides (5). By binding to this site in *trp* leader RNA, tryptophan-activated TRAP promotes formation of a terminator structure, as opposed to the alternative antiterminator structure (6). This causes transcription termination in the leader region upstream of the *trp* operon structural genes (7). TRAP binding has a second effect on *trp* operon expression; when bound to the leader segment of the *trp* operon read-through transcript, it stabilizes an RNA hairpin structure that sequesters the *trpE* Shine-Dalgarno sequence, reducing *trpE* translation (8, 9). This may influence expression of the entire operon, via translational coupling and transcriptional polarity (10). Regulation of *trpG*, and presumably *yhaG* and *ycbK*, also occurs at the translational level. In the translation initiation segments of these coding regions, the TRAP binding site overlaps the Shine-Dalgarno sequence; thus, TRAP binding would directly impede ribosome binding (3, 4, 11, 12).

The three-dimensional structures of tryptophan-activated *trp* (13, 14) and of TRAP bound to RNA (15) have been determined. TRAP interactions with tryptophan and with RNA have been studied in great detail (13–21). TRAP is composed of 11 identical subunits arranged as a doughnut-shaped molecule. Eleven tryptophan binding sites are formed by segments of adjacent TRAP subunits. Tryptophan binds to these 11 sites cooperatively. Tryptophan is believed to induce a conformational change on TRAP’s surface, enabling it to bind cooperatively to specific sequences in RNA TRP binding sites and wrap the messenger RNA around its periphery.

In many bacterial species, both the level of free tryptophan and the extent of charging of tRNA\(^{\text{trp}}\) are sensed as regulatory signals (22). *B. subtilis* also senses both of these signals (23), with the accumulation of uncharged tRNA\(^{\text{trp}}\) leading to the synthesis of the anti-TRAP protein, AT (24), via the T-box transcription antitermination mechanism (4, 25). When present, AT forms a complex with tryptophan-activated TRAP, inhibiting TRAP’s RNA binding ability and thereby abolishing TRAP regulation of gene expression.

To identify the structural features of TRAP necessary for AT binding, we examined mutant TRAPs known to have reduced ability to bind tryptophan and/or RNA (19, 20). We also examined one mutant TRAP that does not require tryptophan for RNA binding (20). Each mutant protein was initially tested for residual transcription termination activity, and was classified as lacking or retaining termination activity. These two classes of mutant TRAPs were then analyzed for their ability to interact with AT, using in vitro transcription assays and cross-

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* This work was supported by National Institutes of Health Grant GM62750 and National Science Foundation Grant MCB-9982652 (to P. G.), by National Institutes of Health Grant GM52840 (to P. B.), and by National Science Foundation Grant MCB-0093023 (to C. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TRAP, *trp* RNA-binding attenuation protein; AT, anti-TRAP; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl))ethylglycine.

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linking analyses. The findings obtained indicate that AT competes with RNA for the same TRAP surface; therefore, AT binding to TRAP appears to be dependent on TRAP forming its tryptophan-induced RNA binding domain. We also observed that the TRAP features required for AT interaction are conserved in the TRAP proteins from other bacilli. In addition, B. subtilis AT efficiently inhibited B. stearothermophilus TRAP at 60 °C.

EXPERIMENTAL PROCEDURES

Protein Purification—The AT protein was purified following a modification of the original procedure (24). E. coli strain BL21 (DE3) (Invitrogen), containing the expression vector pT7-7-TEV A (24), was grown overnight in LB medium supplemented with 100 μg/ml ampicillin. The sample was then equilibrated with buffer B. A 0.6- to 1.0-fold volume contained 412 μg of wild-type or mutant TRAP and 2.1 μM AT, in the presence or absence of 0.5 mM L-tryptophan, in 20 mM Tris-HCl, pH 8, 20 mM NaCl, 4 mM MgCl₂. After incubating at room temperature for 20 min, glutaraldehyde (0.5 μl of 1 M solution) was added and allowed to react for 5 min at room temperature. Reactions were terminated by adding SDS sample buffer and boiling for 1 min. Samples were electrophoresed on a 5% polyacrylamide, 7 M urea gel. Radiolabeled RNA bands were quantified with a phosphor imager (Molecular Imager System GS 363; Bio-Rad) and the standard assay are described in the figure legends.

In Vitro Transcription Termination Assay—In vitro transcription termination assays followed previously published procedures (6, 7). Transcription reaction mixtures (10 μl) contained B. subtilis vegetative (σ⁺) RNA polymerase, 10 nM DNA template, 340 nM TRAP protein (wild type, mutant, or from different bacilli species, depending on the experiment), 0.5 mM L-tryptophan, 20 units of RNasin (Promega), and ribonucleoside triphosphates (2.7 mM ATP, 0.7 mM GTP, 1.1 mM CTP, 1.4 mM UTP, 10 μCi = 370 Bq of [α-32P]UTP 3000 Ci/mmol). The TRAP variant protein and, when present, AT (various concentrations), were preincubated in the presence of tryptophan at 30 °C for 5 min. The remaining components were then added, and reactions were carried out at 30 °C for 30 min. Samples were electrophoresed on 10% polyacrylamide, 7 M urea gel. Radiolabeled RNA bands were quantified with a phosphor imager (Molecular Imager System GS 363; Bio-Rad) and the standard assay are described in the figure legends.

AT-TRAP Cross-linking—Cross-linking experiments with glutaraldehyde followed a published procedure (24). Reaction mixtures (20-μl final volume) contained 412 μg of wild-type or mutant TRAP and 2.1 μM AT, in the presence or absence of 0.5 mM L-tryptophan, in 20 mM Tris-HCl, pH 8, 20 mM NaCl, 4 mM MgCl₂. After incubating at room temperature for 20 min, glutaraldehyde (0.5 μl of 1 M solution) was added and allowed to react for 5 min at room temperature. Reactions were terminated by adding SDS sample buffer and boiling for 1 min. Samples were electrophoresed on 10% polyacrylamide, 7 M urea gel. Radiolabeled RNA bands were quantified with a phosphor imager (Molecular Imager System GS 363; Bio-Rad) and the standard assay are described in the figure legend.

RESULTS

AT Interaction with TRAP Mutant Proteins—From a collection of previously characterized TRAP mutant proteins (19, 20), we selected a representative panel for examination. Proteins T25A, T30A, and T49A have alterations of residues that participate directly in tryptophan binding (Fig. 1, A and B). Proteins E36A, K31A, and K40A have alterations of residues crucial for tryptophan binding; however, these residues do not contact tryptophan directly. Each of these mutant proteins was shown previously to be partially or completely defective in RNA
binding when tested in vitro (19, 20). Proteins K37A, K56A, and R58A and the doubly altered protein K37A/R58A have residue changes in the KKR RNA binding motif of TRAP (Fig. 1, A and B). These mutant proteins bind tryptophan as well as wild type TRAP, but they bind RNA with reduced affinity, or not at all in the case of the double mutant protein. Proteins E42A and E60A have residue changes on opposite surfaces of TRAP and, aside from a slightly increased cooperativity of tryptophan binding, they do not show significantly altered affinity for either tryptophan or RNA (19). Mutant K71A also has a substitution on the surface of the protein. This change has no major effect on the characteristics of the protein (19). The T30V substitution permits the TRAP protein to bind RNA in the absence of added tryptophan; however, this mutant protein has a lower affinity for RNA than does the wild type protein (20).

Preliminary examination of the properties of each single mutant protein in this selected panel, using the in vitro transcription termination assay, allowed us to identify two major classes: class I, mutant proteins that lack transcription termination activity (Table I), and class 2, mutant proteins that retain low to moderate transcription termination activity (Table II). To analyze AT interaction with these mutant proteins, we used two different methods: in vitro transcription assays and glutaraldehyde cross-linking analyses. For the first class (Table I), inactive mutant proteins impaired in tryptophan binding and/or RNA binding, we determined whether they could interact with the AT protein and prevent AT from inhibiting wild type TRAP in the in vitro transcription assay (Table I, Fig. 2A). None of these mutant proteins was capable of preventing AT from blocking wild type TRAP activity. This class of TRAP proteins also was subjected to cross-linking with AT in the presence and absence of tryptophan (Table I, Fig. 3). In the absence of tryptophan, none of these mutant proteins could form a complex with AT. In the presence of tryptophan, mutant proteins impaired in tryptophan binding were still incapable of forming a complex with AT (with the exception of a weak complex formed by E36A). However, mutant proteins with reduced affinity for RNA but not for tryptophan did form a complex with AT, yet, except for K56A, the interaction was weaker when compared with the wild type TRAP plus AT. The double mutant protein, K37A/R58A, which completely lacks RNA binding ability but shows normal tryptophan binding, did not form a complex with AT. These findings establish that AT recognizes a TRAP structural feature that is associated with the binding of tryptophan. Since mutant TRAP interaction with AT is still possible when there is a single substitution in the KKR motif but is abolished when two residues are substituted, we conclude that AT recognizes the same tryptophan-activated surface of TRAP that is involved in RNA binding.

Mutant proteins in class 2, those that retain transcription termination activity (Table II), were examined in the in vitro transcription termination assay to determine their relative susceptibility to AT inhibition (Table II, Fig. 2B). They were also subjected to cross-linking analyses with AT, in the presence and absence of tryptophan (Fig. 3). In both series of experiments, we found that the ability to interact with AT correlated with the termination activity of the mutant protein. For example, mutant TRAPs E42A, E60A, and K71A, which retain tryptophan and RNA binding ability similar to that of wild type TRAP, displayed transcription termination activity similar to that of wild type TRAP. They also interacted with AT as well as wild type TRAP. In all mutants of this class, AT-TRAP complex formation was again tryptophan-dependent. It was surprising that K31A (Table II), a protein previously characterized as being totally impaired in tryptophan and RNA binding in vitro, showed weak termination activity and AT interaction in our assays. We believe this is due to the fact that our assays may be more sensitive than the procedures initially used to characterize these mutant proteins (19, 20). Similarly, the weak AT complex formation observed in the cross-linking experiment with E36A (Table I) may be due to a reduced but not eliminated capacity of this mutant protein to bind tryptophan.

We also examined the interaction between AT and protein T30V. This mutant TRAP shows tryptophan-independent RNA binding (20). Indeed, this protein displayed efficient transcription termination activity in the in vitro transcription assay, in the absence of tryptophan (Fig. 4). A 15-fold molar excess of AT, a concentration that completely abolishes wild type TRAP activity, had no effect on T30V termination activity, either in the presence or absence of tryptophan. However, a 50-fold molar excess of AT over T30V TRAP did partially inhibit its activity, with tryptophan addition enhancing this inhibition. T30V was also subjected to cross-linking analysis with AT, without success (data not shown). Presumably, the low AT/T30V TRAP molar ratio employed in this procedure (5:1) is insufficient to produce stable interaction. Thus, the T30V substitution allows this mutant TRAP to terminate transcription and to bind AT in the absence of tryptophan, but the affinity of the protein for RNA and for AT is reduced.

\(^{2}\) P. Gollnick, unpublished observation.
AT-TRAP Interaction

**TABLE II**

*AT interaction with TRAP mutant proteins that retain transcription termination activity*

| Mutant TRAP | Characteristics | In vitro transcription analysis | Cross-linking analysis* |
|-------------|----------------|-------------------------------|------------------------|
|             | Trp binding<sup>b</sup> | RNA binding<sup>b</sup> | Termination activity with AT<sup>c</sup> | (AT/Trp complex formation) |
| Wild type   | wt<sup>d</sup> | wt | ++ + + | – Trp |
| E42A        | ~ wt<sup>e</sup> | ~ wt | ++ + + | – + + + |
| E60A        | ~ wt | ~ wt | ++ + + | – + + + |
| K71A        | ~ wt | ~ wt | ++ | – + |
| K31A        | NB<sup>f</sup> | NB | + | – + |
| K40A        | Reduced affinity | Reduced affinity | + + + | – + + + |

<sup>a</sup> See legends of figs. 2 and 3 for assay procedure.
<sup>b</sup> See Refs. 19 and 20.
<sup>c</sup> Susceptibility to AT inhibition.
<sup>d</sup> wt, wild type.
<sup>e</sup> ++ + +, depending on the analysis, high level of termination activity, inhibition by AT, AT-TRAP complex formation (similar to that of wild type TRAP).
<sup>f</sup> –, no complex formation with AT.
<sup>g</sup> – wt, binding similar to that of wild type TRAP.
<sup>h</sup> ++, reduced level of termination activity, compared with that of wild type TRAP.
<sup>i</sup> NB, no binding detected.
<sup>j</sup> –, depending on the analysis, very reduced level of termination activity, inhibition by AT, AT-TRAP complex formation, compared with that of wild type TRAP.

**Fig. 2. In vitro transcription analysis of AT interaction with TRAP mutant proteins.** Two representative gels are shown. A, TRAP mutant proteins that lack transcription termination activity (Table I) were tested for their ability to prevent AT from inhibiting wild type TRAP. A mixture containing tryptophan (0.5 mM), mutant TRAP (340 nM), and AT (5-fold molar excess) was preincubated at 30 °C for 5 min, and then wild type TRAP (340 nM) was added. After 1 min, the transcription reaction was started. The reaction was carried out at 30 °C for 10 min. B, TRAP mutant proteins that retain termination transcription activity (Table II) were tested for their susceptibility to AT inhibition. Each mutant protein (340 nM) was tested in the presence of 0.5 mM tryptophan and various amounts of AT (AT concentration is expressed as molar excess over that of the mutant TRAP). Numbers at the bottom of the gel indicate the percentage of transcripts that are terminated (%T) in each lane. RT, read-through transcript; T, terminated transcript.

**Fig. 3. Cross-linking analysis of AT interaction with TRAP* mutant proteins.** A representative gel is shown. 412 nM wild type or mutant TRAP was examined for cross-linking to AT (2.1 μM) with 0.2% glutaraldehyde in the presence or absence of 0.5 mM tryptophan. The resulting complex was analyzed on a 4–20% gradient SDS-polyacrylamide gel. M, molecular size standards in kDa, as shown at right.

**AT Competes with trp Leader RNA in Binding to TRAP**—The results obtained from the analysis of the TRAP mutant proteins suggested that AT directly interferes with TRAP’s ability to recognize its target RNA sequences by masking its RNA binding site. To corroborate this finding, we performed RNA gel retardation assays with wild type TRAP, AT, and the trp leader RNA. We compared the outcome of two sets of reactions in which the same concentrations of proteins were employed. In one set, TRAP was first incubated with AT, and subsequently the RNA was added to the reaction mixture (Fig. 5, set A). In the comparison set, the order of AT and RNA addition was reversed (Fig. 5, set B). The results clearly indicate that once TRAP is bound to RNA, AT is not as effective in abolishing the
gel shift caused by TRAP as when AT is incubated with TRAP prior to RNA addition. The percentage of free RNA is lower in the reaction without AT of set B, compared with that of set A (9% versus 24%). This is due to the fact that in the former, RNA and TRAP were incubated together for a total of 20 min, whereas in the latter they were incubated for 10 min.

**AT Interaction with TRAP Proteins from Other Bacilli**—We also examined the ability of AT from *B. subtilis* to recognize TRAP proteins from two additional bacilli, *B. halodurans* and *B. steaothermophilus*. These TRAP proteins have high homology to *B. subtilis* TRAP (71 and 81% amino acid sequence identity, respectively) (Fig. 1A). The residues crucial for tryptophan binding are conserved as well as residues KKR of the RNA binding domain (Fig. 1A). Homology is lower at the amino- and carboxyl-terminal ends of the protein. The three-dimensional structure of *B. steaothermophilus* TRAP in complex with tryptophan has been determined (14). Comparison with the structure of *B. subtilis* TRAP reveals that, although the central cavities in the two 11-subunit complexes have different sizes and opposite charge distributions, tryptophan binding and RNA binding are likely to proceed by the same mechanisms.

*B. halodurans* TRAP was tested in the *in vitro* transcription termination assay and it proved to be very effective in causing termination in the presence of tryptophan (Fig. 6A). AT recognized *B. halodurans* TRAP very efficiently; a 5-fold molar excess of bound TRAP free, RNA free of bound TRAP. Numbers at the bottom of the gel indicate the percentage of RNA that is free in each lane.

The 140-nucleotide leader RNA (data not shown). We confirmed that AT can distinguish between the inactive and tryptophan-activated conformations of TRAP and that it recognizes the latter exclusively. With only two exceptions, all of the mutant and wild type TRAP proteins examined were incapable of interacting with AT in the absence of tryptophan. The two exceptions were the tryptophan-independent mutant protein T30V and *B. steaothermophilus* TRAP when tested at 30 °C. The proteins T25A, T30A, and T49A, known to be completely impaired in tryptophan binding and/or RNA binding (19, 20). In addition, we examined TRAP proteins from two other bacilli, *B. halodurans* and *B. steaothermophilus* (14). This collection of mutants and wild type TRAP proteins has alterations scattered throughout the TRAP polypeptide chain (Fig. 1A). We analyzed the interaction of each of these proteins with *B. subtilis* AT in an effort to define more precisely the residues within the TRAP protein that are essential for AT binding and AT inhibition.

We confirmed that AT can distinguish between the inactive and tryptophan-activated conformations of TRAP and that it recognizes the latter exclusively. With only two exceptions, all of the mutant and wild type TRAP proteins examined were incapable of interacting with AT in the absence of tryptophan. The two exceptions were the tryptophan-independent mutant protein T30V and *B. steaothermophilus* TRAP when tested at 30 °C. The proteins T25A, T30A, and T49A, known to be completely impaired in tryptophan binding, did not interact with AT, even in the presence of tryptophan. The structure of the tryptophan-independent T30V TRAP was hypothesized to have valine-induced changes that allow this mutant protein to undergo a conformational activation comparable with that normally attained in the presence of tryptophan (20). AT did indeed recognize the activated form mimicked by the T30V protein in the absence of tryptophan. Nonetheless, the affinity of T30V TRAP for AT was lower compared with the affinity of tryptophan-activated wild type TRAP (this is also true for RNA affinity). Tryptophan addition improved AT recognition, sug-

**FIG. 5.** Gel retardation analysis of AT and trp RNA interactions with TRAP. The 140-nucleotide trp leader RNA containing the TRAP binding site, labeled by *in vitro* synthesis, was incubated with TRAP and AT. The samples were loaded onto a native polyacrylamide gel. In A, TRAP and AT were first mixed and incubated for 10 min, and then RNA was added, and the mixture was incubated for an additional 10 min before loading on the gel. In B, TRAP and RNA were first mixed and incubated for 10 min, and then AT was added, and the mixture was incubated for an additional 10 min before loading on the gel. Bound, RNA bound to TRAP; free, RNA free of bound TRAP. Numbers at the bottom of the gel indicate the percentage of RNA that is free in each lane.

**FIG. 6.** *In vitro* transcription analysis of AT interaction with the TRAP proteins from *B. halodurans* and *B. steaothermophilus*. Purified TRAP proteins from *B. halodurans* (A) and *B. steaothermophilus* (B), 340 ns each, were tested in the *in vitro* transcription termination assay in the presence or absence of AT and 0.5 mM tryptophan. The concentration of added AT is expressed as molar excess over that of the TRAP protein. Numbers at the bottom of the gel indicate the percentage of transcripts that are terminated (%T) in each lane. RT, read-through transcript; T, terminated transcript. , the percentage of terminated transcripts was calculated ignoring the highest, shifted band. In B, the samples shown in the last three lanes were purified by phenol extraction prior to loading on the gel.
gesting that the T30V mutant protein only partially mimics tryptophan-activated TRAP, and that tryptophan can still bind and stabilize a more active conformation.

We demonstrated that AT recognizes the tryptophan-induced RNA binding domain of TRAP. TRAP mutant proteins carrying the substitution K37A, K56A, or R58A bind tryptophan as well as wild type TRAP does. However, since one of the residues of the KKR RNA binding motif is altered, the conformational change induced by bound tryptophan does not allow strong RNA binding. In fact, the K37A/R58A double mutant protein does not bind RNA at all. Similarly, AT could still recognize the K37A, K56A, and R58A single mutant proteins but did not interact with the double mutant protein. The K37A and R58A proteins did not cross-link to AT as well as K56A did, suggesting that the residues altered in these two mutant proteins are especially crucial for AT recognition. Interactions between the K37A, K56A, and R58A mutant proteins and AT were observed in cross-linking analyses but not in in vitro transcription assays. This may indicate that the interactions are transient and are not stable enough to sequester AT and prevent its action on wild type TRAP in the in vitro transcription experiments. Band shift analyses with wild type TRAP and trp leader RNA provided independent evidence establishing that AT and RNA compete for the same surface of TRAP. Thus, it appears that AT is designed to recognize and bind to the tryptophan-activated RNA binding domain of TRAP and thereby prevent TRAP from binding to RNA.

In agreement with these observations, we found that the structural features recognized by AT are conserved in the TRAP proteins from B. halodurans and B. stearothermophilus: AT efficiently inhibited both proteins. The structure of B. stearothermophilus TRAP is known (14), and it was postulated that its RNA binding surface is very similar to the comparable surface of B. subtilis TRAP (14). The three-dimensional structure of TRAP bound to RNA was in fact solved using B. stearothermophilus TRAP (15). From our results, it also appears that residues in the variable regions of TRAP, at its amino and carboxyl-terminal ends, are not crucial for AT recognition, since they are not for RNA binding (14). We tested AT action at 60 °C with the thermostable B. stearothermophilus TRAP and observed that AT was active at this temperature.

Acknowledgments—We thank Min Yang and Pan Li for providing purified TRAP mutant proteins and Mirela Milescu for the purification of B. stearothermophilus TRAP. We thank Alexander Yakhnin for providing purified TRAP mutant proteins and for interest in this project. We thank Alfred Antson for preparing Fig. 1B.

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