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Alanine Scanning Mutagenesis of Anti-TRAP (AT) Reveals Residues Involved in Binding to TRAP

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SUMMARY

The *trp* RNA-binding attenuation protein (TRAP) regulates expression of the tryptophan biosynthetic (*trp*) genes in response to changes in intracellular levels of free L-tryptophan in many gram positive bacteria. When activated by binding tryptophan, TRAP binds to the mRNAs of several genes involved in tryptophan metabolism, and down-regulates transcription or translation of these genes. Anti-TRAP (AT) is an antagonist of TRAP that binds to tryptophan-activated TRAP and prevents it from binding to its RNA targets, and thereby up-regulates *trp* gene expression. The crystal structure shows that AT is a cone-shaped trimer (AT₃) with the N-terminal residues of the three subunits assembled at the apex of the cone and that these trimers can further assemble into a dodecameric (AT₁₂) structure. Using alanine-scanning mutagenesis we found four residues, all located on the “top” region of AT₃, which are essential for binding to TRAP. Fluorescent labeling experiments further suggest that the top region of AT is in close juxtaposition to TRAP in the AT-TRAP complex. *In vivo* studies confirmed the importance of these residues on the top of AT in regulating TRAP mediated gene regulation.

Keywords

protein-protein interactions; RNA-binding protein; gene regulation; transcription attenuation; protein oligomerization; tryptophan genes

INTRODUCTION

In many gram positive bacteria, an RNA binding protein called TRAP (*trp* RNA-binding attenuation protein) regulates expression of genes involved in tryptophan metabolism (*trp*) in response to changes in intracellular levels of free L-tryptophan^{1;2;3}. TRAP down-regulates both transcription and translation of the *trp* genes by binding to its RNA targets in response to elevated levels of tryptophan³. An antagonist of TRAP, termed anti-TRAP (AT), has been identified in only a subset of the TRAP-containing bacilli including *B. subtilis*, *B. licheniformis*^{4;5}, *B. mojavensis* and *B. spizizenii* (P. Gollnick unpublished observations). AT binds to tryptophan-activated TRAP and prevents it from binding to its RNA targets, and thus up-regulates expression of the *trp* genes^{4;6}. Synthesis of AT is induced by accumulation of

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uncharged tRNA^{Trp}, however, neither tryptophan nor uncharged tRNA^{Trp} appear to bind to AT directly⁷.

Tryptophan-activated TRAP binds to mRNA targets that contain multiple (9 to 11) NAG triplet repeats (N = G ≈ U > A > C) usually separated by two or three nonconserved “spacer” nucleotides^{2; 3}. TRAP regulates transcription of the *trpEDCFBA* operon by binding to the 5' leader region of the nascent *trp* transcript⁸. This binding promotes formation of an intrinsic transcription terminator that halts transcription within the leader region prior to the coding sequence of *trpE*⁹. There is also a stem-loop structure (5'SL) at the 5' end of the leader region of *trp* operons regulated by TRAP²⁴ (J. Simon and P. Gollnick unpublished observations). Moreover, studies by Babitzke and coworkers have shown that the 5'SL is important for proper TRAP mediated control of transcription of the *trp* operon²⁴. Translation of *trpE*^{10; 11}, *trpG*^{12; 13}, *trpP*^{14; 15}, and *ycbK*¹⁶ is also regulated by TRAP. TRAP binding to *trp* operon transcripts that have escaped transcriptional control promotes formation of a stem-loop structure that sequesters the Shine-Dalgarno (SD) sequence of *trpE*, thus inhibiting translation initiation of this gene^{3; 10}. TRAP down regulates translation of *trpG*, *trpP* and *ycbK* by directly competing with ribosomes for binding to the translation start region of these genes³.

TRAP consists of 11 identical 75-residue subunits arranged in a symmetrical ring^{17; 18; 19}. The secondary structure of TRAP is composed of 11 β-sheets, each containing 7 antiparallel β-strands; three strands from one subunit and four from the adjacent subunit¹⁸. 11 tryptophan-binding pockets are formed at the interfaces between adjacent TRAP subunits¹⁸. Structures of tryptophan-activated TRAP in complex with RNAs containing either 11 GAG or 11 UAG repeats show that the single-stranded RNA wraps around the outer perimeter of the protein ring and interacts primarily with residues Glu36, Lys37, Lys56 and Arg58^{17; 20; 21; 22}. The residues that are involved in binding tryptophan and those that bind to the RNA are conserved in all currently available TRAP sequences²³.

AT is a 53-residue polypeptide encoded by the *rtpA* gene, which is the first gene of the two-gene *at* operon⁴. The other gene in this operon, *ycbK*, is located downstream of *rtpA* and encodes a protein that may be involved in tryptophan transport¹⁶. Both transcription and translation of *rtpA* are regulated in response to accumulation of uncharged tRNA^{Trp}. Transcription of the *at* operon is regulated by the T-box antitermination mechanism, in which accumulation of uncharged tRNA^{Trp} enhances transcription of *rtpA*^{16; 25} and ultimately increases expression of the *trp* genes²⁶. Translational control of *rtpA* expression involves a 10-codon open reading frame, *rtpLP*, located in the 5' leader region upstream of *rtpA*^{7; 27}. Efficient translation through three consecutive Trp codons in *rtpLP* allows the ribosome to reach the stop codon, which is 6 nucleotides upstream of the SD sequence of *rtpA*³. This positioning of the *rtpLP*-translating ribosome interferes with translation initiation of *rtpA*²⁷. In contrast, if the translating ribosome stalls on one of the three Trp codons in *rtpLP* due to insufficient levels of charged tRNA^{Trp}, the *rtpA* SD sequence remains exposed and *rtpA* is translated efficiently^{3; 27}.

The amino acid sequence of AT contains two CXXCXGXG (X is any amino acid) Zn-binding motifs similar to those found in the type I family of Hsp40 molecular chaperones⁴. The cysteine residues in these motifs, and the presence of bound zinc are required for AT stability and function²⁸. Crystal structures of *B. subtilis* AT^{26;29} and *B. licheniformis* AT (M. Shevetsov and A. Antson unpublished data) reveal that AT assembles into cone-shaped trimers (AT₃) (Figure 1B). The N-terminal residues of the three subunits assemble at the apex, or “top” of the cone, and three zinc-binding domains extend down toward the base, or “bottom” of the cone. Four AT trimers further associate into dodecamers (AT₁₂) via weak trimer-trimer interactions²⁶. Analytical ultracentrifugation studies showed that AT exists in a reversible equilibrium between trimers and dodecamers in solution, and provided evidence for an AT-

TRAP complex composed of 12 AT subunits and 11 TRAP subunits³⁰. No complexes with mass corresponding to AT₃-TRAP₁₁, AT₆-TRAP₁₁, or AT₉-TRAP₁₁ were detected suggesting that AT binds to TRAP as a preformed dodecamer or that 4 AT trimers bind cooperatively³⁰. However, physiological concentrations of AT are much lower than those required for AT₁₂ formation *in vitro*³¹, suggesting that AT may function as a trimer *in vivo*.

Studies of AT binding to mutant TRAP proteins indicate that TRAP residues involved in binding RNA are also recognized by AT⁶. However, the residues of AT that are involved in binding to TRAP have not been identified. To identify these residues, we individually substituted 26 (including 21 surface residues) of the 53 amino acid residues of AT with alanine, and examined binding of the resulting mutant proteins to TRAP. Our results showed that four residues located on the “top” of the AT₃ cone structure are important for binding to TRAP. The presence of tryptophan-activated TRAP interfered with the labeling of three different residues located near the top of AT₃ with the fluorescent dye 5-Iodoacetamidofluorescein (5-IAF) indicating that these residues are less solvent exposed in the AT-TRAP complex than in free AT. In addition, *in vivo* studies of the activity of mutant AT proteins in *B. subtilis* confirmed the importance of residues near the top of AT₃ in AT function. Together, these studies indicate that residues located on the top of the AT₃ structure are directly involved in AT binding to TRAP. Based on this conclusion and on results from prior studies of the AT-TRAP interaction^{4; 26; 30} we propose a model for the AT-TRAP complex in which 4 AT trimers bind to a TRAP 11mer. In this model each AT trimer binds at or near the RNA binding region of TRAP and thus AT binding is predicted to simply sterically interfere with RNA binding to TRAP.

RESULTS

Alanine-scanning of the surface residues of AT

In this study we utilized AT from two *Bacillus* species, *B. subtilis* and *B. licheniformis*. These two proteins show 66% sequence identity over their 53 amino acid length (Figure 1)²⁶ and both proteins assemble into very similar trimer structures (A. Antson and M. Shevetsov personal communication). Preliminary studies showed that AT from *B. licheniformis* was expressed to higher levels in *E. coli* than the *B. subtilis* protein. This difference was found to be due to a large number of rare codons in the *B. subtilis* gene (C. McElroy and M. Foster, Ohio State University). We performed our initial alanine scanning studies with *B. licheniformis* AT, which allowed easier production and purification of the mutant proteins. The sequence and structural similarities between the proteins from both bacilli suggest that information obtained from studying *B. licheniformis* AT will apply to both systems. This hypothesis was confirmed by making and testing several key Ala substitutions in *B. subtilis* AT.

To probe which amino acid residues of AT are critical for binding to TRAP, we performed alanine scanning mutagenesis of residues with solvent exposed side chains on the surface of the AT₃. These residues are the best candidates for being involved in direct interactions with TRAP and substitution of these residues is unlikely to disrupt the structure of AT₃. Residues with buried side chains or those involved in monomer-monomer interactions in AT₃ were not substituted so as to avoid disrupting the protein's structure. We also avoided changing the cysteines or glycines in the two CXXCXGXG Zn-binding domains (Figure 1A) because previous studies have shown that the bound Zn is required for AT structure and function²⁸. Consistent with this strategy, size exclusion chromatography and CD studies indicate that neither the subunit assembly nor the overall folding was significantly affected in any of the Ala-substituted AT proteins that we studied (See Below).

Residues on top of AT trimer are important for binding TRAP

WT AT and 21 alanine-substituted AT proteins from *B. licheniformis* were tested for binding to tryptophan-activated TRAP. WT AT binds to TRAP with an observed K_d of 3.1 μM (Table 1). This value appears to be high in view of the maximal *in vivo* levels AT having been measured to be approximately 100 nM³¹. The reason for this discrepancy is unclear; nevertheless it represents a useful value to compare to the Ala-substituted AT proteins. Four of the alanine-substituted AT proteins, V02A, I03A, D06A, and D07A showed greater than 20-fold lower affinity for TRAP than WT AT (Table 1), suggesting that Val02, Ile03, Asp06 and Asp07 play important roles in binding to TRAP. The remaining alanine substituted proteins bound TRAP with no more than 3-fold lower affinity than WT AT (Table 1). The four residues implicated in TRAP binding are all located on the top of the AT trimer structure (Figure 2A), suggesting that this region of AT is critical for binding to TRAP.

We made several additional substitutions of these critical residues on the top of AT₃ to further probe the requirements of the side chains at these positions for interacting with TRAP. Changing Val02 to alanine eliminated detectable binding to TRAP (Table 1). This substitution shortens the side-chain and removes its β -branch suggesting that the side chain at position 2 needs to be hydrophobic and either larger than alanine or β -branched. To examine the importance of these features of residue 2 for binding to TRAP, we substituted Val02 with leucine, which is one methyl group larger than valine and maintains branching of the side chain. The V02L mutant protein also showed no detectable binding to TRAP (Table 1) suggesting a strict requirement for Val at residue 2. These observations suggest a tight fit of the two proteins at this location and a need for specific hydrophobic interaction involving the Val side chain.

Replacing Asp06 or Asp07 with alanine reduced the affinity of AT for TRAP by 20–30 fold (Table 1). These substitutions remove the charged polar side chains of the residues at position 6 or 7. We have found that the stability of the AT-TRAP complex is not sensitive to changes in ionic strength, suggesting that ionic interactions are not crucial for stabilizing this interaction (Y. Chen and P. Gollnick, unpublished observations). Together these observations suggest that a polar side chain may be required at residues 6 and 7. To test this possibility we replaced Asp06 and Asp07 with Serine and Threonine respectively, both of which have uncharged polar side chains. In both cases these substitutions were more deleterious than alanine replacement, and eliminated measurable binding to TRAP (Table 1). These data again suggest that a strict requirement of side chains of specific residues (in this case Asp06 and Asp07) on the top region of AT₃ is needed for binding to TRAP.

The Zn-binding arms and the bottom region of AT₃

AT contains two cysteine-rich CXXCXGXG motifs with sequence⁴ and structural²⁶ similarity to the Zn-binding domain of the type I family of Hsp40 molecular chaperones (Figure 1A)^{26; 32}. The Zn-binding domains of members of the Hsp40 family, including *E. coli* DnaJ^{26; 32} and Ydj1 of yeast³³, have been implicated in interacting with other proteins. Hence a model has been proposed for the interaction of AT with TRAP that involves a direct role of the Zn-binding domains of AT²⁶. However, alanine substitution for any residues in this part of AT including 20–25 of the β -hairpin (β_2 – β_3) at the end of the Zn-binding arm; in the small loop adjacent to the Zn-binding domain (Asn14 or Asn16); or in the β -sheet (β_1 – β_4) between the Zn-binding domain and the α -helical core of the protein (Thr10 or Ile35), had little or no effect on AT binding to TRAP (Table 1 and Figure 2B). These observations suggest that the side chains of residues located on the Zn-binding arms of AT do not directly interact with TRAP. However, these data do not rule out the possibility that the Zn-binding domains of AT interact with TRAP via their main-chain atoms.

Side chains of several residues on the bottom of AT₃, including Ile51, His52 and Glu53 are solvent exposed (Figure 2B) and are thus potential candidates for interacting with TRAP. However, alanine substitution of any of these three residues had no significant effect on the affinity for TRAP (Table 1).

Alanine substitution of non-surface residues of AT

The crystal structure of AT indicates that each monomer has a hinge formed by the small antiparallel β -sheet composed of residues 9–11 (β_1) and 34–36 (β_4) (Figure 1B), which suggests that the Zn-binding arm may be flexible relative to the coil-coil core of the trimer²⁶. The side chains of a number of residues, including Glu09, Lys32, Leu36, Lys49, and His50, are located between the arm and the trimer core (Figure 2C) suggesting that these residues might be involved in stabilizing the local structure of AT₃, which could be important for TRAP recognition. To test this hypothesis, we made alanine substitutions for these residues and examined the binding of resulting mutant proteins to TRAP. Two of these mutant proteins, E09A and H50A, showed significantly reduced binding to TRAP (Table 2). The side chains of Glu09 from the Zn-binding arm of one subunit and His50 from the helical core of the adjacent subunit form a hydrogen bond (Figure 2C)²⁶. Hence substituting either of these residues with alanine might destabilize local structure of AT by eliminating this hydrogen bond, and this could consequently alter the surface structure of AT recognized by TRAP. Consistent with this hypothesis, alanine substitution of Glu09 or His50 in AT resulted in either 11-fold reduced binding affinity or in no detectable binding to TRAP respectively (Table 2). However, the observed differences in the TRAP binding activity of these two mutant proteins suggests that the Ala substitutions affect more than just formation of the hydrogen bond between these two residues.

Alanine substitution of Lys32, Leu36 or Lys49 in this region of AT did not significantly alter binding to TRAP (Table 2). The side chains of these residues are not completely buried between the arm and the AT₃ core, and they appear to be less critical than His50 and Glu09 in stabilizing the local structure necessary for TRAP recognition.

Analysis of the secondary and quaternary structure of alanine substituted AT proteins

One potential explanation for the reduced TRAP binding activity seen for the four alanine substituted AT proteins we identified is that these substitutions could disrupt the overall secondary structure of AT rather than directly affect residues that interact with TRAP. To examine this possibility we compared the CD spectra of WT AT and several of the mutant proteins that do not bind TRAP (Figure 3). The spectrum of WT AT shows negative peaks at 208 nm and 222 nm, as well as a positive peak at 195 nm, which are typical for proteins that are largely alpha helical, such as AT (Figure 1)³⁴. The spectra of four mutant AT proteins with alanine substitutions on the top of AT₃ (V02A, I03A, D06A, and D07A) are all very similar to that of WT AT (Figure 3), suggesting that these substitutions did not significantly alter the overall secondary structure of AT. Other mutant AT proteins, including N16A, R20A, E22A, E24A, P25A, S41A and I51A, also had very similar spectra as WT AT (Data not shown). Only two mutant AT proteins, E09A (Figure 3) and H50A (data not shown), showed slightly diminished signal between 195 and 215 nm, which suggests that these mutant proteins contains more random coil than WT AT. As described in the previous section, there is a hydrogen bond between the side chains of Glu09 and His50 in the C-terminal α -helix of AT (Figure 2C). Our CD data are consistent with the proposal that this hydrogen bond is involved in maintaining the local structure of AT, which appears to be important for TRAP binding.

Proper oligomerization of AT is likely critical for binding to TRAP. AT has been shown to exist as both trimers (AT₃) and as dodecamers (AT₁₂) in solution³⁰. Thus alanine substitutions that diminish the activity of AT could do so by altering oligomerization. We therefore examined

the properties of WT and mutant AT proteins on size exclusion chromatography. WT AT (118 μ M AT₃) showed a predominant peak at 8.3 minutes after injection and a smaller peak at 9.5 minutes (data not shown). Based on protein MW standards, these times correspond to MWs of 74.6 kDa and 17.9 kDa respectively, which are close to the predicted MWs of AT₁₂ (67.2 kDa) and AT₃ (16.8 kDa). All mutant AT proteins that we examined, including V02A, I03A, D07A and D07A, which showed diminished affinity for TRAP, as well as E09A, N16A, R20A, E22A, E24A, P25A, S41A, H50A and I51A showed very similar elution patterns as WT AT (data not shown). These results suggest that alanine substitution did not dramatically alter the quaternary structure of these proteins as compared to WT AT under the conditions of these experiments.

Fluorescent labeling AT

The results of alanine substitution studies identified four residues on the top region of AT₃ that are critical for binding to TRAP (Table 1 and Figure 2). A simple interpretation of these observations is that the top region of AT₃ interacts directly with TRAP. However, it is also possible that the deleterious effects of alanine substitution of residues Val02, Ile03, Asp06 and Asp07 are indirect. To further investigate which region of AT directly interacts with TRAP, we examined the solvent accessibility of several surface residues of AT when the protein was free as compared to when it was in complex with TRAP. To do so we used the fluorescent dye 5-Iodoacetamidofluorescein (5-IAF), which reacts with free sulfhydryl groups³⁵. WT AT contains four Cys residues but showed very low reactivity with 5-IAF (Figure 4C), presumably because the side chains of these 4 cysteine residues coordinate Zn and thus do not react with 5-IAF. We made cysteine substitutions for residues located on various region of the AT₃ structure (Figure 4A). These included His52 on the bottom of AT₃, Glu22 on the end of the Zn-binding arm, Thr10 and Asn16 on the arm between the bound Zn and the top of the trimer, and Thr05 near the top of the trimer. These residues were chosen because when they were substituted with alanine AT remained fully functional for binding TRAP (Table 1). Unfortunately it was not possible to place a cysteine at the very top of the AT₃ structure because substituting residues in this region significantly reduced the affinity of AT for TRAP (Table 1). The rate of labeling these Cys-substituted proteins with 5-IAF in the absence and presence of tryptophan-activated TRAP was examined. Figure 4B shows an example of 5-IAF labeling T05C AT. In this case the rate of labeling was slower in the presence of tryptophan-activated TRAP than in its absence suggesting that residue 5 is less solvent accessible in the AT-TRAP complex than in free AT. The rate of labeling T05C AT was not affected if the reaction was performed with TRAP in the absence of tryptophan (data not shown). A simple interpretation of these observations is that residue 5 is near TRAP in the AT-TRAP complex. Similar studies showed 25–35% reduced labeling for T05C, T10C, and N16C in the presence of tryptophan-activated TRAP (Figure 4C); the Cys residues in these substituted proteins are near the top of AT₃ (Thr05 and Thr10) or on the upper part of the arm (Asn16) of AT₃ (Figure 4A). Two other mutant AT proteins that contain cysteine residues on the end of the Zn-binding arm (E22C) or on the bottom (H52C) of AT₃ showed little or no difference in reactivity with 5-IAF in the absence or presence of TRAP (Figure 4C), indicating that these residues are equally solvent accessible in free AT and in the AT-TRAP complex. Together, these results are consistent with the alanine substitution results described above and further suggest that AT interacts with TRAP through the region on the top of the trimer.

Alanine substitution of residues in *B. subtilis* AT

The amino acid sequences and the trimer structures of AT from *B. licheniformis* and *B. subtilis* are very similar (Figure 1). Moreover, all four residues that were identified as being important for *B. licheniformis* AT binding to TRAP (Val02, Ile03, Asp06, Asp07) are conserved in both proteins (Figure 1A). These observations suggest that both proteins likely bind to TRAP by a similar mechanism. To test this hypothesis, we made four alanine

substitutions in *B. subtilis* AT and examined their effects on binding to TRAP (Table 3). As seen with *B. licheniformis* AT, alanine substitutions of residues on the top region of *B. subtilis* AT₃ (V02A and D06A) eliminated binding to TRAP, whereas substitutions of residues at the end of the Zn-binding arm (E22A) or on the bottom (N52A) of AT₃ had little or no effect on binding (Table 3).

***In vivo* studies of AT binding to TRAP using mutant AT proteins**

We also examined the importance of residues on the top region of the AT trimer for AT function *in vivo*. To do so, we expressed WT and three mutant *B. subtilis* AT proteins (D06A and D07A on the top of AT₃, and E16A on the Zn-binding arm of AT₃) in *B. subtilis* CYBS410pJS673, which carries a chromosomal deletion of the *rtpA-ycbK* region (*rtpA* encodes AT) and has a *trpE*'-'*lacZ* gene fusion under control of the *trp* promoter and regulatory leader region^{16; 36}. The AT proteins to be tested for their ability to modulate TRAP-mediated regulation of the *trpE*'-'*lacZ* fusion were expressed from the IPTG-inducible *spac* promoter on pDG148⁴. TRAP regulated β -galactosidase expression 281-fold in response to tryptophan in this strain carrying the empty vector pDG148, and this regulation was not affected by IPTG (Table 4). As seen previously⁴, IPTG induced expression of WT AT from pDG148 eliminated TRAP-mediated control of the *trpE*'-'*lacZ* fusion, resulting in elevated β -galactosidase expression in the absence or presence of tryptophan (Table 4). The presence of this plasmid also resulted in slightly elevated β -galactosidase expression in cells grown with tryptophan in the absence of IPTG, presumably due to a small amount of leaky expression of AT from the *spac* promoter. Expressing either D06A or D07A AT, which contain alanine substitutions for residues on the top of AT₃ resulted in dramatically less inhibition of TRAP-mediated regulation of the *trpE*'-'*lacZ* fusion than seen with WT AT (Table 4). In contrast expressing E16A AT (alanine substitution in the Zn-binding arm) resulted in similar effects as expressing WT AT. These results together with those in the previous section are summarized in Figure 5, and are again consistent with the conclusion that the residues on top portion of AT₃ are critical for binding to TRAP.

DISCUSSION

Several studies have demonstrated that AT specifically binds to tryptophan-activated TRAP^{4; 6; 30}. Moreover, Valbuzzi *et al.* provided evidence that AT recognizes residues in the RNA binding region of TRAP, including Lys37, Lys56, and Arg58, which are located on the outer perimeter of the TRAP ring (Figure 6A)⁶. Less is known about how AT functions in the AT-TRAP complex. For example, the oligomeric state of AT in this complex is not clear. Ultracentrifugation studies have shown that AT exists in solution as both trimers (AT₃) and dodecamers (AT₁₂)³⁰. These studies also provided evidence for an AT:TRAP complex containing 12 AT subunits/TRAP 11mer. Since no complexes were detected with 3, 6 or 9 AT subunits/TRAP 11mer, it was suggested that AT binds to TRAP as a preformed 12mer³⁰. However, the concentrations of AT₃ used in these studies, 12–48 μ M (with equal fractions of AT₃ and AT₁₂ seen at 20 μ M), are three orders of magnitude higher than have been measured for AT *in vivo* (~30 nM AT₃)³¹, suggesting that AT may function as a trimer *in vivo*. In crystals of AT, four trimers further assemble into a dodecamer (Figure 6B)²⁶, although there is no evidence that this is a biologically relevant assembly of AT.

The alanine substitution studies described here identified four residues (Val02, Ile03, Asp06 and Asp07) in AT that are important for interaction with TRAP (Table 1 and Table 3). These residues are located on the top region of the AT trimer structure (Figure 2). This region of the each trimer is pointed inward toward the center of the tetrahedral dodecamer structure such that Val02, Ile03, Asp06 and Asp07 are not exposed to interact with TRAP (Figure 6C). If AT interacts with TRAP as the dodecamer seen in the crystal structure, then it is possible that

alanine substitutions of these residues interfere with TRAP binding indirectly by interfering with formation of the dodecamer. However, several observations suggest that this is not the case. First, alanine substitution for residues in all other parts of the protein, including the Zn-binding arms and the bottom region, had little or no effect on TRAP binding (Table 1 and Figure 2). Thus if the deleterious effects of substituting residues on the top of AT₃ are indirect, we were unable to identify any other region of the protein in which the amino acid side chains directly interact with TRAP. Secondly, none of the alanine substitutions that affected TRAP binding altered the behavior of the mutant proteins on size exclusion chromatography as compared to WT AT suggesting they do not alter the oligomerization properties of AT. Finally fluorescent labeling studies suggest that Thr05, Thr10 and Asn16, which are near the top region of AT₃, are less solvent exposed in the presence of tryptophan-activated TRAP than in free AT. These residues, particularly residue 10, are buried in the *B. subtilis* AT₁₂ structure and hence it is unlikely they would be even less exposed in the complex with TRAP if this dodecamer binds to TRAP.

Taken together the available data support the proposal that AT binds to TRAP as trimers via residues on the “top” of the of the trimer structure (Figure 1B & 2A). Based on this conclusion, we propose the model for the AT-TRAP complex shown in Figure 7. In this model 4 AT trimers bind to the outer perimeter of the TRAP ring at or near the residues that bind RNA. This model is consistent with the results presented here, as well as with data from previous studies. These include analytical ultracentrifugation studies that provided evidence for an AT-TRAP complex containing 12 AT subunits per TRAP 11mer³⁰, as well as studies of AT binding to mutant TRAP proteins, which suggest that AT binds to the same region of TRAP that binds to RNA⁶. A similar, although less detailed, model has been previously proposed by Valbuzzi and Yanofsky⁴.

An important feature of the model shown in Figure 7 is that AT binding would simply sterically interfere with the binding of RNA to TRAP. This feature differs from a previously proposed model in which AT binds to TRAP as dodecamer via the Zn-binding arms with the top region of each AT trimer buried within the dodecamer²⁶. In that model AT does not directly interact with the RNA binding region of TRAP and would not directly interfere with RNA binding to TRAP. To reconcile this model with prior studies showing that substitutions in the RNA binding region of TRAP interfere with AT binding⁶, it was proposed that these substitutions in the RNA binding residues alter the conformation of TRAP in distant regions that AT recognizes²⁶. Similarly it was proposed that AT binding alters the conformation of the RNA binding region of TRAP indirectly through conformational changes such that it doesn't bind RNA²⁶. Consistent with these proposals, recent studies have shown that tryptophan binding to TRAP³⁷ as well as substitution for an amino acid residue in the tryptophan binding pocket of TRAP²³ alter the conformation of distant regions of the protein. Nevertheless it is simpler to suggest that the residues in both AT (Val02, Ile03, Asp06, and Asp07) and in TRAP (Lys37, Lys56, and Arg58) that have been identified as being crucial for complex formation interact directly as we propose in Figure 7.

The suggestion that multiple AT trimers bind to the TRAP 11mer (Figure 7) raises interesting questions about the mechanism of AT's action to prevent TRAP from binding RNA in order to regulate its target genes. For example it is not clear how many AT trimers must bind in order to inactivate TRAP. TRAP binds to 11 (G/U)AG repeats in the leader region of the *trp* operon to regulate transcription attenuation^{3; 38}. The bound RNA wraps around the outside of the TRAP ring with each triplet repeat interacting with two adjacent TRAP subunits^{17; 19; 22}. This binding prevents formation of an antiterminator RNA structure and allows an overlapping intrinsic terminator to form. TRAP may have to move along the nascent RNA transcript to wrap the RNA around the protein as part of its regulatory mechanism. Hence it is conceivable that as few as one or two bound AT trimers would interfere with this mechanism and prevent

TRAP function. Consistent with this proposal, Yang and Yanofsky have shown that *in vivo* TRAP mediated regulation of the *trp* operon is completely alleviated under conditions when there are approximately 2 AT trimers per TRAP 11mer present³¹.

It is also unclear at what point during transcription of the *trp* operon that AT can function to control TRAP. Prior studies have shown that AT cannot bind to TRAP after it has bound an RNA containing 11 (G/U)AG repeats⁶ (Y. Chen and P. Gollnick unpublished observations). However, it is not known whether AT can bind to TRAP when it has bound less than 11 RNA triplets, such as would be the case if TRAP initiates binding to the RNA during transcription of the *trp* operon leader region when less than 11 triplet repeats are available. Moreover, studies by Babitzke and coworkers have shown that an RNA stem-loop that forms at the 5' end of the *trp* mRNA just upstream of the first UAG repeat plays a role in attenuation control of transcription²⁴. Their current model proposes that during transcription of the *trp* leader region, TRAP first associates with the 5' Stem-loop prior to binding to the (G/U)AG triplet repeats²⁴. It is possible that AT could bind to TRAP when it is associated with the 5' Stem-loop prior to its interaction with the triplet repeats.

One awkward aspect of this new model is the symmetry mismatch for 4 AT₃ binding to a TRAP 11-mer (Figure 7). However examples of such symmetry mismatch have been observed previously in other protein-protein interactions^{39; 40; 41}. Moreover, preliminary NMR studies of *B. subtilis* AT binding to TRAP are consistent with AT being symmetrical in the absence of TRAP and losing this symmetry upon forming a complex with TRAP (C. McElroy, M. Foster Ohio State University and P. Gollnick, unpublished observations). To reconcile this new model with the ultracentrifugation data³⁰, we suggest that under the conditions of those experiments (4–12 μM AT₃) four AT trimers bind cooperatively to TRAP and hence complexes containing 3, 6 or 9 AT subunits per TRAP 11-mer were not observed. Further studies are clearly needed to further the models for the AT-TRAP interaction as well as its mechanism of action and we are currently pursuing such studies.

MATERIALS AND METHODS

Plasmids

Wild-type and mutant *Bacillus licheniformis* AT coding sequences were inserted between the NdeI and EcoRI sites of the expression plasmid pET17b (Novagen), to generate pETBIAT. *B. subtilis* AT proteins were expressed from pETBsAT, which contains the AT coding sequence, with codons optimized for *E. coli*, inserted between the NdeI and EcoRI sites of pET24a (Novagen). This plasmid was kindly provided by C. McElroy and M. Foster at Ohio State University. The native *B. subtilis* AT coding sequence was also inserted between the HindIII and SalI sites of the *E. coli/B. subtilis* shuttle plasmid pDG148^{42; 43} to create pDG148*trpA*, which was transformed into CYBS410pJS673 (see below) to express AT in *B. subtilis*. Expression of AT from this plasmid is controlled by the IPTG-inducible *spac* promoter³⁶.

Bacteria strains

E. coli strain K802 was used to propagate all plasmids. *E. coli* Rosetta(DE3) (Novagen) was used for expression of wild-type and mutant AT proteins. The *B. subtilis* strain used in this study, CYBS410pJS673-*amyE*::[P_{trp}(*trpE'*-'*lacZ*)]C_m^R · S_p^R · Δ*rtpA-ycbK*, was generously provided by Dr. Charles Yanofsky at Stanford University. The *rtpA* gene, which encodes AT, is deleted in this strain, which also carries a *trpE'*-'*lacZ* reporter fusion under control of the *trp* promoter and regulatory leader region.

Site-directed mutagenesis PCR

Mutant *rtpA* genes with amino acid substitutions in AT were created with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) using pETBIAT, pETBsAT or pDG148*rtpA* as templates. Each mutation was introduced using a pair of complementary mutagenic oligonucleotides (Integrated DNA Technologies) with the desired substitutions. PCR products were treated with DpnI (New England BioLabs) at 37°C for 1 hour to digest the original methylated plasmid template. The resulting PCR generated plasmids were transformed into *E. coli* K802, and individual clones were sequenced to confirm the presence of the desired substitution.

Purification of AT and TRAP proteins

Wild-type and mutant *B. licheniformis* and *B. subtilis* AT proteins were purified by slight modifications of a previously described procedure⁴. *E. coli* Rosetta™ (DE3) (Novagen) transformed with the appropriate expression plasmid was grown in LB medium at 37°C until OD₆₀₀ reached 0.4. AT expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to 1mM. After growth was continued for an additional 3 hours, cells were collected by centrifugation, resuspended in buffer A (100 mM Tris-Cl, pH 7.8, 1 mM dithiothreitol (DTT)). Cells were disrupted by two passages through a French Press cell at 10,000 psi (AMINCO). The lysate was cleared by centrifugation at 30,000 × g for 20 min. Streptomycin sulfate powder was added to the supernatant with stirring on ice until the final concentration was 3%, followed by stirring for an additional 20 min. After centrifugation at 30,000 × g for 20 min to remove the precipitate, the supernatant was heated to 90 °C for 8 min. The heat-treated extract was centrifuged at 30,000 × g for 20 min and the supernatant was dialyzed overnight at 5 °C versus buffer B (50 mM Tris-Cl, pH 7.8, 1 mM DTT). The sample was applied to a DEAE A-25 column (Pharmacia Fine Chemicals) equilibrated with Buffer B and eluted with a gradient from 0 to 0.5 M NaCl in Buffer B. Fractions containing AT were identified by electrophoresis on 15% SDS polyacrylamide gels, pooled and concentrated using Amicon Ultra-15 centrifugal concentrators (Millipore). After dialysis against buffer A, the sample was further purified over Sephadex G-75. AT-containing fractions were pooled, concentrated as described above, then dialyzed into buffer A containing 50% glycerol and stored at -20 °C. The purity of the protein was monitored by 15% SDS polyacrylamide gel electrophoresis and the concentration of AT was determined by BCA protein assay using BSA as standard (Pierce).

Wild-type *B. stearothermophilus* TRAP was expressed from pTZstmtrB in *E. coli* BL21(DE3) and purified as described previously¹⁹. Wild-type *B. subtilis* TRAP was expressed in *E. coli* BL21(DE3) and purified by chromatography on phenyl agarose as described previously^{44; 45}.

RNA synthesis

(GAGUU)₁₁ RNA was transcribed from HindIII-linearized pTZ18U-GAGTT₁₁ template using T7 RNA polymerase and labeled with [α -³²P] UTP (PerkinElmer Life Sciences)⁴⁶. The RNA was purified on a 6% polyacrylamide gel containing 8 M urea and extracted by a crush and soak protocol⁴⁷.

Filter binding assay of AT binding to TRAP

To measure AT binding to TRAP a previously developed filter binding assay for RNA binding to TRAP⁴⁷ was modified to incorporate competition between AT and RNA. In the presence of 1 mM tryptophan, RNA binding to TRAP reaches ~90% saturation at 20 nM TRAP. For the AT binding assay, 0 to 60 μM AT trimer was mixed with 20 nM TRAP in 95 μl Filter Binding Buffer (FBB) consisting of 16 mM HEPES pH8.9, 250 mM potassium glutamate, and 1 mM

L-tryptophan. The binding reaction was incubated for 10 minutes at room temperature, which was determined to be sufficient to reach equilibrium. 1 fmol (10 pM) of 32 P-labeled (GAGUU)₁₁ RNA ($\approx$ 5000 dpm) and 10 μ g of yeast tRNA were then added in 5 μ l FBB and the reaction mixtures were incubated for an additional 10 min at room temperature. 50 μ l aliquots of each binding reaction were filtered through a two membrane layer using a 96-well dot blot mini fold apparatus (Schleicher and Shuell)⁴⁸. The membranes consisted of 0.45 μ m nitrocellulose (Schleicher and Shuell) on top, which retains the bound RNA, and Hybond-N+ (Amersham) on the bottom, which retains the unbound RNA. Each well was washed with 100 μ l of FBB, both membranes were dried and exposed to a phosphorstorage screen (Molecular Dynamics), which was developed with a Storm phosphorimager (Molecular Dynamics). AT binding to TRAP was measured by its ability to inhibit RNA binding to TRAP. We defined % inhibition as $\text{RNA}_{\text{free}} / (\text{RNA}_{\text{bound}} + \text{RNA}_{\text{free}})$, where RNA_{free} is the unbound RNA, and $\text{RNA}_{\text{bound}}$ is the RNA in complex with TRAP. The background of RNA_{free} in the absence of AT (20 nM TRAP) was subtracted from the RNA_{free} value for each sample. The data were normalized by setting the % inhibition at saturation (≥ 60 μ M AT trimer) to 100%. The normalized % inhibition data were analyzed by fitting to a nonlinear single binding-site regression algorithm (Prism, GraphPad Software Inc.) to determine apparent dissociation constant (K_d) values.

Circular dichroism spectra

CD spectra (195–255 nm) of 200 μ g/ml wild type and mutant AT proteins were recorded in 50mM sodium phosphate buffer (pH 8.0) at 37°C using a JASCO model J715 spectropolarimeter.

Fluorescent labeling assay

5- Iodoacetamidofluorescein (5-IAF, Molecular Probes-Invitrogen) labels solvent-accessible sulfhydryl groups such as the side chain of cysteine. To test the solvent accessibility of various AT residues, 30 μ M WT or Cys-substituted AT₃ were incubated for 10 minutes in the absence and presence of 8.8 μ M TRAP in 10 μ l of 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM tryptophan. 2.5 μ M 5-IAF was then added and labeling reactions were incubated at room temperature. Aliquots of the labeling reactions were removed at various times and the reactions were stopped by adding 10 μ l SDS-PAGE loading buffer containing 50 mM DTT, which reacts with the unincorporated 5-IAF. The samples were run on 15% SDS gels to separate labeled AT from unincorporated 5-IAF. Images of the gels were obtained with a 312-nm transilluminator and a Kodak DC290 digital camera. Intensities of the labeled AT bands were quantified using ImageQuant software (Molecular Dynamics).

β -galactosidase assay

B. subtilis CYBS410pJS673 transformed with pDG148*rtpA* containing the WT or mutant *rtpA* genes was grown at 37°C in medium consisting of VB minimal salts⁴⁹, 10mM Glucose, 0.2% acid-hydrolyzed casein, 1.32mM K₂HPO₄, 10 μ M FeCl₃, 5 μ g/ml phleomycin (Sigma), in the absence or presence of 50 μ g/ml L-tryptophan. Cells were harvested when the OD₆₀₀ reached 0.4–0.6 and β -galactosidase activity was measured as described previously^{8; 22}.

Size exclusion chromatography

To compare the oligomerization states of WT and mutant AT proteins, size exclusion chromatography of AT was performed using a Biosep SEC-S2000 size exclusion column (300 $\times$ 7.8 mm; Phenomenex) and a Millipore Waters 501 HPLC system. Samples of 2 mg/ml WT or alanine substituted AT in 50 mM sodium phosphate (pH 8.0) were run on the column at a flow rate of 1 ml/min. Proteins were detected using a Millipore Waters 484 absorbance detector at wavelength of 215 nm.

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Figure 1.

Amino Acid Sequence alignment and trimer structure of AT. **(A).** Alignment of amino acid sequences of *B. licheniformis* AT (B1) and *B. subtilis* AT (Bs)⁵⁰. The cysteine and glycine residues of the Zn-binding CXXCXGXG motif are highlighted in cyan. The identical and non-identical residues between the two sequences are marked in black and gray, respectively.

(B). Ribbon diagram of the top view (left) and side view (right) of the *B. subtilis* AT₃ structure. The three subunits are shown in red, green and blue ribbons. Zn atoms are shown as yellow spheres. The regions of the protein defined as top, bottom, and the Zn-binding arm are labeled. Individual β -strands (β_1 – β_4) and the putative hinge region are labeled on the green subunit.

Figure 2.

Locations of the AT residues examined by alanine substitution. The backbone of each subunit is shown as a solid gray ribbon and Zn atoms are shown as yellow spheres. **(A)** Side chains of AT residues for which alanine substitution significantly reduced the affinity for TRAP (Val02, Ile03, Asp06 and Asp07) are shown as red sticks on the top (left side) and side (right side) views of AT₃. **(B)** The side chains of AT residues for which alanine substitution did not significantly alter interaction with TRAP are shown as green sticks on the Zn-binding region and orange on the bottom region of AT₃. **(C)** The location of AT residues potentially involved in anchoring the Zn-binding arms to the AT₃ core is circled with black dashed line. The side

chains Glu09, Lys32, Leu36, Lys49 and His50 are shown in magenta sticks and a dotted line indicates the hydrogen bond between His50 and Glu09.

Figure 3.

Circular dichroism spectra of AT proteins. CD spectra of WT and several Ala-substituted AT proteins are shown in WT (black), V02A (red), I03A (yellow), D06A (magenta), D07A (cyan), and E09A (blue) lines. Each AT protein was 200 $\mu\text{g/ml}$ in 50 mM sodium phosphate buffer (pH8.0) and the spectra were recorded at 37 $^{\circ}\text{C}$.

Figure 4.

Labeling AT with 5-Iodoacetamidofluorescein (5-IAF). **(A)** The location of the side chains of AT residues Thr05, Thr10, Asn16, Glu22 and His52, which were substituted with cysteine for 5-IAF labeling studies are shown as blue sticks on a gray ribbon diagram of the backbone of AT₃ with the Zn atoms shown as yellow spheres. **(B)** Example of the time course of 5-IAF labeling T05C AT in the absence (circles/ solid line) and presence (triangles/dashed line) of tryptophan-activated TRAP. The fluorescence intensity measured after labeling 5-minutes in the absence of TRAP was set to be 1.0 and all other measurements were normalized to this value. Each point represents the average of at least two independent experiments. **(C)**. Comparison of the relative fluorescence intensity of WT and Cys-substituted AT proteins

labeled with 5-IAF in the absence (solid column) and presence (striped column) of tryptophan-activated TRAP for 5 minutes. The average fluorescence intensity for the cysteine mutant AT proteins labeled in the absence of TRAP was set to be 1.0 and other measurements were normalized accordingly. Each column represents the average of at least three independent experiments.

Figure 5.

Summary of the effects of Ala substitution of residues in *B. subtilis* AT. The effects of alanine substitution of residues V02, D06, E22 and N52 were tested *in vitro* for binding to TRAP, and the effects of alanine substitution of D06, D07 and E16 were tested *in vivo* for the ability to affect TRAP mediated control of a *trpE*'-*lacZ* gene fusion. The backbone of each AT subunit is shown as a solid gray ribbon and Zn atoms are shown as yellow spheres. Residues for which alanine substitution significantly affected interaction with TRAP are marked in red stick diagrams, whereas those for which alanine substitution did not significantly alter interaction with TRAP are shown as green sticks.

Figure 6.

Structures of TRAP and AT dodecamer. **(A)** The backbone of the TRAP 11mer is shown in blue cartoons with the bound tryptophan molecules shown in Van der Waals spheres. The side chains of residues Lys37, Lys56, and Arg58, which interact with bound RNA are shown as red sticks. **(B and C)** Ribbon diagrams of the dodecamer structure of *B. subtilis* AT. In panel **B** four AT trimers are shown in red, green, blue and magenta with the Zn atoms shown as yellow spheres. In panel **C** three of the AT trimers are shown in green ribbons and one trimer is highlighted in blue on which residues Val02, Ile03, Asp06, and Asp07 on the top region are shown as red sticks.

Figure 7.

Model of AT-TRAP interaction. A TRAP 11mer and four AT trimers are shown as green and blue spacefilling models respectively and are drawn to scale. On TRAP, residues (Glu36, Lys37, Lys56, and Arg58) which interact with bound RNA are highlighted in yellow. On the four AT trimers, residues (Val2, Ile3, Asp6, Asp7), which are critical for binding to TRAP are highlighted in red. Four AT trimers are positioned around the outer perimeter of TRAP such that the AT residues (red) on top region of the trimers interact with the TRAP residues (yellow) that interact with the bound RNA.

Table 1Substitution of surface residues on *B. licheniformis* AT₃

AT protein	Residue location on AT ₃ [¶]	K_d (μM) [‡]
W.T.	wild type	3.1 ± 0.3
V02A	top	n.b.*
I03A	top	n.b.*
T05A	close to the top	3.8 ± 0.8
D06A	top	101.7 ± 8.5
D07A	top	64.3 ± 6.8
T10A	arm	1.6 ± 0.2
N14A	arm	3.7 ± 0.3
N16A	arm	4.1 ± 0.5
R20A	end of the arm	6.2 ± 0.7
E21A	end of the arm	1.9 ± 0.1
E22A	end of the arm	3.0 ± 0.3
P23A	end of the arm	1.4 ± 0.1
E24A	end of the arm	3.5 ± 0.3
P25A	end of the arm	1.0 ± 0.1
I35A	arm	6.7 ± 0.8
T37A	near the top	2.3 ± 0.3
S41A	side	6.9 ± 0.8
H45A	side	9.6 ± 0.8
I51A	bottom	4.0 ± 0.7
H52A	bottom	1.7 ± 0.1
E53A	bottom	2.8 ± 0.2
V02L	top	n.b.*
D06S	top	n.b.*
D07T	top	n.b.*

[¶] Location of AT residues examined by alanine substitution (See Figure 2A&B).[‡] Each value is the average ± the standard deviation of at least two independent binding measurements, each done in triplicate.* n.b., no binding detected with AT₃ up to 60 μM.

Table 2Alanine substitution of residues between the arm and the core of AT₃

AT protein	K_d (μM) [‡]
W.T.	3.1 ± 0.3
E09A	35.9 ± 3.4
K32A	6.6 ± 1.0
L36A	6.5 ± 0.7
K49A	12.6 ± 1.8
H50A	n.b. [*]

[‡] Each value is the average $\pm$ the standard deviation of at least two independent binding measurements, each done in triplicate.

^{*} n.b., no binding detected using AT₃ up to 60 μM .

Table 3*B. subtilis* alanine substituted AT proteins binding to TRAP

<i>B.subtilis</i> AT	Residue location on AT ₃	K_d (μ M) [‡]
W.T.	wild type	3.1 ± 0.1
V02A	top	n.b. [*]
D06A	top	n.b. [*]
E22A	end of the arm	5.9 ± 1.0
N52A	bottom	2.2 ± 0.5

[§] Location of AT residues tested for alanine substitution (See Figure 5A).

[‡] Each value is the average ± the standard deviation of at least two independent binding measurements, each done in triplicate.

^{*} n.b., no binding detected with AT₃ up to 60 μ M.

Table 4
Effects of expressing WT and Alanine substituted AT on *trp*⁺*E*⁻*lacZ* expression *in vivo*.

AT expressed	β-galactosidase activity (Miller units)					
	-IPTG			+IPTG		
	-Trp	+Trp	Fold Regulation *	-Trp	+Trp	Fold Regulation *
None (plasmid only)	281	1	281	275	1	275
WT	219	6	36	375	382	1
D06A	253	3	84	252	7	36
D07A	246	1	246	352	3	117
E16A	258	6	43	644	575	1

* Fold regulation is defined as the ratio of β-galactosidase units of -Trp/+Trp.