The anti-TRAP protein (AT) of Bacillus subtilis regulates expression of the trp operon and other genes concerned with tryptophan metabolism. AT acts by inhibiting the tryptophan-activated trp RNA-binding attenuation protein (TRAP). AT is an oligomer of identical 53-residue polypeptides; it is produced in response to the accumulation of uncharged tRNA<sub>Trp</sub>. Each AT polypeptide has two cysteine-rich clusters that correspond to the signature motif of the cysteine-rich zinc-binding domain of the chaperone protein DnaJ. Here we characterize the putative zinc-binding domain of AT and establish the importance of zinc for AT assembly and activity. AT is shown to contain Zn(II) at a ratio of one ion per monomer. Bound zinc is necessary for maintenance of the quaternary structure of AT; the removal of zinc converts the AT complex into inactive monomers. All four cysteine residues in the AT polypeptide are involved in Zn(II) coordination. Chemical cross-linking analyses indicate that the AT functional oligomer is a hexamer composed of two trimers. Substituting alanine for any cysteine residue of AT results in rapid degradation of the mutant protein <i>in vivo</i>. We propose a model for the AT trimer in which three AT chains are held together by three zinc atoms, each coordinated by the N-terminal segment and the C-terminal segment of separate AT polypeptides.

In <i>Bacillus subtilis</i> the genes of tryptophan metabolism are coordinately regulated in response to the cellular level of free tryptophan and the extent of charging of tRNA<sub>Trp</sub> (<i>trp</i>). Tryptophan biosynthesis requires expression of the <i>trp</i> operon (<i>trp-ECDFBRA</i>) and an unlinked <i>trp</i> gene, <i>trpG</i> (2). When the concentration of tryptophan is sufficiently high tryptophan binds to and activates the <i>trp</i> RNA-binding attenuation protein (TRAP).<sup>1</sup> Activated TRAP recognizes and binds to (G/U)AG repeat sequences in the <i>trp</i> operon leader RNA, promoting formation of an RNA terminator structure and transcription termination (3–5). Activated TRAP also inhibits translation initiation on four coding regions: <i>trpE</i> and <i>trpG</i> (6, 7), <i>yhaG</i>, a gene believed to encode a tryptophan transport protein (8), and <i>ycbK</i>, a gene of unknown function (9). The structures of tryptophan-activated TRAP and of an active TRAP-RNA complex have been reported (10, 11). TRAP has 11 identical subunits arranged as a doughnut-shaped molecule. Tryptophan binds cooperatively to 11 binding sites in TRAP (12) and is believed to promote a conformational change on the periphery of each TRAP subunit, activating its RNA-binding surface (13).

The extent of charging of tRNA<sub>Trp</sub> is also sensed in <i>B. subtilis</i> (9, 14). Uncharged tRNA<sub>Trp</sub> accumulation induces expression of an operon containing the gene <i>rtpA</i> (regulator of the TRAP protein, previously named <i>yczA</i>) encoding the protein AT. Induction occurs by the T-box transcription antitermination mechanism (9, 16). AT is a multimeric protein composed of identical 5.6-kDa subunits (15). AT binds to the tryptophan-activated form of TRAP (15). By masking the RNA-binding surface of TRAP, AT blocks the regulatory action of TRAP and induces <i>trp</i> operon expression (17).

The predicted 53-residue sequence of AT shows remarkable similarity to the sequence of the cysteine-rich domain of the chaperone protein DnaJ (18). In particular the signature motif CXXCXXGXG, which is repeated four times in DnaJ, is present twice in the AT polypeptide (15). The cysteine-rich domain of DnaJ is believed to be involved in the binding of unfolded protein substrates (19). Structure-function analyses with DnaJ of <i>Escherichia coli</i> revealed that this domain contains two Zn(II) ions, each coordinated to four cysteine residues. The removal of zinc ions from DnaJ resulted in destabilization of its tertiary structure (20). Deletion of the cysteine-rich region (20) or substitution of pairs of cysteine residues by serine residues (19) significantly affects the activity of the protein. The spacing between the cysteine residues of DnaJ that chelate zinc is similar to that found in a family of C4 zinc finger proteins (19). However, the structure of the cysteine-rich domain of <i>E. coli</i> DnaJ reveals a unique fold in which an extended V-shaped structure is formed, and each Zn(II) ion is coordinated by pairs of cysteine residues that are far apart in the primary sequence (18, 21).

In this article we present the results of studies on the possible roles of zinc and the cysteine residues of AT in determining the structure and function of the AT. We show that AT is indeed a metalloprotein and that zinc removal converts the protein into inactive monomers. The Zn(II) content of AT was determined, and the zinc/AT monomer molar ratio was found to be 1. All four cysteine residues appear to be essential for the integrity of the AT complex; substitution of any of the cysteine residues of AT by an alanine residue destabilizes the protein. The AT complex is shown to be a hexamer that appears to be composed of two trimers of AT chains. We propose a model for AT structure in which three Zn(II) ions are coordinated to cysteine residues from three polypeptide chains to assemble the trimer. Two trimers subsequently associate to form the hexamer.

**EXPERIMENTAL PROCEDURES**

**Preparation of Zinc-free AT**—Purified native AT was prepared as described previously (17). Zinc-free AT was prepared as follows: 8 molar

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† These abbreviations are used: TRAP, trp RNA-binding attenuation protein; AT, anti-TRAP protein; HMB, p-hydroxymercuribenzoate; DTT, dithiothreitol; Tricine, N(2-hydroxyethyl)-1,1-bis(hydroxymethyl)-ethyglycine; PAR, 4,2-pyridylazoresorcinol; IPTG, isopropyl-β-D-thiogalactopyranoside; wt, wild type; ReAT, zinc-reconstituted AT.
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The atomic absorption spectroscopy analysis for zinc in our purified AT preparation was performed by the Galbraith Laboratories. Mercurial promoted Zn(II) release experiments were performed in metal-free 25 mM Tris–HCl, pH 7.8. In the HMB titration experiments, 1.25-μl aliquots of 1 mM HMB were added to a 500-μl cuvette containing 6 μM AT (monomer concentration). The reactants were mixed by inversion, and the absorbance at 250 or 500 nm was measured. When measurements were performed at 500 nm, 0.1 mM 4-(2-pyridylazo)resorcinol (PAR) was present in the cuvette (ε = 6.6 × 10^3 M^−1 cm^−1 at 500 nm for the PAR–Zn(II) complex) (20).

**Chemical Cross-linking—**Cross-linking experiments were performed as described previously (15), except that 0.5 μg of AT (various preparations) and 1 μl of 5% w/v glutaraldehyde were used in each reaction (10 μl total volume) in the presence of 10 mM Tris–HCl, pH 7.8. In the time course experiment with native AT, 30 mM Tris–HCl, pH 7.8, 20 mM NaCl, and 4 mM MgCl₂ were present in each reaction mixture.

**Zinc Content Measurement—**The atomic absorption spectroscopy analysis for zinc in our purified AT preparation was performed by the Galbraith Laboratories. Mercurial promoted Zn(II) release experiments were performed in metal-free 25 mM Tris–HCl, pH 7.8. In the HMB titration experiments, 1.25-μl aliquots of 1 mM HMB were added to a 500-μl cuvette containing 6 μM AT (monomer concentration). The reactants were mixed by inversion, and the absorbance at 250 or 500 nm was measured. When measurements were performed at 500 nm, 0.1 mM 4-(2-pyridylazo)resorcinol (PAR) was present in the cuvette (ε = 6.6 × 10^3 M^−1 cm^−1 at 500 nm for the PAR–Zn(II) complex) (20).

**AT Mutagenesis—**Cys → Ala substitutions were introduced in the AT sequence of a multicopy expression plasmid by multiple steps of PCR. For each substitution, in the first step two oligonucleotides were used that are complementary to each other and anneal to the rtpA gene sequence but carry the desired nucleotide change. Each was used in combination with an external oligonucleotide that anneals to the vector sequence to perform PCR using the plasmid pDGrtPavet (15) as template. The two amplified products corresponding to the 5′ and 3′ portions of the rtpA gene and partially overlapping in the central mutagenized region were gel-purified. They were then mixed, annealed, and used as template for the second step of PCR performed with the two external oligonucleotides. The product obtained, carrying an rtpA gene sequence with the desired substitution, was cloned in pDG148, and the sequence was verified by sequencing. Each modified vector was transformed into a B. subtilis strain carrying a chromosomal deletion of the rtpA-ycbK region and a tcp promoter-leader-trpEF-lacZ translational fusion (9).

**β-Galactosidase Assays—**β-Galactosidase activity was determined as described previously (15).

**Western Blot Analysis—**Cultures were grown under the conditions employed for β-galactosidase assays (15) and were diluted to an A_{600 nm} reading of 0.5. Cell pellets were resuspended in 20 μl of SDS-Tris-Tricine sample buffer and boiled for 5 min. 8.7 μl of sample were electrophoresed on SDS-15% polyacrylamide gels in Tris-Tricine buffer and then electrophoretically transferred to a nitrocellulose membrane (Protran BA79, Schleicher & Schuell). Immunoblotting was performed following the supplied instructions using rabbit polyclonal antibodies directed against AT prepared by the Covance company. Bound antibody was visualized by use of horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham Biosciences) and SuperSignal West Pico chemiluminescent detection reagents (Pierce).

**RESULTS**

**AT Is a Metalloprotein: Removal of the Intrinsic Zinc of AT and Reconstitution of the Native Protein—**The AT protein contains a putative zinc-binding domain similar to a portion of the cysteine-rich zinc-binding domain of DnaJ (15). Atomic absorption spectroscopy was performed with a sample of pure AT (1 mg in 5 ml of 100 mM Tris–HCl, pH 7.8, 1 mM DTT) that was previously dialyzed overnight against Chelex X-100 (Bio-Rad) to remove free zinc from the buffer. The results obtained (Galbraith Laboratories) indicated that zinc was indeed present in the protein at a level of 1.5 ppm. This corresponds to 0.6 Zn(II) atoms per AT monomer. Zinc appeared to be tightly bound as an aggregate of AT with Chelex X-100 or chelators such as EDTA or N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) had no noticeable effect on the stability of the protein (analyzed by native gel electrophoresis) or on its anti-TRAP activity (tested in the in vitro transcription termination assay) (data not shown). Based on AT-DnaJ similarity we expected the cysteine residues of AT to be involved in Zn(II) coordination. Therefore, we attempted to displace the intrinsic zinc by treating the protein with HMB. This compound, a strong sulfhydryl-binding reagent, has been widely used to remove zinc from metalloproteins and is generally incorporated at one molecule per -SH group (22, 23). Pure native AT was treated with HMB to produce HMB-AT. The displaced zinc was removed by addition of EDTA and multiple washings, and the treated AT was concentrated using a Microcon YM-3 filtration device. These steps were all performed in the presence of EDTA. Subsequently, the mercurling agent was removed by reversing the reaction with DTT to generate zinc-free AT. In a parallel experiment an aliquot of the same HMB-AT preparation was subjected to DTT treatment without the steps designed to remove the labilized zinc being performed, producing zinc-reconstituted AT. We examined AT subunit association in native AT, HMB-AT, zinc-free AT, and zinc-reconstituted AT using cross-linking with glutaraldehyde (Fig. 1). Under the conditions employed, the most efficiently cross-linked species observed with native AT was a trimer (predicted M₆, 16,800; Fig. 1, lane 1). After treatment with HMB, AT was reduced to the monomeric state (HMB-AT), indicating that displacement of zinc and incorporation of the mercuorial agent destroyed the AT complex (Fig. 1, lane 2). However, when the HMB reaction was reversed in the presence of the labilized zinc, ReAT, the protein appeared to return to its original structure (Fig. 1, lane 3). Conversely, when the reaction was reversed following zinc elimination, most of the AT protein remained as monomer or formed aggregates of high molecular weight (Fig. 1, lane 4). Some trimer species were observed in the presumed zinc-free...
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AT sample. This could be caused by incomplete removal of the zinc (Fig. 1, lane 4). Further incubation of zinc-free AT with 100 μM ZnCl₂ did not restore the original trimeric structure, suggesting that the cysteine residues had been rendered incapable of coordinating Zn(II) (Fig. 1, lane 5). We believe that in the absence of zinc the free cysteine residues in the AT monomer undergo oxidation-forming Cys-Cys bridges that promote aggregation (in the various AT preparations the DTG concentration was lowered prior to analysis). In fact, when zinc-free AT was incubated with ZnCl₂ in the presence of DTT, a pattern resembling that obtained with native AT was observed (Fig. 1, lane 6).

We also compared the activities of these different AT preparations using an in vitro transcription termination assay (Table I). As expected, zinc-free AT had reduced ability to inhibit TRAP-dependent transcription termination in the leader region of the trp operon. The residual activity observed is probably caused by the fraction of native protein remaining in the preparation, because not all the zinc had been removed. From the values obtained with the native AT preparation we derived a saturation curve describing the inhibition by AT of TRAP-dependent transcription termination (15). Based on this curve we estimate that 27% of the AT molecules in the zinc-free preparation are still functional. Incubation of zinc-free AT with ZnCl₂ did not increase its activity. However, zinc-reconstituted AT was fully active (Table I), demonstrating that AT can regain both its original structure and function upon zinc re-incorporation.

**Determination of the Zinc Content of AT**—To obtain an independent and accurate measure of the zinc content of AT, we took advantage of the effective reaction between AT and HMB. It is possible to follow formation of the mercaptide bond between the free cysteine residues of AT and HMB by monitoring absorbance at 500 nm. If this assay is conducted in the presence of the high affinity metallochromic indicator PAR, release of Zn(II) ions, concomitant with HMB reaction with the sulphydryl groups of AT, can be detected by monitoring absorbance at 500 nm (20, 23). In our experiments, the absorbance at both 250 and 500 nm reached a plateau at a HMB/AT monomer molar ratio of 4.1 (Fig. 2). Because the same amount of HMB is required to saturate the cysteine residues and to exhaustively remove zinc from AT, it appears that all four cysteine residues are involved in tetrahedral coordination with Zn(II) ions. From the absorbance at 500 nm measured for HMB/AT mixtures at saturation, A_{500 nm} = 0.397, we derived the concentration of the (PAR)₂Zn(II) complex formed as 6.015 ± 0.015 μM, which corresponds to the concentration of the zinc ions released by 6 μM AT (Δε = 6.6 × 10^4 M⁻¹ cm⁻¹ at 500 nm for (PAR)₂Zn(II) complex). Therefore, we unambiguously established the zinc/AT monomer molar ratio to be 1.

**Structural Organization of AT**—Chemical cross-linking with bifunctional reagents can help elucidate important details about the structure of oligomeric proteins (24). As previously mentioned, in a standard 5-min reaction of glutaraldehyde with pure native AT, most of the protein appears to be cross-linked as a trimeric species. We decided to allow the cross-linking reaction to proceed for longer periods, analyzing identical AT samples incubated with glutaraldehyde for different lengths of time (Fig. 3). After 30 s of incubation the trimer was already visible, and at all subsequent time points it appeared to be the most abundant product. Starting with 10 min of incubation, a new complex became evident, with a size matching the predicted molecular weight for an AT hexamer.
Fig. 3. Cross-linking analysis of the native AT oligomer. Identical 0.5-μg aliquots of pure native AT protein were cross-linked with 0.8% glutaraldehyde for different time intervals (time in minutes is indicated at the top of each gel lane). The resulting complexes were analyzed by electrophoresis on a 4–20% gradient SDS-polyacrylamide gel. M, molecular size standards; units at left are kilodaltons.

Fig. 4. Analysis of the in vivo stability of specific AT mutant proteins. Total cell extracts were prepared from B. subtilis strains overexpressing wild type (wt) or mutant (C12A, C15A, C26A, C29A) AT proteins from an IPTG-inducible multicopy plasmid construct. The recipient strain used had a chromosomal deletion of the rtpA-ycbK region. Extracts were electrophoresed on an SDS-15% polyacrylamide gel, and AT was visualized by immunoblotting. Cultures were grown in Vogel-Bonner minimal medium containing 0.5% glucose, and IPTG (1 mM) was added in mid-exponential phase. On the left part of the gel, cells collected 2 h after IPTG addition were analyzed. On the right part of the gel, cells collected 1 h after IPTG addition were removed from the medium (by centrifugation, washing, and resuspension) and analyzed. The cellular content from 435 μl of culture (A_{600nm} = 0.50) was examined in each lane. Detection of unspecific bands by the anti-AT antibody confirms that the same amount of cell protein was loaded in each lane (upper panel). Because 1 h after IPTG removal the densities of the cultures showed a 16% increase, an approximate 10% loss of AT protein would be expected to occur because of a dilution of the cellular content. (M_{r} = 33,600). The progressive increase of the hexamer concentration with incubation time was paralleled by the gradual disappearance of the monomer and dimer species. Note that no intermediate species between trimer and hexamer are visible. This biphasic cross-linking pattern suggests that the fastest unit to be cross-linked is a trimer of AT chains. However, upon continued incubation the native form of AT, a hexamer composed of two trimers, is detectable. It is unlikely that in solution AT exists both as trimer and hexamer species, because native AT behaves as a single species when examined by gel-filtration chromatography or native gel electrophoresis (data not shown).

AT Mutagenesis: Importance of the Four Cysteine Residues of AT—The crucial role of zinc for the maintenance of AT stability and activity was confirmed by mutagenesis experiments. Mutant AT proteins, each lacking one of the four cysteine residues, were overproduced in B. subtilis using an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter in a multicopy plasmid construct (15). Each of the mutant proteins had one of its four cysteine residues substituted by alanine. These substitutions were obtained by introducing a maximum of four nucleotide changes in the sequence of the wild type rtpA gene. Cell extracts of cultures presumably overexpressing wild type or mutant AT proteins were subjected to Western blot analysis using an antisera specifically directed against AT (Fig. 4). Visual inspection of the blot indicated that the AT levels in strains expressing Cys → Ala variant proteins were dramatically lower than the level observed in a strain producing the wild type protein. A possible explanation for this finding is that these variant proteins, each lacking one cysteine residue, are rapidly degraded. To examine AT lability further, the inducer IPTG was removed from each growing culture, and the cultures were reincubated for 1 h without IPTG. In these 1-h samples

TABLE II

| Plasmid       | β-Galactosidase activity^a |
|---------------|---------------------------|
| pDGrtpAwt     | 615                       |
| pDGSTOPrtpA   | 13                        |
| pDGrtpAC12A   | 12                        |
| pDGrtpAC15A   | 13                        |
| pDGrtpAC26A   | 13                        |
| pDGrtpAC29A   | 13                        |

^a The activity assayed is β-galactosidase expressed from the trpE'-lacZ fusion and is given in Miller units.

Fig. 5. Schematic representation of the proposed quaternary structure of AT. Each AT polypeptide chain is depicted as a black curved line with stars marking the locations of cysteine residues. Spheres represent the three Zn(II) ions in a trimer. The native protein is schematized as a dimer of AT trimers (see text for details).
following IPTG removal, which had only a 16% increase in mass per culture, the mutant proteins were no longer detectable (Fig. 4). Thus, degradation is most likely responsible for the low levels of mutant AT proteins observed in the induced samples. In contrast, wild type AT was maintained at a stable level (Fig. 4). Two hours after IPTG removal, the wild type AT concentration decreased; this decrease was caused by dilution associated with cell growth (data not shown). Therefore, all four cysteine residues in the conserved DnaJ motif of AT appear to be essential for the stability of the AT polypeptide in vivo.

The instability of the Cys → Ala mutant proteins does not exclude the possibility that these variant proteins are still able to interact with TRAP and exert an inhibitory action. Hence, we compared the activities of the mutant proteins with that of the corresponding mutant AT protein would exist as an unstable monomer. Apparently AT monomers are incapable of inactivating TRAP.

Analysis of cross-linking of the subunits of native AT with glutaraldehyde indicated that the AT oligomer is a hexamer composed of two identical trimers. Based on the information collected in this study, and taking into consideration the peculiar fold of the DnaJ zinc-binding domain, we postulate a model for the AT complex (Fig. 5). We propose that three 5.6-kDa AT chains are associated by three Zn(II) ions, which are coordinated by pairs of cysteine residues belonging to different chains. A single type of interaction involving the cysteine residues near the N terminus of one chain and near the C terminus of an adjacent chain would be the simplest way to bring the three subunits together into a trimer. Two assembled trimers would then interact to produce the AT hexamer. The disruptive effect of single cysteine substitutions in the AT mutagenesis experiments is consistent with each Zn(II) ion being coordinated by only two cysteine residues from the same chain; the equal importance of each of the four cysteine residues would favor our proposed symmetrical structure.

In the highly reducing intracellular environment of the cell, ubiquitous zinc represents a convenient and economic vehicle for assembly and stabilization of protein domains or oligomers (25). The AT protein adds to the growing number of zinc-binding proteins that are distinct from the classical zinc-finger proteins in that they present novel types of folding (21) and bind proteins rather than nucleic acids (25). We anxiously await a determination of the structure of AT and analyses of how AT forms specific inhibitory complexes with the 11-subunit tryptophan-activated TRAP regulatory protein.

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