The nature of the TRAP–Anti-TRAP complex

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Edited by Charles R. Cantor, Sequenom Inc., San Diego, CA, and approved December 18, 2008 (received for review February 3, 2008)

Tryptophan biosynthesis is subject to exquisite control in species of *Bacillus* and has become one of the best-studied model systems in gene regulation. The protein TRAP (*trp* RNA-binding attenuation protein) predominantly forms a ring-shaped 11-mer, which binds cognate RNA in the presence of tryptophan to suppress expression of the *trp* operon. TRAP is itself regulated by the protein Anti-TRAP, which binds to TRAP and prevents RNA binding. To date, the nature of this interaction has proved elusive. Here, we describe mass spectrometry and analytical centrifugation studies of the complex, and 2 crystal structures of the TRAP–Anti-TRAP complex. These crystal structures, both refined to 3.2-Å resolution, show that Anti-TRAP binds to TRAP as a trimer, sterically blocking RNA binding. Mass spectrometry shows that 11-mer TRAP may bind up to 5 AT trimers, and an artificial 12-mer TRAP may bind 6. Both forms of TRAP make the same interactions with Anti-TRAP. Crystallization of wild-type TRAP with Anti-TRAP selectively pulls the 12-mer TRAP form out of solution, so the crystal structure of wild-type TRAP–Anti-TRAP complex reflects a minor species from a mixed population.

protein complex | X-ray crystallography | transcription regulation | analytical ultracentrifugation | mass spectrometry

TRAP (*trp* RNA-binding attenuation protein) plays a central role in the highly intricate regulation of transcription and translation of the *trp* operon in several species of *Bacillus*, and this system has provided important insights into several mechanisms of gene regulation (1–5). The protein forms an 11-mer ring that binds 11 molecules of tryptophan (Trp) at symmetry-related sites (6–8), and the TRAP–Trp–RNA complex has been solved for the *Bacillus stearothermophilus* protein (9). With tryptophan bound, TRAP can bind *trp* mRNA and induce transcription termination. In *Bacillus subtilis*, when levels of charged tRNA^{Trp} fall, the protein Anti-TRAP (AT) is expressed, which blocks RNA binding to Trp-bound TRAP and relieves expression of the *trp* operon (10, 11). AT forms a stable trimer, 4 copies of which can further associate into a dodecamer form (12, 13). Each 53-residue polypeptide chain carries a zinc-binding site made of 4 cysteine residues, and chemical cross-linking studies suggested that the active form of AT may be a hexamer (14). Analytical ultracentrifugation data have been interpreted to indicate that the AT dodecamer is the active (TRAP-binding) form, giving an AT₁₂–TRAP₁₁ complex, although it was noted that the data did not rule out alternative stoichiometry (12). Antson and colleagues (13) further proposed, on the basis of their X-ray structure of AT, a model in which the AT dodecamer may bind up to 4 TRAP 11-mer rings by aligning their 11-fold symmetry axes with its 3-fold axes. In this model, AT would not directly block RNA binding, or contact TRAP residues Lys-37 and Arg-58 that are known to be required for TRAP–RNA (15) and TRAP–AT interaction (10). Instead it penetrates the TRAP ring, and it is suggested that there is indirect communication between AT and the RNA-binding residues of TRAP. One difficulty with the proposed model is the known binding of AT to TRAP from both *B. subtilis* and *B. stearothermophilus*, even though these have very different charge in the center of the ring (alanine 66 in *B. subtilis* TRAP is

replaced by arginine 66 in the *B. stearothermophilus* protein). *B. stearothermophilus* itself does not appear to produce AT.

Although the structures of both binding partners are known, the 12-mer/11-mer symmetry-mismatch between them suggests that crystallographic analysis of the TRAP–AT complex would be virtually impossible. Experimental tests of the current model (13) are, however, highly desirable, because there is no ready explanation for the ability of AT to compete with RNA binding to TRAP. This lack of mechanistic understanding is rather unusual in such a well-studied system, and we have addressed this issue with a variety of biophysical methods including crystallography. To favor cocrystallization of TRAP and *B. subtilis* AT, we have used not only wild-type *B. stearothermophilus* TRAP but also an artificial 12-mer form of TRAP that has suitable point-group symmetry to crystallize readily.

Results

Artificial 12-mer TRAP Rings. We have previously constructed artificial 12-mer forms of TRAP that are ideal for testing different models of the TRAP–Anti-TRAP interaction, because the outer surface is unchanged from the wild-type, except for the slight ring expansion. These 12-mer rings are made by joining copies of *B. stearothermophilus* TRAP in tandem with linkers of alanine residues. Crystal structures show that placing 3 or 4 subunits of TRAP on the same polypeptide causes the protein to associate with subunit interfaces virtually identically to wild-type TRAP but with 12 subunits per ring (16). The peptide linkers lie within the cavity of the ring and would hinder interactions with any protein in this region. In this report, we have used a mutant carrying 3 copies of *B. stearothermophilus* TRAP and 2 linkers of 7 alanine residues (“T3A7,” PDB ID code 2ZCZ), which is more thermostable than other 12-mer forms described previously (17). The binding of AT to 12-mer rings of TRAP was initially tested with analytical ultracentrifugation. This method clearly demonstrates the size difference of 11-mer and 12-mer TRAP (Fig. 1). Comparison of TRAP–AT mixtures in the centrifuge shows that the 12-mer T3A7 binds AT with very similar affinity to wild-type 11-mer TRAP (Fig. 2), despite the increased diameter of the ring, which might not be expected from the dodecamer AT model. The preservation of functionality in the 12-mer TRAP was further shown by gel-shift assays, confirming that 12-mer TRAP binds RNA with similar

Author contributions: S.A. and J.R.H.T. designed research; M.W., J.G.H., K.K., S.U., S.A., S.-Y.P., and J.R.H.T. performed research; M.W., J.G.H., K.K., S.U., S.A., and S.-Y.P. analyzed data; and J.G.H. and J.R.H.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2ZP8 and 2ZP9).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0801032106/DCSupplemental.

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Fig. 1. Sedimentation velocity analysis of TRAP and AT. All experiments were carried out at 20 °C and a rotation speed of 50 krpm. The buffer used was 50 mM Tris (pH 8.5), 300 mM NaCl, 1 mM DTT. Protein was detected by using interference optics. The sedimentation coefficient distribution $C(S)$ is derived from solution of the Lamm equation, and represents relative concentration of material with a given sedimentation coefficient S . L-tryptophan (0.5 mM) was added wherever TRAP was present, and the experiments were repeated with protein concentrations of 1, 0.5, or 0.2 mg/ml (shown as red solid, orange dashed, and blue dotted lines, respectively). (A) *B. subtilis* TRAP alone. (B) *B. stearothermophilus* TRAP alone. (C) T3A7 12-mer TRAP alone. The higher molecular mass of the 12-mer TRAP is apparent. (D) AT alone, at concentrations 1, 0.5, or 0.1 mg/ml. Under these conditions, AT trimers show very little association into higher-order oligomers.

affinity to the wild-type protein (17). The RNA-binding site is not, therefore, disturbed by introducing an extra subunit into the ring.

Crystal Structure of the T3A7–AT Complex. The 12-mer TRAP ring has point-group symmetry highly advantageous for crystallization, and the T3A7–AT complex readily forms crystals in space group $P6$. Diffraction data were collected to 3.2 Å, and the structure was readily solved by molecular replacement. The crystal structure of an artificial 12-mer TRAP has already been refined to 1.8 Å (16) and that of the AT dodecamer to 2.8 Å (13), so a model could easily be

constructed from the previously refined parts. Refinement of the complex led to a rather high R factor, and the AT shows considerable disorder around the zinc-binding sites, although the relative positioning of the TRAP ring and AT trimers is unambiguous. This model of the 12-mer-TRAP–AT complex is radically different from those previously proposed, but immediately explains the ability of AT to block RNA binding to TRAP since AT contacts the RNA-binding residues directly (see below). To confirm this model crystallographically, an additional structure was obtained by using wild-type TRAP.

Fig. 2. Sedimentation velocity analysis of mixtures of TRAP and AT. All experiments were run under identical conditions to those shown in Fig. 1. TRAP concentration was fixed at 0.5 mg/ml final, and varying amounts of AT were added. (A–C) The data obtained from 4 experiments are overlaid. The molar ratios (by monomers) of TRAP–AT are 1:0 (blue), 1:1 (green), 1:4 (orange dashed) and 1:16 (red). (A) Wild-type *B. stearothermophilus* 11-mer TRAP and AT. (B) Wild-type *B. subtilis* 11-mer TRAP and AT. (C) T3A7 and AT. Free AT trimers sediment with an S value of 1.6. Under the conditions used, the wild-type 11-mer TRAP reached a maximum of 6.5 S with excess AT, but 12mer TRAP (T3A7) shows a greater increase in molecular mass. Molecular mass of the complex increases incrementally with AT concentration, and a greater apparent number of AT molecules bind to 12-mer TRAP than to 11-mer TRAP. (D) Sedimentation velocity analysis of mutant TRAP F32A (shown in red) compared with wild-type TRAP (blue). The molar ratio (by monomer) of TRAP(F32A)/AT is 1:16. Phe 32 is distant from the binding site previously proposed for an AT dodecamer (13), yet mutating this residue to alanine abolishes AT binding.

Fig. 3. Structure of the wild-type TRAP-AT complex. (A) Ribbon diagram of the wild-type TRAP-AT complex (*R32* form), showing the TRAP 12-mer ring (colored by subunit) and the AT molecules around it (blue), looking along the ring axis, and an orthogonal view. Helices are shown as coils and β -strands as arrows. TRAP has no α -helices. (B) A stereo diagram showing the contents of the asymmetric unit. Chains are colored separately and shown as $C\alpha$ traces; zinc ions are shown as red spheres. One AT trimer is labeled A, B, and C to indicate which subunits form major, minor, or no contact with TRAP, respectively.

Crystal Structure of Wild-Type TRAP–AT. To demonstrate that the structure obtained with artificial 12-mer TRAP is not an artifact, we also crystallized AT mixed with wild-type TRAP. This complex also formed crystals that diffracted to 3.2 Å, and allowed a clear molecular replacement solution to be determined in space group *R*32. The crystal contacts are quite different from the T3A7–AT complex, but the TRAP–AT interface is the same. Refinement proceeded more smoothly than with the T3A7 complex, giving a lower final *R* factor. This model shows the wild-type TRAP has also formed 12-mer rings without the aid of subunit linker peptides.

Three AT trimers and 6 TRAP subunits are found in the asymmetric unit of the *P6* crystal form and 2 AT trimers and 4 TRAP subunits in the asymmetric unit of the *R32* form (Fig. 3). Omit maps were calculated for each structure, with an AT trimer removed from the model, and show excellent fit to the missing atoms (Fig. 4). Overlaying an AT trimer and its associated TRAP subunit pair from each crystal form gives a rmsd of 0.63 Å over 1,144 main-chain atoms and 0.56 Å over 286 C α atoms. Because both models are essentially identical, we describe the TRAP–AT interface only once. One AT trimer binds to 2 neighboring TRAP subunits within a ring, with the majority of the contacts being between 1 AT chain and 1 TRAP subunit (which we name “A” and “A” respectively). The neighboring TRAP subunit (“B”) makes quite different contacts with another chain in the AT trimer (“B”), and the third chain in the AT trimer (“C”) points into solvent. The modeling tool PISA (18) shows AT subunits of type *A* and *B* bury ≈ 480 Å² and 300 Å² with their closest TRAP subunit in the complex, significantly less than the interfacial area between TRAP subunits, which is 1,070 Å². This

Fig. 4. Weighted Fo-Fc omit maps covering the TRAP-AT interface. Electron density maps were calculated by omitting an AT trimer from each model and refining for 10 further cycles with REFMAC, with no NCS restraints applied, to remove phase bias. The maps were calculated with the output coefficients DELFT and phases PHDELFT, and both maps are displayed at 3.0 σ over the final model (TRAP in gray and AT in pink). The omitted AT trimer is shown as a simple α trace, and the neighboring trimers are shown as ribbons. (A) Wild-type TRAP-AT complex. The omitted trimer appears clearly in the map, except for the zinc-binding loop of the subunit pointing away from TRAP, which is the least ordered part. The map is very clear, the main noise peak being small and on the TRAP symmetry axis. The central helices of the AT trimer appear strongly, making the fit of the known trimer structure into the map unambiguous. (B) An equivalent figure to A but using the T3A7-AT complex.

is consistent with the relative ease of AT dissociation from TRAP rings, which are themselves very stable (16).

The contacts between TRAP and AT include both hydrophobic interactions and salt bridges (see below). Phe-32A of TRAP, a residue close to bound RNA (9), sits in an apolar pocket formed by *Pro-13A*, *Ala-28A* and *Ile-35A* of AT. Phe-32B makes extensive contact with *Met-1B*, the sulfur atom lying over the benzene ring. Lys-37A sits between *Asp-6A* and *Asp-7A*, making salt bridges with both side chains. Arg-58A forms an apolar contact with *Pro-13A*, and Arg-58B forms an apolar contact with *Val-2B*. The model immediately explains the known importance of Lys-37 and Arg-58 residues to TRAP-AT binding (10) and how AT sterically blocks RNA binding to TRAP. Phe-32 appears from the model to be a key residue because 2 copies contact each AT trimer, and replacing it with alanine abolishes binding to AT (Fig. 2). This result cannot be explained by the dodecamer AT model, in which AT contacts the central cavity of TRAP, but agrees well with the structure presented here.

Mass spectrometry confirms that a small proportion of wild-type TRAP exists in a 12-mer ring form in solution (Fig. 5). A

distort the N terminus by steric repulsion of the 3 bulkier residues, and close the Phe-32-binding pocket. Although Chen and Gollnick concluded from the *Ala-2* and *Leu-2* mutants that *Val-2* of wild-type AT must fit tightly into the TRAP–AT interface, the much weaker interaction in the crystal structures is nevertheless perfectly compatible with their experimental results. The *Ile-3* side chain points into the hydrophobic core of the AT trimer in the complex and is barely surface exposed, but its main chain does contact Phe-32 (Fig. 6). Apart from its proximity to Lys-37A, the *Asp-7* side-chain hydrogen bonds the nitrogen atom of *Ala-4* in each AT subunit, an interaction that will be lost in the *Asp7Ala* mutant. Because Phe-32 lies against the main chain of *Ile-3* and *Ala-4*, this mutation would clearly greatly weaken TRAP–AT binding (Fig. 6B). Chen and Gollnick deduced from their results a model in which AT trimers bind around the rim of the TRAP ring, not entirely unlike the crystal structures presented here. However, the model they present is based merely on bringing *Val-2*, *Ile-3*, *Asp-6*, and *Asp-7* into proximity with Lys-37, Lys-56, and Arg-58, and offers no atomic detail. In the simple space-filling model presented (20), it appears the rotation axis of AT lies perpendicular to that of TRAP, and 1 11-mer TRAP ring may bind up to 4 AT trimers because of steric hindrance. The mass spectrometry results shown in Fig. 5, however, clearly show that not only may 11-mer TRAP bind 5 AT trimers, but 12-mer TRAP may bind 6. This result is in complete agreement with our AUC data (measured at pH 8.5 with 300 mM salt), suggesting that it cannot be dismissed as an artifact of the technique. Chen and Gollnick note one “awkward” aspect of their model is the symmetry mismatch between TRAP and AT. The 2 crystal structures described here, however, solved at pH 7.0 and 9.0, show that each AT trimer covers exactly 2 TRAP subunits. Chen and Gollnick mention preliminary NMR data showing that AT loses its symmetry upon binding TRAP. Such a result appears to us more compatible with our model of the AT–TRAP interaction because their model appears to have a rather small interface area, inconsistent with tight binding, and leaves much of the AT trimer exposed to solvent. In our model, 1 AT subunit of each trimer makes no interaction with TRAP and the other 2 make defined but very distinct interactions with it.

The AUC data of Snyder and colleagues (12) suggested AT dodecamers bind to TRAP, but did not rule out the possibility that individual AT trimers bind with high cooperativity. Chen and Gollnick reconcile their model with these data by suggesting that the 4 AT trimers do indeed bind in this way. Their model, however, shows the AT trimers to be widely spaced, with no direct contact, and offers no explanation of the considerable cooperativity reportedly required by the AUC data (20). Our model places the *Glu-22A* side-chain of 1 AT trimer within salt-bridging distance of the N-terminal nitrogen atom of *Met-1C* of the adjacent AT trimer. *Thr-24A* also contacts *Leu-36B* and *Thr-37B* of the neighboring trimer. After our demonstration that AT is wholly trimeric at pH 8.5 (SI), we are now in a position to address the question of cooperativity without the complication of trimer–dodecamer equilibrium.

In conclusion, our crystal structures, analytical centrifugation analysis, and mass spectrometry show that AT dodecamer formation is not required for interaction with TRAP, and that individual AT trimers may bind around the TRAP ring. Further work is required to determine whether a single AT trimer bound to TRAP is sufficient to relieve transcription termination, and whether a 12-mer form of TRAP could function equally well in vivo. More generally, our results show the importance of using

a variety of biophysical methods and solution conditions in studies of protein complexes.

Materials and Methods

Gene construction, cloning and mutagenesis are described with protein expression and purification in SI.

Analytical Ultracentrifugation. Sedimentation velocity experiments with TRAP alone, AT alone, and the TRAP–AT complex, were carried out by using an Optima XL-I analytical ultracentrifuge (Beckman–Coulter) using a Beckman An-50 Ti rotor. Four hundred microliters of sample and 420 μ L of reference buffer were loaded into cells with a sapphire windows and a standard Epon 2-channel center piece. Experiments were carried out at 20 °C, the rotor temperature being equilibrated for 2 h before starting each run. Sedimentation was carried out at 50×10^3 rpm and monitored by using interference optics or absorption at 280 nm. The partial specific volume of the protein, solvent density, and solvent viscosity were calculated from standard tables by using the program SEDNTERP (21). Results were analyzed by using the continuous distribution $c(s)$ analysis in the program SEDFIT (22).

Sedimentation equilibrium experiments were carried out with AT alone and T3A7 alone. The buffer and protein concentrations were the same as for the sedimentation velocity experiments (but no tryptophan was added). Absorbance data were collected at 280 nm. All runs were carried out at 20 °C. For AT, data were obtained at 15, 20, and 25×10^3 rpm, and a final speed of 40×10^3 rpm for baseline measurements. T3A7 data were obtained at 6, 8, 10, and 20×10^3 rpm. A total equilibration time of 14 h was used for each speed, with a scan taken at 12 h to ensure that equilibrium had been reached. Data analysis was performed by global analysis of the datasets obtained at different loading concentrations and rotor speeds using XL-A/XL-I Data Analysis Software Version 4.0.

Nano-ESI Mass Spectrometry. Samples for Nanoflow ESI (NanoESI) were prepared by extensive dialysis against 50 mM (for TRAP) or 0.5 or 1 M (for AT and TRAP–AT) ammonium acetate. The final concentration of TRAP was estimated as $\approx 10 \mu$ M (rings) whereas that of anti-TRAP was $\approx 60 \mu$ M (trimers). NanoESI mass spectra were acquired by Q-ToF-2 (Waters) with a nanoESI source. The mass spectra were calibrated with $(\text{CsI})_n\text{Cs}^+$ ions from m/z 2,500 to m/z 8,000. MassLynx version 3.5 software (Waters) was used for data processing and peak integration. The temperature of the ion source was set to 80 °C. An aliquot of 3 μ L of the sample solution was placed in a Waters nanospray tip and electrosprayed with an applied capillary voltage of 0.7–0.8 kV. The pressure in the quadrupole ion guide of Q-ToF-2 was kept relatively high ($\approx 7.5 \times 10^{-3}$ Pa) by throttling down the Speedivalve fitted to the rotary pump. Each mass spectrum was acquired in 4 s, and >10 spectra were accumulated and smoothed with the Savitzky–Golay method.

Data Collection and Refinement. All crystal were cryocooled to -180 °C before collecting data. Diffraction data for T3A7–AT complex were collected at beamline AR-NW12A at the Photon Factory, Tsukuba, Japan, using radiation of 1.0-Å wavelength. A total of 200° of data were collected in 0.5° oscillations. The data were processed to 3.2-Å resolution with HKL2000 (23). X-ray diffraction data for the wild-type TRAP–AT complex were collected at the microfocus beamline BL17A, also using radiation of 1.0-Å wavelength. A total of 120 images were collected by using 1.0° oscillations. Data were also processed to 3.2-Å resolution with HKL2000 for this complex. Data handling was carried out by using the CCP4 suite (24), and molecular replacement was performed with PHASER (25). Molecular replacement was carried out by using a pair of neighboring TRAP subunits, and an AT trimer, as search models. Refinement was carried out by using REFMAC (26), and manual adjustments were made with COOT (27). The stereochemical properties of the models were checked with PROCHECK (28) and the validation tools of COOT. The validation tool MOLPROBITY (29) suggests that the wild-type TRAP–AT complex structure is in the 99–100th percentile. Figures were created with PYMOL (30). Data collection and refinement statistics are shown in SI. The coordinates and X-ray data have been deposited in PDB (codes 2ZP8 and 2ZP9).

ACKNOWLEDGMENTS. We thank Kanako Sugiyama for technical assistance and useful discussions. J.G.H. was supported by a Japan Ministry of Science and Technology (JST) CREST research fellowship. J.R.H.T. was supported by a JST International Collaboration grant.

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