Crystal Structure of the Apo Forms of Ψ55 tRNA Pseudouridine Synthase from Mycobacterium tuberculosis

A HINGE AT THE BASE OF THE CATALYTIC CLEFT

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The three-dimensional structure of the RNA-modifying enzyme, Ψ55 tRNA pseudouridine synthase from Mycobacterium tuberculosis, is reported. The 1.9-Å resolution crystal structure reveals the enzyme, free of substrate, in two distinct conformations. The structure depicts an interesting mode of protein flexibility involving a hinged bending in the central β-sheet of the catalytic module. Key parts of the active site cleft are also found to be disordered in the substrate-free form of the enzyme. The hinge bending causes the active site to act as a clamp to position the substrate. Our structural data further the previously proposed mechanism of tRNA recognition. The present crystal structure emphasizes the significant role that protein dynamics must play in tRNA recognition, base flipping, and modification.

Post-transcriptional modifications of various forms of RNA are essential cellular processes (1). Naturally occurring RNA molecules (tRNA, rRNA, and small nuclear RNA) contain a variety of nonstandard ribonucleosides such as ribothymidine and pseudouridine. Pseudouridine (Ψ), the C-glycoside isomer of uridine, is the most abundant of the modified bases found in RNA (2).

Pseudouridine is formed by the site-specific enzymatic modification of uridine present in tRNA, rRNA, and small nuclear RNA by an ancient and universal class of enzymes called pseudouridine synthases (PUS; EC 4.2.1.70, 2). Based on protein sequence analysis, PUSs were originally classified into four major sequence families (5): (i) TruB (specific for the nearly universal Ψ55 in tRNA); (ii) Rua (rRNA-specific); (iii) RluA (rRNA-specific); and (iv) TruA (tRNA anti-codon loop-specific). In addition, a new sequence family (TruD, Ψ13 tRNA-specific) of PUS was identified recently (4).

Several studies have shed light on the biological implications of pseudouridine bases. Conserved Ψ residues are believed to play important roles in RNA stability, codon recognition, spliceosomal assembly, and other functions (2, 5–7). The importance of Ψ residues is underscored by their tendency to occur in RNA molecules near functionally important regions such as near the peptidyl transfer site in rRNA (8). It has been demonstrated that the deletion of PUS genes results in impaired growth (RluD) and competitive disadvantage (TruB) in Escherichia coli (9–11). The human homolog of TruB (dyserin) is implicated in an X-linked hematopoietic disorder called dyskeratosis congenita (12). Suggestions have been put forward that, apart from their usual catalytic role, certain PUS enzymes (e.g. TruB) may also act as chaperones for RNA folding (11, 13).

Crystal structures have been determined for TruA (14), TruB (15, 16), Rua (17), and RluD (18, 19) from E. coli and TruB from Thermatoga maritima (16). A comparison of those structures indicates a common ancestral linkage and a shared enzymatic mechanism involving a catalytic aspartate residue (17, 20). The structures of TruB from E. coli (EC-TRUB) and T. maritima (TM-TRUB) in complex with an RNA segment mimicking the T stem-loop of tRNA demonstrate that TruB accesses the target U55 of its tRNA substrate by a mechanism involving a base-flipping conformational change (15, 16). A combination of rigid docking followed by induced fit binding was proposed for tRNA recognition based on the significant conformational change observed between the structures of RNA-bound and -free forms of EC-TRUB (16).

Here we present the structure of Ψ55 tRNA pseudouridine synthase from Mycobacterium tuberculosis (MTB-TRUB) at 1.9-Å resolution in two substrate-free forms. The structures depict an interesting mode of β-sheet bending movement affecting the shape of the catalytic cleft as well as a major disordering of the active site in the absence of the RNA substrate. Implications of the above observations are discussed in relation to protein function. The structure of MTB-TRUB was determined as part of the M. tuberculosis structural genomics initiative (21).

EXPERIMENTAL PROCEDURES

Cloning—The Rv2793c gene was amplified by PCR using M. tuberculosis H37Rv genomic DNA as the template, with a forward primer (5'-CCATATGGCTAGCGCAACCGGCCCCGGAATCGTGGTTATCGA-3') that introduced an NdeI site (underlined) and a reverse primer (5’-GGCCGACGATGCACCCCGGGGGTGTACCACGAGGTAAGCTT-3’) that introduced a HindIII site (underlined). The forward primer also inserted an alanine codon (GCT) immediately following the start codon to enhance protein expression (22), while the reverse primer introduced a thrombin recognition sequence to the C terminus. The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen). Following sequence confirmation, the gene was subcloned into pET22b (Stratagene), which added a hexahistidine tag to the expressed protein, trailing the thrombin recognition sequence. The resulting C-terminal extension had the...
amino acid sequence of GVPBGKLAALEHHHHHH immediately following the natural protein sequence.

Expression and Purification—The recombinant protein was expressed at ~10 mg/liter E. coli strain BL21(DE3)-Gold (Stratagene) in enriched buffered Luria-Bertani medium (10 g of NaCl, 40 g of tryptone, 20 g of yeast extract per liter of medium, 5% glycerol, and 10 mM MOPS, pH 7).

Each gram of cell pellet was resuspended and lysed in 5 ml of lysis buffer (20 mM Tris, pH 8, 0.3 M NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol, 2 μg/ml DNase I, 0.2 mg/ml lysozyme, and 1:100 protease inhibitor mixture (Sigma)). The lysate was clarified by centrifugation at 27,000 × g for 30 min. The soluble, recombinant protein was initially purified using nickel-nitrilotriacetic acid Superflow resin (Qiagen). Before the final stages of the refinement, 110 mM (NH₄)₂SO₄, 0.1 M Tris, pH 8.5, and 0.3 M NaCl after purification. The dialyzed sample was concentrated using Centricon YM-10 concentrators (Millipore) and further purified on a Superdex 75 column (Amersham Biosciences) equilibrated with 20 mM Tris, pH 8, and 0.3 M NaCl. The peak fractions were pooled and concentrated to 20 mg/ml. The purified protein was ≥95% pure as estimated by SDS-PAGE. Liquid chromatography-mass spectrometry confirmed the molecular weight of the recombinant protein and the purity of the sample.

Crystalization—The protein was initially crystallized as rod clusters after 4 weeks at room temperature by sitting drop vapor diffusion in which 1 μl of protein concentrate was mixed with 1 μl of crystallization reagent (0.2 M (NH₄)₂SO₄, 0.1 M Tris, pH 8.5, and 250 mM (w/v) PEG 3350 (Hampton index screen formulation 69)) at room temperature. Diffraction-quality crystals were grown with microseeding. In the optimization trial, pre-equilibrated drops of 10 μl of protein mixture with 10 μl of reservoir reagent gave larger and better diffraction crystals. The final crystallization conditions were 110 mM (NH₄)₂SO₄, 0.1 M Tris, pH 7.5, and 17% (w/v) PEG 3350. Crystals grew to ~700 × 400 × 80 μm.

Data Collection—Prior to shock-freezing the crystals in the nitrogen stream for data collection, each crystal was soaked for 5 min in a series of reagents for cryoprotection. The reagents comprised of the same components as the crystallization reservoir solution with the addition of PEG 3350 at a concentration of 35%, followed by 30 and 35%. It was noted that PEG concentrations >35% caused crystals to fracture. All data were collected at the Advanced Light Source beamline 5.0.1 (Richetin YM-10 concentrators (Millipore)) and further purified on a Superdex 75 column (Amersham Biosciences) equilibrated with 20 mM Tris, pH 8, and 0.3 M NaCl. The peak fractions were pooled and concentrated to 20 mg/ml. The purified protein was ≥95% pure as estimated by SDS-PAGE. Liquid chromatography-mass spectrometry confirmed the molecular weight of the recombinant protein and the purity of the sample.

Refinement—Rigid body refinement was performed in REFMAC5 (25) prior to the atomic refinement step using data to 3 Å. An initial attempt to build an atomic model using the ARP (26) automated re-building protocol with data to 1.9 Å failed. A conformational difference between the two chains was obvious from an inspection of the electron density map. Both chains were manually rebuilt in the core region, and the BRUTE (27) option of LSQMAN (28) was subsequently used to build the rest of the model. Manual rebuilding and chain selection were done using the graphical package O (27). A small portion of the data set was used to calculate Rmerge for cross-validation (25). Moderate restraints were initially used for non-crystallographic symmetry restraints applied.

In an attempt to understand the molecular motions in terms of rigid body vibrations, we performed three parallel TLS refinements in REFMAC5 under the following conditions: (i) case I, an entire protein molecule as a rigid body (two TLS groups); (ii) case II, the catalytic and the hinge-based flexibility of the protein is not captured by the minor rigid body vibrations manifested by the proteins in their crystalline environment.

Model Quality—The quality of the final model was quite satisfactory (Table I, Part B). The WHAT-IF (31), PROCHECK (32), and ERRAT (33) programs and several tools from the O program were used for model evaluation. A Ramachandran plot was satisfactory with only 0.8% of the non-glycine residues lying outside the core region (as defined in MOLEMAN; Ref. 34). Residues 54 and 162 are in the disallowed region. The coordinates have been deposited in the Protein Data Bank with the accession code 1SGV.

Surface Area Computation—The solvent-excluded surface area of the molecule was calculated using the MSMS program (35) with a probe radius of 1.4 Å. All hydrogen atoms were generated using the CNS program (36) prior to the surface area computation. The DINO graphics package (http://www.dinodog.org) was used to visualize and inspect the surface.

Software Used for Structure Analysis and Illustration—The BRUTE option of LSQMAN (34), the FIT program (bioinfo1.mbfys.lu.se/~guoguang/fit.html), the LSQ option of O, and several programs from the CCP4 suite (37) were used for structural superposition and analysis. DSSP (38) was used for secondary structure assignments. The DINO and MOLSCRIPT (39) programs were used for making the illustrations.

Results

Overall Structure of MTB-TRUB—The crystal structure of MTB-TRUB was determined with two molecules in the asymmetric unit. Here, we refer to the two molecules as MTB-TRUB-A and MTB-TRUB-B (A and B correspond to the chain names of the two polypeptides in the Protein Data Bank coordinate set). The final structure contains residues 3–292 and 1–294 from 298 residues in the A and B chains, respectively. Residues 115–142 in MTB-TRUB-A and residues 112–139 in MTB-TRUB-B are missing in the electron density map (henceforth referred to as the disordered region) and are not modeled. The structure is composed of two domains (Fig. 1), namely the

### Table I

Data collection and refinement statistics

| Dataset       | MTB-TRUB |
|---------------|----------|
| Part A        |          |
| Space group   | P2        |
| Cell dimension (Å and °) | 45.9, 94.1, 75.8; β = 100.4 |
| Resolution range (Å) | 50.1–1.9 (1.97–1.9) |
| Rmerge (%)    | 0.046 (0.034) |
| Total no. of reflections | 184,403 |
| Multiplicity  | 3.6 (3.2) |
| Completeness (%) | 99.5 (99.5) |
| Average I/σ(I) | 12.4 (2) |

Part B

| No. of reflections | 48,859 |
| Resolution range (highest shell) (Å) | 40.2–1.9 (1.95–1.9) |
| No. of atoms refined | 4,381 |
| Rmerge (%) | 0.20 (0.28) |
| Rmax (%) | 0.24 (0.33) |
| Rfactor (%) | 0.097 |
| Rfree (%) | 0.97 |
| R.m.s.d. bond angle (°) | 5.5 |
| R.m.s.d. dihedral angle (°) | 15.1 |
| Average B (A²) protein atom | 17.1 |
| Average B (A²) protein main chain | 19.2 |
| Average B (A²) protein side chain | 0.8 |
| Average B water (A²) | 43.8 |

### Notes

* Calculated in REFMAC5 (25).
* Calculated in MOLEMAN (34).
* Individual isotropic B-factor in REFMAC5 (25).

only marginally better in case III than in case II or case I. Evidently the hinge-based flexibility of the protein is not captured by the minor rigid body vibrations manifested by the proteins in their crystalline environment.
large N-terminal catalytic domain (residues 1–226) and the short C-terminal RNA-binding domain (residues 227–298). Both domains are of the (α/β) type. The catalytic domain contains 12 β-strands (11 β-strands in the B chain), four α-helices, and two 3_10 helices (Fig. 1). A mostly anti-parallel, mixed, multiply bifurcated β-sheet (henceforth, the central β-sheet) forms the core of the catalytic domain. The C-terminal domain contains a four-stranded mixed β-sheet flanked by one α-helix on each side.

Hinge Bending—A key observation from the crystal structure is the presence of a significant conformational difference, occurring in the central β-sheet, between the two copies of the enzyme visualized in the crystal asymmetric unit (Fig. 1B). As a result, a portion of the catalytic domain (residues 72–199; segment I) moves essentially as a rigid body with respect to the remainder of the catalytic domain (residues 1–71 and 200–226; segment II) between the two chains (Table II and Fig. 2). The observed rigid body motion can be described by an −13° rota-
The LSQMAN (34) program was used to compute the Cn atom-based r.m.s.d. in Å units (corresponding Cn atoms within 8 Å distance are accepted as aligned). The two short stretches for which the rigid body definition breaks down (the β-α-3 loop covering residues 175 to 180 and residues 112 to 148) are omitted for the r.m.s.d. computation.

| Range of residues used for superposition | R.m.s.d. computed for the residue range | R.m.s.d. | No. of Co atoms aligned |
|-----------------------------------------|----------------------------------------|---------|------------------------|
| Segment II (3–71, 200–226)             | Segment II (3–71, 200–226)             | 0.42    | 96                     |
| Segment I (72–111, 149–174, 181–199)   | Segment I (72–111, 149–174, 181–199)   | 0.4     | 85                     |
| PUA domain (227–292)                   | PUA domain (227–292)                   | 0.3     | 66                     |
| Segment II (3–71, 200–226)             | Segment I (72–111, 149–174, 181–199)   | 3.8     | 85                     |
| Segment II (3–71, 200–226)             | PUA domain (227–292)                   | 0.92    | 66                     |

The Active Site—The observed conformational variability leads to certain differences in the active sites of the two MTB-TRUB molecules. The active site cleft is occupied by a few water molecules in both the A and B protein molecules (Fig. 3, A and B). The wall of the cleft is lined mainly by hydrophobic residues (Tyr-70, Leu-199, Tyr-178, Arg-180, Asp-42, Lys-68, Cys-173, Ile-179, Thr-177, Arg-146, and Thr-40; the underscored residues are conserved in the TruB family). Those residues from the shifted segment I that contribute to the catalytic cleft are repositioned in the B chain with respect to the A chain. The Tyr-70 side chain orientation and the Asp-42/Arg-180 salt link, as observed in the structures of other TruB homologs, is maintained in both molecules of MTB-TRUB. It has been suggested that this salt link serves to keep the nucleophilic aspartate in the deprotonated charged form in an otherwise low dielectric environment (15). Based on the different orientations of Asp-42 and Arg-180 in the RNA-bound and substrate-free forms of EC-TRUB, a substrate-induced “conformational switch” has been proposed to align the Asp-42 side chain for catalysis (16). However, our structure suggests that such a conformational change may not necessarily be substrate-induced, because a comparable side chain reorientation of Asp-42 and Arg-180 is observed between the A and B molecules of substrate-free MTB-TRUB (Fig. 3). The observed structures of MTB-TRUB suggest that the two Arg-180 and Asp-42 side chain orientations represent alternate conformations with similar energies that are capable of interconverting by normal protein “breathing.”

Significant differences between the two conformations of MTB-TRUB are also observed for the β-α-3 loop housing the conserved CXXGXX motif (Cys-173/Gly-176/Tyr-178, henceforth called the CGY loop). The alternate conformation of the Lys-68 and Tyr-178 side chains, the CGY loop movement, and the above mentioned bending motion combine to make the uridine substrate (U55) binding pocket in MTB-TRUB less accessible (Fig. 3). The pocket is more open to the bulk solvent in the MTB-TRUB-A conformation. Movement of the Tyr-179 residue (which corresponds to Tyr-178 in MTB-TRUB) was also observed in the substrate-free EC-TRUB, resulting in a more accessible U55 pocket (16).

The Disordered Thumb Region—One of the key observations from the present structure is that a significant portion of that part of the enzyme that grips the tRNA molecule in the major groove (the “thumb”) is fully disordered in both molecules of MTB-TRUB (Fig. 4, A and B). Residues 121–152 in EC-TRUB form a protrusion or so-called thumb that constitutes one side of the binding cleft and accommodates the flipped bases of the substrate (15). The highly conserved thumb region makes numerous interactions with the flipped bases and the cognate sugar/phosphate backbone in the major groove of bound RNA. This protruding part is mostly disordered in both molecules of the MTB-TRUB as well as the apo form of EC-TRUB (16). The disordered thumb in the MTB-TRUB structure reinforces the previous suggestion (16) that this region is likely unstructured in solution and apparently undergoes a disorder-to-order transition upon binding RNA.
The C-terminal Domain—A recurring theme in RNA modification enzymes is the acquisition of small RNA binding domains to form an extended RNA binding surface (40). The C-terminal domain of the MTB-TRUB is a PUA (40) domain, which is commonly found fused to RNA modification enzymes (PUS, RNA methylases, and archaeosine tRNA-guanine transglycosylase (ATG); Ref. 41), but occasionally as a single domain protein. This short domain contains several exposed basic residues. In the search for structures similar to the C-terminal domain of MTB-TRUB using DALI (42), apart from EC-TRUB (Z_score = 7.8, 1.9 Å r.m.s.d. for 64 aligned Ca atoms) the most similar structure identified was the RNA-modifying enzyme archaeosine tRNA-guanine transglycosylase (Protein Data Bank code 1J2B, Z_score = 6, 2.9 Å r.m.s.d. for 63 aligned Ca atoms).

The apo form of EC-TRUB also shows a hinge-bending motion with respect to the RNA-bound EC-TRUB involving a significant movement of the C-terminal domain (Fig. 4) (16).
The overall orientation of the C-terminal domain with respect to the rest of the protein is marginally different for the two MBT-TRUB molecules (1 A r.m.s.d.; Table II; Fig. 1B). This domain is envisaged as another flexible element of the TruB structure that assists in tRNA recognition (16).

The RNA Binding Surface—A plot of the acidic and basic residues in the solvent-excluded substrate binding surface of MBT-TRUB shows the parts of the surface that would form the tRNA binding surface (Fig. 1D). The RNA binding surface is quite similar in all of the reported structures of TruB (EC-TRUB, TM-TRUB, and MBT-TRUB). TruB enzymes do not necessarily require an entire tRNA molecule for recognition and catalysis (43, 44). However, the extensive tRNA binding surface in TruB apparently serves in vivo to stabilize parts of the tRNA molecule beyond the recognition element.

DISCUSSION

The crystal structure of MTB-TRUB at atomic resolution provides two views of the substrate-free enzyme. The two quite different conformations observed in the present structures apparently have similar energies with the distinct conformations being preferred in the two different packing environments in the crystal asymmetric unit. Both molecules make about the same number of intermolecular contacts (contact distance of \( \leq 4 \) A, computed using CONTACT; Ref. 37) with the surrounding protein chains in the crystal, whereas the B form makes a few additional intra-molecular interactions at the catalytic cleft. The two alternate conformational substates that are stabilized by weak crystallographic packing interactions almost certainly reflect a range of conformations to which the enzyme has access in vivo. Interestingly, the different conformers reveal the presence of a hinge region at the bottom of the active site cleft. In support of this, a preliminary structure of MBT-TRUB has also been determined at 3- \( \AA \) resolution from a monoclinic crystal form with four molecules in the asymmetric unit (data not shown). Although those structures are not described in detail here because of the relatively poor resolution, the certain reflect a range of conformations to which the enzyme must encounter as they attempt to flip the bases out of their pairing regions.

Thus, the crystal structures of TruB strengthen the view that protein flexibility and dynamics play key roles in tRNA recognition and modifying reactions involving base-flipping mechanisms.

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