RNA helicase module in an acetyltransferase that modifies a specific tRNA anticodon

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Post-transcriptional RNA modifications in the anticodon of transfer RNAs frequently contribute to the high fidelity of protein synthesis. In eubacteria, two genome-encoded transfer RNA (tRNA) species bear the same CAU sequence as the anticodons, which are differentiated by modified cytidines at the wobble positions. The elongator tRNA^{Met} accepts an acetyl moiety at the wobble base to form N⁴-acetylcytidine (ac⁴C): an inherent modification ensures precise decoding of the AUG codon by strengthening C–G base-pair interaction and concurrently preventing misreading of the near cognate AUA codon. We have determined the crystal structure of tRNA^{Met} cytidine acetyltransferase (TmcA) from *Escherichia coli* complexed with two natural ligands, acetyl-CoA and ADP, at 2.35 Å resolution. The structure unexpectedly reveals an idiosyncratic RNA helicase module fused with a GCN5-related N-acetyltransferase (GNAT) fold, which intimately cross-interact. Taken together with the biochemical evidence, we further unravelled the function of acetyl-CoA as an enzyme-activating switch, and propose that an RNA helicase motor driven by ATP hydrolysis is used to deliver the wobble base to the active centre of the GNAT domain.

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Introduction

Most cellular RNAs require post-transcriptional chemical modifications to be mature and functional (McCloskey and Crain, 1998). Among more than a hundred modified ribonucleosides identified to date, the vast majority are found in transfer RNA (tRNA), particularly embedded in the anticodon loop region (Grosjean *et al*, 1996). Modified bases at the first (wobble) position of the tRNA anticodons are involved in maintaining the fidelity of genetic information transfer by

stabilizing specific codon–anticodon interactions to prevent misreading of non-cognate codons, and to ensure the precise reading frame (Björk *et al*, 1999; Suzuki, 2005; Agris *et al*, 2007). Additionally, wobble modifications coincidentally serve as determinants for recognition by cognate aminoacyl-tRNA synthetases (aaRSs) (Muramatsu *et al*, 1988a; Sylvers *et al*, 1993; Senger *et al*, 1997). In γ -proteobacterial cells including *Escherichia coli*, the decoding system for AUR (R = A or G) codons is dominated by a pair of the modifications at the wobble positions of the two tRNAs that share the same CAU anticodons: the AUA codon-specific isoleucine tRNA (tRNA^{Ile2}) and the elongator tRNA^{Met} responsible for AUG decoding (Supplementary Figure S1). Modifying the wobble cytidine base of tRNA^{Ile2} to lysidine (k²C) results in a conversion of codon and amino-acid specificities from AUG to AUA and methionine to isoleucine, respectively (Muramatsu *et al*, 1988a,b). The essential enzyme that synthesizes lysidine at the first letter of the anticodon of tRNA^{Ile2} was previously identified as TILS (tRNA^{Ile}-lysidine synthetase) and characterized (Soma *et al*, 2003; Ikeuchi *et al*, 2005). Meanwhile, the elongator tRNAs^{Met} contain specific N⁴-acetylcytidine (ac⁴C) at the same position. This modified wobble base has been reported to stabilize the C3'-endo puckering conformation of ribose, encouraging the C–G base-pair interaction, thereby ensuring the decoding of the AUG codon as methionine (Kawai *et al*, 1989). Moreover, *in vitro* translation experiments using *E. coli* tRNA^{Met} suggested that ac⁴C also has an important function in preventing errors that may arise during protein synthesis due to misreading of isoleucine AUA codon by tRNA^{Met} (Stern and Schulman, 1978). Hence, these two modification enzymes that generate a pair of wobble modifications essentially determine the accuracy of the decoding property of each tRNA, and the protein synthesis as well.

Most recently, Suzuki and colleagues (Ikeuchi *et al*, 2008) have successfully identified a non-lethal gene, *ypfI*, responsible for ac⁴C formation at the wobble base of the elongator tRNA^{Met}, which therefore has been renamed *tmcA* (tRNA^{Met} cytidine acetyltransferase). *E. coli tmcA*, which belongs to the orthologous cluster COG1444, encodes an uncharacterized enzyme of 671 amino-acid residues with a molecular mass of 75 kDa. Cautious inspection of the sequence alignments revealed a pair of highly conserved regions: the DUF699 and Acetyltransf_1 Pfam domains near the amino and carboxyl termini, respectively. DUF699 is approximately 150 amino-acid residues long and appears to have a distinctive 'AxRGRGKS' P-loop (also known as Walker A) motif, implying inherent ATPase activity. Indeed, *in vitro* synthesis of ac⁴C revealed that TmcA exploits acetyl-CoA as an acetyl group donor, which is transferred in an ATP-dependent manner (Ikeuchi *et al*, 2008). Neither structural information nor likely roles of DUF699 ATPase activity in ac⁴C synthesis have so far been elucidated. In this study, to provide the precise mechanistic insight into site-specific RNA acetylation that maintains the decoding fidelity in bacterial translation system,

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we solved the crystal structure of *E. coli* TmcA bound with acetyl-CoA and ADP at a resolution of 2.35 Å. In the structure, TmcA reveals a unique four-domain configuration in which acetyl-CoA- and nucleotide-binding sites are unexpectedly separated by 30 Å. Strikingly, with no detectable sequence similarity, the peripheral N-terminal and DUF699 domains structurally mimic the DEAD-box RNA helicases, forming duplicated RecA-like fold in a non-canonical inverted topology as the P-loop resides in the C-terminal part. Combining our structural interpretation with a wealth of biochemical studies, we propose an unprecedented model for an RNA-modification enzyme that incorporates an RNA helicase module designed to remodel the substrate structure, and give access to the modified sites.

Results

Overall structure of *E. coli* TmcA complexed with Acetyl-CoA and ADP

Our initial attempts to crystallize the full-length *E. coli* TmcA fused with a hexahistidine tag at either the N or C terminus were unsuccessful. Both tag removal and supplementation with acetyl-CoA were indispensable for yielding quality crystals (Supplementary Figure S2). Finally, the structure of TmcA was solved in the orthorhombic space group $P2_12_12_1$ at 2.35-Å resolution using a selenomethionine-substituted protein, and refined to an R_{free} of 0.274 and R -factor of 0.234 (Table I). The two TmcA molecules in an asymmetric unit (labelled A and B to distinguish them) were essentially identical, with a root mean square deviation (r.m.s.d.) of 0.9 Å for all corresponding C α atoms. ATP and MgCl₂ were also included in the crystallization conditions with the aim of characterizing the ATP-binding and hydrolysis activities of TmcA. Unexpectedly, there was only one detectable ADP molecule, presumably resulting from the TmcA-catalysed

ATP hydrolysis, bound to monomer A. In contrast, the site that was occupied by the β -phosphate in monomer A bore obvious density for a sulphate ion in monomer B (Supplementary Figure S2). The absence of a nucleotide cofactor in monomer B likely gave rise to higher temperature B -factors in the DUF699 domain, and thus we hereafter focus on monomer A for structural description, unless otherwise stated.

Overall, the current structure is well ordered except for 11 residues (55–65) in the central portion of the extended loop flanked by the β 2- and β 3-strands, which were not modelled. TmcA is apparently a four-domain protein in an L-shaped morphology with the dimensions of 60 Å × 70 Å × 70 Å, composed of three mixed α/β -domains and a single exclusively helical domain at the C terminus (Figure 1B). TmcA represents a novel structure with unique domain organization based on a search for similar structures by the DALI engine. The peripheral N-terminal globular domain (hereafter referred to as ‘the head domain’) is formed by the N-terminal residues 1–171 and residues 321–336 from the middle, constructing a central parallel five-stranded β -sheet (β 1– β 5) sandwiched by α -helices on both sides (Figure 1B and F). The juxtaposed α/β -domain is the Pfam-classified DUF699 domain (residues 172–320), which is connected to the head domain by a lengthy linker (~60 Å) that folds back onto the C terminus of the α 1 helix. Apparently, the size and conformation of the DUF699 domain closely resembles those of the head domain with a few additions of a β -strand and two flanking short helices on one side. Superposition of the head and DUF699 domains of TmcA gives a moderate r.m.s.d. of 3.4 Å for 108 pairs of C α atoms compared. This observation was hardly predictable from sequence comparison as DUF699 and the head domains share only 12% amino-acid sequence identity.

The C-terminal tail of DUF699 domain folds back again into the head domain and sticks an α 12 helix on the backside of the β -sheet in the head domain (Figure 1F). A short helix

Table I Summary of crystallographic data and refinement statistics

Data collection	Selenium crystal 1			Selenium crystal 2
Data set	Peak	Edge	Remote	
Space group		$P2_12_12_1$		$P2_12_12_1$
Unit cell (Å)		$a = 61.1, b = 101.2, c = 263.5$		$a = 61.3, b = 101.0, c = 263.1$
Wavelength (Å)	0.9791	0.9797	0.9645	1.0000
Resolution (Å) ^a	50–2.90 (3.00–2.90)	50–2.90 (3.00–2.90)	50–2.90 (3.00–2.90)	50–2.35 (2.43–2.35)
Observed total/unique reflections	266 351/36 980	268 698/36 912	265 695/36 883	425 934/66 310
Completeness (%) ^a	100.0 (100.0)	100.0 (100.0)	100.0 (100.0)	96.6 (92.1)
R_{merge} (%) ^{a,b}	13.5 (64.0)	13.2 (65.5)	13.6 (67.0)	5.6 (37.3)
I/σ (I) ^a	9.1 (2.4)	9.0 (2.4)	8.9 (2.3)	25.3 (4.7)
Redundancy ^a	7.2 (7.3)	7.3 (7.4)	7.2 (7.3)	6.4 (6.1)
Refinement				
Resolution (Å)				20–2.35
Number of reflections (working/free)				61 032/5079
R -factor/ R_{free} (%) ^c				23.4/27.4
Protein/ligand/ion/water atoms				10 411/129/5/350
Ramachandran (%) ^d				88.5/11.3/0.0/0.2
r.m.s.d. bonds (Å)/angles (deg)				0.015/1.99
Average B -value (Å ²)				47.5

^aValues in the parentheses refer to the highest resolution shell.

^b $R_{\text{merge}} = \sum_j \sum_i |I_j - I_{j,i}| / \sum_j \sum_i I_{j,i}$, where $\langle I_j \rangle$ is the average intensity of reflection j for its symmetry equivalents.

^c R -factor = $\sum \|F_{\text{obs}} - |F_{\text{calc}}|\| / \sum |F_{\text{obs}}|$. R_{free} is calculated for a randomly chosen 7% of total reflections that were not used for structure refinement.

^dFractions of residues in the most favoured/allowed/generously allowed/disallowed regions of the Ramachandran plot.

Figure 1 Overall structure of *E. coli* TmCA. (A) Sequence alignment of the seven consensus motifs found in TmCA and its homologues from all kingdoms. *E. coli* TmCA used for structure determination is in the top row and is numbered. Seven other homologues from *Haemophilus influenzae*, *Vibrio vulnificus*, *Pyrococcus horikoshii*, *Haloarcula marismortui*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Homo sapiens* are listed from the top. (B) Ribbon representation of the TmCA crystal structure shown in a top-down view. Each domain is differently coloured and labelled. Bound ADP and acetyl-CoA are depicted in a ball-and-stick representation. (C) An edge-on view reveals an L-shaped pot with an acetyl-CoA plug. (D) Solvent-accessible surface is shown in the same orientation as (B) and is coloured according to the electrostatic potential calculated by the program GRASP (blue for positively charged and red for negatively charged). Bound ADP and acetyl-CoA are drawn as spherical models. (E) Coordination of ADP by the DUF699 domain. Side chains involved in interactions with ADP are depicted in a ball-and-stick representation and are labelled. For clarity of the hydrogen-bonded network (cyan dots), the backbones of the motif I (P loop) are drawn as sticks. Water molecules are shown as cyan spheres. Nitrogen atoms are coloured in blue, and oxygen in red. The omit $F_o - F_c$ difference electron density map (3.5σ , magenta) of ADP is superimposed onto the refined coordinates. (F) Topology diagram of TmCA. The domains are coloured as in (B) and secondary elements are labelled.

($\eta 3$) following $\alpha 12$ connects two N-terminal repeated α/β -domains with the middle catalytic 'body' domain. The body domain of TmCA is made by a planar mixed α/β -topology

containing a central seven-stranded β -sheet ($\beta 12$ – $\beta 18$) bordered by six α -helices ($\alpha 13$ – $\alpha 17$, and $\alpha 20$) and backed by two α -helices ($\alpha 18$ and $\alpha 19$). It possesses an Acetyltransf_1 Pfam

Figure 2 Structural comparison to well-known RNA helicases. **(A)** Superposition of the DUF699 domain of TmcA onto the N-terminal RecA-like domains of various DEAD-box RNA helicases. The C-terminal domains of both Vasa and HCV NS3 helicases rotate towards the N-terminal domains revealing a closed conformation upon binding of the substrate RNAs (both 5' and 3' termini are marked). **(B)** In the upper panel, the universally conserved sequence motifs among the DEAD-box helicases are mapped onto the Vasa structure. Each motif is differently coloured and amino-acid sequences are given. Symbols used are as follows. x: any amino-acid residue; o: small hydrophilic residues such as serine or threonine; h: hydrophobic residues. Bound poly(U) RNA is illustrated as dark sticks. Mg^{2+} is shown as a green sphere. The canonical helicase motifs are structurally superimposed onto the conserved sequences in the N-terminal region of TmcA (lower panel). Note that the two N-terminal domains of TmcA are in an inverted topology compared with Vasa (i.e. the P-loop resides in the N-terminal domain of Vasa but it resides in the C-terminal domain of TmcA).

signature, indicating the acetyltransferase catalytic centre. Indeed, acetyl-CoA binds to a wedge-like opening in the centre of the domain, which is made by breaking the two parallel β 15 and β 16 strands by halves. A characteristic 'β-bulge' that contributes to this distortion is formed by a bifurcated hydrogen bond between the main chains of Trp410 and the two adjacent Ser459–Arg460 residues. At the end of the body, the bordering α 20 helix makes hydrogen-bonding interactions with the capping 'tail domain', and is kinked at Arg550 triggered by a salt bridge with Asp577 on the α 21 helix in the tail domain. The tail domain is an antiparallel bundle of six helices with alternating up–down topology (α 21– α 26), forming a bulky helical bundle together with α 16, α 17, and η 4 of the body domain. This creates a shallow positively charged inner concave by clusters of arginine

and histidine residues, which plausibly contribute to the tRNA-binding activity (Figure 1D).

The N-terminal head and DUF699 domains structurally mimic DEAD-box helicases

The two tandemly repeated α/β -domains at the N terminus of TmcA seemingly reveal structural duplication of a module within the molecule with a great deal of amino-acid residue substitution. They are oriented in such a way that the β -sheets lie approximately 60° to each other, and are in close proximity, so that they can be arranged to form a deep interdomain cleft where ADP binds. The presumable phosphate-binding P-loop (Walker A) motif resides in the DUF699 domain flanked by α 7 and β 6, and is absent in the head domain. This appearance is reminiscent of the

DEAD-box RNA helicases that are composed of two successive RecA-like domains. As expected, the highest structural similarity to the head and DUF699 domains was found in a DEAD-box protein from the hyperthermophile *Methanococcus jannaschii* (1HV8; Story *et al*, 2001), with a C_{α} r.m.s.d. of 3.1 Å (Figure 2A). The relative orientation of the head and DUF699 domains in the TmcA structure differs significantly from that in the 'closed' form of the DEAD-box protein complexed with poly(U) RNA and non-hydrolysable AMPPNP (Kim *et al*, 1998; Sengoku *et al*, 2006). The structure most likely represents an alternative 'open' state prior to accommodating RNA substrate as observed in other core structures of DEAD-box proteins, such as eIF4A, MjDEAD, and UAP56 (Caruthers *et al*, 2000; Story *et al*, 2001; Shi *et al*, 2004). Bearing this in mind, binding of tRNA^{Met} to TmcA possibly causes a drastic movement of the DUF699 domain towards the head and body domains, which may be necessary for the recognition and/or the positioning of tRNA.

To date, all characterized DEAD-box proteins carry a core composed of two RecA-like domains with the P loop always occurring in the N-terminal domain. Remarkably, in contrast to the universal topology of the DEAD-box helicases, TmcA harbours the ATPase site in the DUF699 domain C-terminal to the head domain, that is, in a permuted topology (Figure 2B). This is ascribed to a stretched turn loop between $\beta 5$ and $\alpha 6$, by which the head and DUF699 domains are linked. This dramatic topological difference between TmcA and the DEAD-box helicases explains why earlier sequence analyses could not envisage their structural resemblance.

A number of well-known conserved motifs in the DEAD-box helicases are lined along the interdomain interfaces between the two RecA-like domains (Figure 2B) (Tanner and Linder, 2001; Cordin *et al*, 2006), where mutual inter-motif interactions have an important function in coupling ATP hydrolysis to helicase activity (Sengoku *et al*, 2006). Although the sequences in these regions are hardly preserved in the N-terminal domains of TmcA, most elements can be annotated in terms of an equivalent structural relationship. It is noted that these pseudo-helicase motifs are also strictly conserved in TmcA among species (Supplementary Figure S3). Accordingly, we readily noticed a motif II (Walker B motif)-like sequence at the end of the $\beta 9$ strand with a remarkable DEAA sequence. Furthermore, we discovered three unrecognized sequence motifs that we designated TmcA-specific (TS) motifs 1, 2, and 3, following the aberrant DEAA motif in TmcA (Figure 2B; Supplementary Figure S3). On the basis of the structural relationship, the TS1 (YEGxG) and 2 (hRW/Y) motifs correspond to motifs III and VI of the DEAD-box proteins, respectively. The invariant Ser-Ala-Thr (SAT) sequence in motif III, which is essential for RNA-unwinding activity but not ATPase activity for DEAD-box helicases (Pause and Sonenberg, 1992), is no longer present in the TS1 motif. The large-scale amino-acid substitution in the corresponding helicase motifs of TmcA could be rationalized by intimate interactions among motifs II, III, and VI (DEAD, SAT, and HRIGR) seen in the complex structures of DEAD-box helicases with bound synthetic poly(U) RNA (Bono *et al*, 2006; Sengoku *et al*, 2006).

Long-range communication between the two remote active sites

The nucleotide-binding pocket is formed within a cleft between the head and the DUF699 domains. TmcA has

intrinsic hydrolysis activity as implied in the crystal structure, and described in recently published paper (Ikeuchi *et al*, 2008). The adenine base is held in a hydrophobic pocket between Phe236 and Ile318 (Figure 1E). Ile318 is a conserved hydrophobic side chain in the TS2 motif, and also makes a van der Waals interaction with the ribose ring, together with Val232 and the methylene group of Arg319. The adenine base is specifically selected through bifurcated hydrogen bonds with Gln180 in the Q motif, and further assured through hydrogen bonding with Gln211. From the TS2 motif, Arg319 contacts the ribose 3'-OH. TmcA may not be able to rigorously differentiate dATP or GTP from ATP as the 2'-OH is not discerned and the interacting glutamine side chain is capable of acting both as the hydrogen acceptor and the donor against the C6 exocyclic groups of adenine and guanine, respectively. As predicted, these were possible substrates for the hydrolysis reaction (Ikeuchi *et al*, 2008). The more promiscuous use of NTPs has so far been reported in cases of DEAH-box proteins (for example, see Tanaka and Schwer, 2006). On the other hand, the phosphate moiety is directly recognized by the invariant Lys205 and Ser206 residues in the P loop, and is anchored by the backbone amide nitrogens of residues 202–207 in the P loop. ADP binds to the hydrolysis pocket of TmcA in a way compatible with that of Vasa except for a rotation of the adenine base by 48° around the N-glycosidic bond. It is notable that the second arginine residue in motif VI (HRxGRxGR) of DEAD-box proteins, which has been proposed to function as an 'arginine finger' stabilizing the transition state of the hydrolysed intermediate (Scheffzek *et al*, 1997; Caruthers and McKay, 2002), is replaced by an extra Arg201, the first arginine in the P loop (AxRGRGKS) of TmcA.

Around 30 Å away from the hydrolysis site is an acetyl-CoA-bound slot, where the transfer of the acetyl group to RNA should be carried out. The region encompassing the cofactor-binding site, 'the body domain', revealed the structure most homologous to the ubiquitous GCN5-related N-acetyltransferase (GNAT) superfamily, which comprises the nuclear histone acetyltransferases (HATs) and others (Supplementary Figure S4) (Vetting *et al*, 2005). The two sequence motifs of the GNAT folds, motifs GNAT A and GNAT B, form the essence of the acetyl-CoA-binding site in TmcA. The cofactor acetyl-CoA is trapped through direct hydrogen bonds between its pyrophosphate group and main chain amides of residues Arg469–Arg474 at the amino end of $\alpha 18$ in the GNAT A motif, and between its 3'-phosphate group and Arg506 in the GNAT B motif (Supplementary Figure S4). The pantetheine group lies on the hydrophobic platform made by both $\beta 15$ and $\beta 16$ in the GNAT motifs A and B, respectively, and is sharply bent, probably due to a specific interaction with Gln468. The adenine ring is recognized by Glu499 in motif B, whereas the acetyl group on the other end is barely constrained. The nearest atom to the carbonyl oxygen of acetyl-CoA is the backbone amide nitrogen of Ile461, which is 3.7 Å away.

In the RNA modification process, ATP is often utilized by enzymes for the activation of premodified sites through an adenylate intermediate prior to nucleophilic substitution by the donors. Recently solved structures of tRNA-modifying enzymes (e.g. TilS and MnmA) hold both ATPs and substrates within the immediate vicinity to prevent undesirable hydrolysis of the labile intermediate (Numata *et al*, 2006). As

acetyl-CoA is an activated carrier, it is intuitive that TmcA should not require ATP for RNA base activation to synthesize ac⁴C. However, *in vitro* analysis of TmcA demonstrated that ATP hydrolysis was essential for ac⁴C formation (Ikeuchi *et al*, 2008). In addition, we were aware of a channel that is flanked on both sides by a line of the most characteristic conserved motifs, which orchestrates an extensive and complicated interaction network combining two remote active sites (Figure 4A). From the ATPase centre, Arg203 in the middle of the P loop is in a van der Waals contact with Pro317, and forms a salt bridge with Glu327 in the TS2 motif, which in turn is hydrogen-bonded to Trp320. The P loop also directly interacts with the TS1 motif through a putative arginine finger (Arg201) sandwiched by Tyr291 and Thr287, and is further bridged indirectly to the TS3 motif by electrostatic interactions formed along the path Arg201 (I)–Glu292 (TS1)–Arg379 (TS3). There is a characteristic threonine cluster (residues 285, 286, 287, and 294) at the bottom of the TS1 hairpin, where the P loop, the motif II, and the TS1 motif are linked together through Ser206 (I)–Asp262 (II)–Tyr285 (TS1) with a water molecule. Finally, two residues, His 377 and Asp384, in the TS3 motif cooperatively pin Arg460, the residue juxtaposed to Ile 461 in the GNAT A motif. It is noteworthy that almost all relevant residues listed above are unique to and evolutionarily conserved in TmcA; therefore, this long-range interaction network is quite different from that of DEAD-box helicases (Sengoku *et al*, 2006), and may represent an unparalleled remote communication that has an important function in coupling ATPase with acetyltransferase activities in TmcA (see below).

Acetyl-CoA switches on both ATPase- and tRNA-binding activities

Several lines of evidence have indicated that typical RNA helicases hydrolyse ATP in an RNA-dependent manner (Cordin *et al*, 2006). At the same time, HATs and probably other GNAT proteins have an ordered Bi-Bi mechanism for acetyl-CoA-dependent substrate-specific binding (Lau *et al*, 2000; Tanner *et al*, 2000). What is the case with TmcA, in which both modules are integrated? To seek out the undefined roles of cofactors in tRNA^{Met} binding, we carried out electrophoretic mobility shift assays (EMSAs) using *in vitro* transcribed unmodified tRNAs as substrates (Figure 3A). The results confirmed a high potential ability of TmcA to discriminate tRNA^{Met} from tRNA^{Ile2} (Figure 3B). However, specific binding activity was quite low in the absence of cofactors, and was not turned up by additions of either ATP or its non-hydrolysable derivative, ADPCP. In contrast, the acetyl-CoA cofactor boosted tRNA-binding activity, and was essential for the formation of a stable TmcA–tRNA^{Met} complex. Supplying this prereaction complex with ATP, but not ADPCP, resulted in the release of tRNA from the complex as the reaction was completed. Coenzyme A alone could not retain tRNA-binding activity, nor did CoAs with bulky thioesters (Figure 3A and B). These results, therefore, underline an achievement of high selectivity for both RNA substrate and cofactor by TmcA, and suggest that the acetyl moiety has a key function in the rational ‘up- and downregulation’ of tRNA-binding affinity during the course of RNA acetylation.

Regarding hydrolysis activity, TmcA has, unlike the DEAD-box helicases, an intrinsic basal ATPase activity regardless of tRNA binding as recently reported (Ikeuchi *et al*, 2008).

ATP binds to TmcA even more tightly (K_m value $\sim 3 \mu\text{M}$) compared with the DEAD-box helicases or other proteins that bind both ATP and RNA (Cordin *et al*, 2006). Most intriguingly, although the two factor-binding sites in TmcA are $\sim 30 \text{ \AA}$ distant, acetyl-CoA accelerates ATP hydrolysis by promoting a three-fold increase in the turnover rate to near maximum (Ikeuchi *et al*, 2008). To conclude, binding of acetyl-CoA to TmcA simultaneously activates both ATPase- and tRNA-binding activities.

Mutational studies

To examine the function of conserved sequence motifs of TmcA in RNA acetylation, we carried out alanine-substituted mutational analyses (Figure 4D; Supplementary Figure S5). First, we introduced a series of TmcA variants into an *E. coli* strain that contained a deletion of its endogenous TmcA (*tmcAΔ*), and subsequently evaluated the presence of ac⁴C by mass spectrometric analyses of the total RNA isolated from those cells (Figure 4B). Over half of the TmcA mutants we tested could not effectively alleviate the defect in ac⁴C formation *in vivo* (Figure 4D). Besides the expected vital functions of residues inside the P loop (Arg201; an alternative arginine finger, and Lys205) and the Walker B motif (Asp262), complementation experiments also unveiled significant roles of all the three characteristic TS motifs in RNA acetyltransferase activity. Dissecting their biological functions at each stage in the reaction, we further assayed ATPase- and tRNA-binding activities using purified enzyme variants. All mutant proteins, except for E414A, which was hardly soluble and was not pursued further, were highly expressed and soluble comparable to the wild-type protein, promising their proper folding in solution. The *in vitro* assays definitively categorized the essential TmcA mutations into two groups with the obliteration of either ATPase- or tRNA-binding activity. Point mutations in the P-loop and the Walker B motif caused drastic reductions in ATPase activity, whereas tRNA binding was far less affected. The two essential residues, Asp262 and Glu263, do not make direct contact with ADP in the structure but are presumably indispensable for anchoring Mg^{2+} ion during the hydrolysis reaction, as observed in other helicase structures (Sengoku *et al*, 2006). The R319A and E327A mutants similarly abolished ATPase activity without influencing tRNA-binding capacity. Arg319 in the TS2 motif directly interacts with the ribose 3'-hydroxyl group, and Glu327 makes a salt bridge with Arg203 (P loop) that is necessary for efficient ATPase activity. This salt bridge is not involved in ADP recognition in the current structure and its precise role in the hydrolysis reaction remains elusive. Corroborated by recent structural studies on Upf1 helicase, the local conformational alterations are coupled with the release of γ -phosphate and undergo about 4 Å displacement of motifs V and VI away from the ATP-binding site (Cheng *et al*, 2007). As the TS2 motif is structurally related to motif VI in the DEAD-box proteins, this suggests a regulatory role for the Glu327–Arg203 salt bridge in the hydrolysis cycle upon ATP binding. Defective ATPase activity was also detected due to T287A, Y291A, and E292A mutations in the TS1 motif. It was reported earlier that alanine substitution of the corresponding motif III (SAT) in the eIF4A DEAD-box helicase abolished RNA unwinding, but instead enhanced ATPase activity (Pause and Sonenberg, 1992). It is noteworthy that two mutations, R296A and K301A, of conserved basic

Figure 3 Electrophoretic mobility shift assays (EMSAs) using *in vitro* transcribed tRNAs. **(A)** EMSA with the natural substrates. Non-denaturing polyacrylamide gels were stained with Coomassie Brilliant Blue (upper panel) or ethidium bromide (lower panel) to visualize proteins and RNA, respectively. Bands corresponding to 50 pmol recombinant TmcA protein and tRNA^{Met} are shown in the first and second lanes, respectively. Loaded protein was fixed at 50 pmol throughout the experiments, whereas the amount of tRNA ranged from 25 ($\times 0.5$ times excess of enzyme) to 200 ($\times 4$) pmol. The chemical structure of CoA is illustrated at the top of its lane but only the terminal moiety is shown for acetyl-CoA. Arrows indicate the positions of TmcA–tRNA complex bands. Asterisk indicates uncharacterized bands possibly responsible for the nonspecific binding of TmcA to RNA due to the extensive positively charged surface. **(B)** EMSA with bulky thioester groups as well as tRNA^{Ile}.

residues at the end of the TS1 motif possessed moderate ATPase activity but impaired tRNA-binding activity, revealing a bilateral character of the TS1 motif. On the other hand, the R387A mutant of the TS3 motif completely abolished its tRNA-binding capacity but still sustained its function *in vivo*. We have no plausible explanation for this apparent discrepancy between the *in vivo* and the *in vitro* results at this time. Moreover, both ATPase- and tRNA-binding activities were significantly reduced by the critical H377A mutations in the TS3 motif. Interestingly, His377 stacks on and is hydrogen-bonded to an invariant Arg460 in the GNAT A motif, whose alanine-substituted mutant also failed to bind tRNA. As His377 is ~ 20 Å apart from the β -phosphate of ADP, atypical long-range inter-motif interactions should have a key function in regulating a cycle of conformational changes coupled to ATP binding and hydrolysis by TmCA.

Is TmcA a tRNA^{Met}_{CAU}-specific DEAA-box RNA helicase?

To address this challenging issue, we have developed a sensitive assay by modifying traditional *in vitro* unwinding experiments (Rogers *et al*, 2001). tRNA^{Met} was mixed with an

abundance of TmcA at 298 K, and subsequently, the reactions were quenched and the tRNA configuration was concurrently locked by immediately adding adequate amounts of EDTA and Proteinase K, and then the mixtures were analysed on a gradient native gel at 277 K (Figure 5A). The results showed that TmcA hydrolysed ATP, triggering an unbiased band shift of tRNA^{Met} but not tRNA^{Ile2}. The presence of this distinctive slow-migrating band was not reproducible when ATP was removed or replaced by the non-hydrolysable ADPCP. These results suggest that structural changes in substrate tRNA occur during the course of ATP hydrolysis before the acetyl group is transferred. We further attempted to explore the transient rearrangement of the tRNA conformation by utilizing the method of enzymatic RNA probing. Although those experimental results confirmed the rigorous identity elements on tRNA^{Met} for TmcA recognition that were identified earlier by tRNA mutational studies (Supplementary Figure S1), we could not gain a conclusive evidence for the structural rearrangement of tRNA through the hydrolysis of ATP by TmcA (data not shown). Our failure might be due to the rapid conformational conversion of tRNA during the course

of hydrolysis of ATP, which alleviated differences in the cleavage patterns, and the requisite magnesium ions in the reactions most likely drove the refolding of tRNA soon

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Figure 5 Biochemical evidence supports RNA-unwinding activity of TmcA and proposed reaction model. (A) Modified RNA-unwinding activity assay (see Materials and methods for details). Red arrows and cross emphasize the positions of the extra-shift bands observed only in the case of tRNA^{Met}. (B) *In vitro* acetyltransferase assay with the wild-type (open circle) or A31U mutant (filled circle) of tRNA^{Met}, which was detected by liquid scintillation counting of ¹⁴C-acetyl group. (C) Model of RNA acetylation by TmcA. The molecular surfaces of TmcA are coloured light grey. Both ligands, ADP and acetyl-CoA, are depicted as spheres. Point mutations severely affecting tRNA-binding activity are coloured red on the protein surface. At the tRNA selection step, the anticodon helix of tRNA is coloured green, and bases that serve as the positive determinants are in magenta. At the tRNA-unwinding step, red arrows indicate potential conformational changes driven by the hydrolysis of ATP to deliver the modified base into the active site.

that TmcA recognizes and discriminates the identity elements present only in the anticodon helix of tRNA (Ikeuchi *et al*, 2008), and the distance between the hydrolysis and the acetyltransferase sites of TmcA can enfold the whole anticodon helix of tRNA, we propose that TmcA binds exclusively to the anticodon helix of tRNA, and the transient conformational rearrangement should be localized in this region. To verify our speculation, we further performed the *in vitro* acetylation assay with a tRNA mutant (A31U) that had the bottom A31–U39 base pair of the anticodon stem (Supplementary Figure S1) substituted by U31–U39. A31U mutant markedly elevated the acetylation activity, raising the initial rate about three times compared with that of the wild-type tRNA^{Met} (Figure 5B). This stem base-pair caps the anticodon loop, and therefore, disruption of the base pair might lower the energetic cost and help deformation of the anticodon helix by TmcA.

Discussion

Our combined structural information and biochemical evidence from EMSA, unwinding assay, and mutational studies lead us to the natural conclusion of the existence of an RNA helicase-like activity with an unusual DEAA (Walker B) motif in TmcA. The substitutions of the DEAD-box motifs III and VI by the TS1 and 2 motifs in TmcA most likely restore its helicase function in a specific manner for acetylase tRNA anticodon.

TmcA reveals several idiosyncratic features that are deviated from the canonical DEAD-box RNA helicases. TmcA does not require ATP for RNA binding or recognition (Figure 3A) as observed in Vasa or others (Sengoku *et al*, 2006), and in turn, the binding of RNA substrate is not a prerequisite for ATP hydrolysis in TmcA. The regulation of

hydrolysis reaction of TmcA depends on an acetyl-CoA cofactor that binds 30 Å away inside the neighbouring domain. Up to now, only the yeast Dbp9 DEAD-box protein, required for 60S ribosomal subunit biogenesis, has exhibited an RNA-independent ATPase activity (Kikuma *et al*, 2004). These characteristic behaviour of TmcA can be ascribed to the redefinition of the sequence motifs of the DEAD-box proteins as well as their mutual interactions (Figures 2B and 4A). Moreover, functional specialization of DEAD motors is often derived from auxiliary ‘specificity domain’ or additional subunits that have the potential to interact with different ligands or protein partners (Caruthers and McKay, 2002; Cordin *et al*, 2006). Therefore, the long-range inter-domain communications involving the body domain most likely have a critical function in the regulation of hydrolysis and tRNA-binding activities of TmcA. In our structure, Arg460 is immediately upstream of Ile461 that has its backbone amide nitrogen in the proximity of the carbonyl oxygen of acetyl-CoA, and is hydrogen-bonded to an essential His377 in the TS2 motif. Moreover, the R460A mutation extinguished tRNA-binding activity. We therefore speculate that Arg460 in the GNAT A motif could function as a sensor that monitors the acetyl group of bound acetyl-CoA to coincidentally trigger both the hydrolysis and tRNA-binding activities.

It is not clear why the helicase module would be required as TmcA modifies a cytidine in the tRNA anticodon where bases are exposed and easily accessible; however, a plausible explanation could be rationally given from the standpoints of both the structure and the reaction mechanism. The acetyl group of acetyl-CoA is confined in the splayed strands of the GNAT A and B motifs, and is weakly hydrogen-bonded to the backbone amide of Ile461. This backbone amide probably polarizes the carbonyl group of the thioester before nucleophilic attack of the C34 amino group and stabilizes the

negative charge that develops on the oxygen in the tetrahedral transition state. In contrast to the proposed mechanism for *Tetrahymena* GCN5 HAT (Rojas *et al*, 1999), there are no ideal candidates for either a general acid or base in the close vicinity of the acetyl group, and we speculate that the C34 amino group is precisely positioned at the active site close enough for direct nucleophilic attack of the thioester linkage. Moreover, the active site has a net positive electrostatic potential constructed by a characteristic arginine cluster (residues 379, 387, 433, 457, and 460), which may serve to help in interactions with the RNA backbone and to lock it rigorously in the active site, simultaneously enhancing the nucleophilicity of the C34 amino group as it enters the active site.

Although the positioning of the nucleophilic amino group in the active site is most likely a key mechanism for acetylation of RNA, that site is at the bottom of the basin with a narrow vestibule, and thus offers limited access. Docking a tRNA model on TmcA according to the biochemical data, results in severe steric clashes with the tRNA anticodon before it reaches the active site (Figure 5C). These observations imply that prior to acetyl group transfer to C34, tRNA^{Met} must undergo substantial conformational remodelling, particularly in the anticodon helix, to gain access to the acetyl group of acetyl-CoA bound at the active site. The minimal rearrangement of the structure emerging by induced-fit or base-flipped-out mechanisms as in the case of other RNA-modifying enzymes (Hoang and Ferre-D'Amare, 2001; Losey *et al*, 2006) would not be capable of delivering a to-be-modified base to the active site. Hence, we believe that TmcA acquired an RNA helicase activity through the evolutionary process to successfully remodel the substrate structure, and allow accessibility to the modified sites.

On the basis of the crystal structure combined with previous and our biochemical data, we can extract a simple rationale for a sequential mechanism of RNA acetylation that is essential for maintaining decoding fidelity by TmcA in *E. coli* (Figure 5C). Initially, the cofactor acetyl-CoA binds to TmcA, triggering a conformational change that switches on both ATP hydrolysis and tRNA-binding activities, and as a consequence, TmcA binds to the elongator tRNA^{Met} by discerning two checkpoints in the anticodon helix: the first C27–G43 base pair and its juxtaposed G44, and the C30–G40 base pair (Ikeuchi *et al*, 2008). Soon after tRNA accommodation, ATP hydrolysis takes place, by which energy is expended to unwind, albeit partially, the anticodon helix to protrude the wobble C34 base into the active site. Spontaneous nucleophilic attack of the C34 amino group on the thioester carbonyl occurs and results in ac⁴C formation. Once the acetyl group is detached from CoA, the affinity of TmcA for tRNA is attenuated, possibly controlled by an Arg460 sensor in the GNAT A motif. Following dissociation of tRNA, CoA leaves the enzyme making it ready for a new reaction cycle.

This study has elucidated the first structure of RNA acetyltransferase and provided the implication of the usage of the DEAD-box helicase module to deform the RNA substrate, and subsequently decorate it with the chemical modification. The acetyl-CoA cofactor functions both as the donor and the regulator of the reaction catalysed by TmcA. Intriguingly, TmcA homologues are found ubiquitously in archaeal and eukaryotic organisms, the tRNAs of which do

not contain ac⁴C in the anticodons (see Supplementary data). The precise functions of ac⁴C modification as well as TmcA homologues in eukaryotes may deserve further investigation.

Materials and methods

Preparation of protein and RNA

The *E. coli* *ypfI* gene encoding TmcA was amplified and cloned into the expression vector pET-28a (Novagen) between the *NcoI* and *HindIII* restriction sites, fused with a hexahistidine tag and a thrombin cleavage sequence at the N terminus. Expression of TmcA in *E. coli* BL21 (DE3) cells was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM at $A_{600} = 0.6$ and proceeded overnight at 28°C. All purification processes were carried out at 4°C and kept under a reductive environment owing to the labile nature of TmcA. Recombinant protein was one-step purified to near homogeneity by a nickel-attached HiTrap chelating column (GE Healthcare Life Sciences) with a linear gradient of 25–350 mM imidazole. For crystallization, the impeding histidine tag was removed by adding 1 U thrombin per mg protein during dialysis against 20 mM HEPES (pH 8.0), 0.2 M NaCl, 15 mM imidazole, 10 mM MgCl₂, 5% (v/v) glycerol, and 5 mM β -mercaptoethanol for 2 days. The protease was inactivated by adding PMSF to a final concentration of 0.2 mM, prior to passing the tag-free TmcA through an additional round of affinity chromatography. The final step of purification was performed using a Superdex 200 gel-filtration chromatography (GE Healthcare Life Sciences) in crystallization buffer (10 mM HEPES (pH 8.0), 0.2 M NaCl, 5% glycerol, and 2 mM DTT), and purified recombinant protein was concentrated by ultrafiltration (Ultra-15; Amicon) to approximately 20 mg/ml, and then divided into small aliquots, flash-frozen with liquid nitrogen and stored at –80°C. The selenomethionine (Se-Met) TmcA derivative was expressed in a methionine auxotrophic *E. coli* B834 strain grown in a minimal medium containing 25 mg/l Se-Met. The purification procedure for Se-Met-labelled TmcA was essentially the same as that for the native enzyme.

In vitro runoff-transcribed tRNAs were prepared as described earlier (Nakamura *et al*, 2006). The oligo DNAs used to construct *E. coli* tRNA^{Met} were as follows: 5'-primer: ***TAATACGACTCATATAGGCTACGTAGC*** (forward); middle template: ***CACATATAGGCTACGTAGCTCAGTTGGTTAGAGCACATCACTCATAATGATGGGGTCACAGGTTCC*** (reverse); 3'-primer: ***TGmGTGGCTACGACGGGATTCCGAACCTGTGACCCC*** (reverse); in which the T7 promoter sequence is in bold and italic character and complementary regions are underlined. Gm represents 2'-O-methyl deoxyguanosine, used for manipulating the uniform 3'-end of transcribed products (Sherlin *et al*, 2001).

Crystallization, data collection and structure determination

Se-Met-labelled TmcA was cocrystallized with ATP and acetyl-CoA using 15% (w/v) PEG 5000 monomethyl ether as a precipitant at 18°C. The crystals were soaked in a cryoprotectant including 25% (v/v) glycerol prior to the data collection under cryogenic condition (100 K). The structure was determined by a combination of multi-wavelength anomalous diffraction and molecular replacement methods (see Supplementary data for details). The final refinement was performed on LAFIRE (Yao *et al*, 2006), yielding the model with a crystallographic *R*-factor of 23.4% and an *R*_{free} factor of 27.4% (Table I).

In vivo complementation activity analysis

The wild-type *E. coli* TmcA and its variants were subcloned into the expression vector pQE-80L (Qiagen) harbouring an N-terminal hexahistidine tag, and transformed into the TmcA-defective *E. coli* strain (*tmcAΔ*). All mutants were constructed using the Quik-Change site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene), and were verified by DNA sequencing. The transformants were grown in LB medium supplemented with IPTG at 37°C, and cells were harvested during log phase growth. The RNAs were extracted from *E. coli* cells basically as described earlier (Ikeuchi *et al*, 2006), and were digested into nucleosides and analysed by liquid-chromatography/mass-spectrometry using ion-trap mass spectrometry (Soma *et al*, 2003).

In vitro activity assays

ATP hydrolysis. ATPase assays were performed at 37°C in 20 µl of a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 10 mM KCl, 10 mM MgCl₂, 10 mM DTT, 25 µM [α -³²P]ATP (GE Healthcare Life-Sciences), and 5 µM recombinant TmcA variants. An aliquot (0.5 µl) of the reaction was quenched by formic acid and spotted onto a PEI (polyethyleneimine)-cellulose TLC plate (POLYGRAM CEL 300 PEI/UV254, Macherey-Nagel) and developed with 0.75 M KH₂PO₄ (pH 3.5) at room temperature. The radioactivity was visualized by a FLA7000 bioimaging analyzer (Fujifilm).

RNA binding. The protocol of EMSA was basically similar as described earlier (Nakamura *et al*, 2006) with a slight modification. A hundred picomoles of recombinant enzyme with or without 2 mmol of each ligand were incubated at 20°C for 30 min in 8 µl of a reaction mixture consisting of 50 mM HEPES (pH 7.6), 30 mM KCl, 10 mM MgCl₂, 5% glycerol, 1 mM DTT, 0.1% Nonidet P-40, and various amounts of transcript. Bands of complexes were analysed on a 5% non-denaturing polyacrylamide gel composed of 50 mM Tris-acetate (pH 8.0), 10 mM magnesium acetate, 1 mM DTT, and 0.1 mM EDTA. Gels were stained with both ethidium bromide and Coomassie Brilliant Blue to visualize RNA and protein, respectively.

RNA unwinding. To detect potential tRNA-unwinding activity of TmcA, the traditional RNA helicase assay was modified as follows. Here, 10 pmol of transcribed tRNA was mixed with the indicated amounts of recombinant TmcA in a buffer consisting of 50 mM Tris (pH 8.0), 2 mM MgCl₂, 50 mM KCl, 10% glycerol, 0.5 mM DTT, and 0.01 mM EDTA, with or without 0.2 mM acetyl-CoA. The reactions were initiated by the addition of 2 mM ATP (or ADPCP), 5 mM phospho(enol)pyruvate, and 2.25 U of pyruvate kinase (Sigma) to the final 10 µl reaction mixture and stopped by the addition of 10 µl of 50 mM EDTA (pH 8.0), 20% glycerol, 1% SDS, 0.02% xylene cyanol, and 1 mg/ml Proteinase K (Roche) after 1 h incubation at 15°C. The mixtures were left at 15°C for another 10 min to complete

proteolytic degradation before being subjected to native PAGE on a 10–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan). Samples were electrophoresed at 50 mA in a cold room (4°C).

Acetyltransferase reaction. The *in vitro* ac⁴C formation was basically performed as described earlier (Ikeuchi *et al*, 2008) except for the reaction mixtures (50 µl) that contained 10 µM tRNA^{Met} transcript and 20 µM recombinant TmcA, and were incubated at 30°C.

Accession numbers

Atomic coordinates and the structure factors of *E. coli* TmcA-ADP-acetyl-CoA complex have been deposited in the Protein Data Bank with the ID code 2ZPA.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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References

- Agris PF, Vendeix FA, Graham WD (2007) tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol* **366**: 1–13
- Björk GR, Durand JM, Hagervall TG, Leipuviene R, Lundgren HK, Nilsson K, Chen P, Qian Q, Urbonavicius J (1999) Transfer RNA modification: influence on translational frameshifting and metabolism. *FEBS Lett* **452**: 47–51
- Bono F, Ebert J, Lorentzen E, Conti E (2006) The crystal structure of the exon junction complex reveals how it maintains a stable grip on mRNA. *Cell* **126**: 713–725
- Caruthers JM, Johnson ER, McKay DB (2000) Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. *Proc Natl Acad Sci USA* **97**: 13080–13085
- Caruthers JM, McKay DB (2002) Helicase structure and mechanism. *Curr Opin Struct Biol* **12**: 123–133
- Cheng Z, Muhrad D, Lim MK, Parker R, Song H (2007) Structural and functional insights into the human Upf1 helicase core. *EMBO J* **26**: 253–264
- Cordin O, Banroques J, Tanner NK, Linder P (2006) The DEAD-box protein family of RNA helicases. *Gene* **367**: 17–37
- Grosjean H, Edqvist J, Straby KB, Giege R (1996) Enzymatic formation of modified nucleosides in tRNA: dependence on tRNA architecture. *J Mol Biol* **255**: 67–85
- Hoang C, Ferre-D'Amare AR (2001) Cocrystal structure of a tRNA P_{si}55 pseudouridine synthase: nucleotide flipping by an RNA-modifying enzyme. *Cell* **107**: 929–939
- Ikeuchi Y, Kitahara K, Suzuki T (2008) The RNA acetyltransferase driven by ATP hydrolysis synthesizes N⁴-acetylcytidine of tRNA anticodon. *EMBO J* **27**: 2194–2203
- Ikeuchi Y, Shigi N, Kato J, Nishimura A, Suzuki T (2006) Mechanistic insights into sulfur relay by multiple sulfur mediators involved in thioridine biosynthesis at tRNA wobble positions. *Mol Cell* **21**: 97–108
- Ikeuchi Y, Soma A, Ote T, Kato J, Sekine Y, Suzuki T (2005) Molecular mechanism of lysidine synthesis that determines tRNA identity and codon recognition. *Mol Cell* **19**: 235–246
- Kawai G, Hashizume T, Miyazawa T, McCloskey JA, Yokoyama S (1989) Conformational characteristics of 4-acetylcytidine found in tRNA. *Nucleic Acids Symp Ser* **21**: 61–62
- Kikuma T, Ohtsu M, Utsugi T, Koga S, Okuhara K, Eki T, Fujimori F, Murakami Y (2004) Dbp9p, a member of the DEAD box protein family, exhibits DNA helicase activity. *J Biol Chem* **279**: 20692–20698
- Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C, Caron PR (1998) Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* **6**: 89–100
- Lau OD, Courtney AD, Vassilev A, Marzilli LA, Cotter RJ, Nakatani Y, Cole PA (2000) p300/CBP-associated factor histone acetyltransferase processing of a peptide substrate. Kinetic analysis of the catalytic mechanism. *J Biol Chem* **275**: 21953–21959
- Losey HC, Ruthenburg AJ, Verdine GL (2006) Crystal structure of *Staphylococcus aureus* tRNA adenosine deaminase TadA in complex with RNA. *Nat Struct Mol Biol* **13**: 153–159
- McCloskey JA, Crain PF (1998) The RNA modification database—1998. *Nucleic Acids Res* **26**: 196–197
- Muramatsu T, Nishikawa K, Nemoto F, Kuchino Y, Nishimura S, Miyazawa T, Yokoyama S (1988a) Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. *Nature* **336**: 179–181
- Muramatsu T, Yokoyama S, Horie N, Matsuda A, Ueda T, Yamaizumi Z, Kuchino Y, Nishimura S, Miyazawa T (1988b) A novel lysine-substituted nucleoside in the first position of the anticodon of minor isoleucine tRNA from *Escherichia coli*. *J Biol Chem* **263**: 9261–9267
- Nakamura A, Yao M, Chimnarong S, Sakai N, Tanaka I (2006) Ammonia channel couples glutaminase with transamidase reactions in GatCAB. *Science* **312**: 1954–1958
- Numata T, Ikeuchi Y, Fukai S, Suzuki T, Nureki O (2006) Snapshots of tRNA sulphuration via an adenylated intermediate. *Nature* **442**: 419–424

- Pause A, Sonenberg N (1992) Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. *EMBO J* **11**: 2643–2654
- Rogers Jr GW, Lima WF, Merrick WC (2001) Further characterization of the helicase activity of eIF4A. Substrate specificity. *J Biol Chem* **276**: 12598–12608
- Rojas JR, Trievel RC, Zhou J, Mo Y, Li X, Berger SL, Allis CD, Marmorstein R (1999) Structure of *Tetrahymena* GCN5 bound to coenzyme A and a histone H3 peptide. *Nature* **401**: 93–98
- Scheffzek K, Ahmadian MR, Kabsch W, Wiesmuller L, Lautwein A, Schmitz F, Wittinghofer A (1997) The Ras–RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* **277**: 333–338
- Senger B, Auxilien S, Englisch U, Cramer F, Fasiolo F (1997) The modified wobble base inosine in yeast tRNA^{Ile} is a positive determinant for aminoacylation by isoleucyl-tRNA synthetase. *Biochemistry* **36**: 8269–8275
- Sengoku T, Nureki O, Nakamura A, Kobayashi S, Yokoyama S (2006) Structural basis for RNA unwinding by the DEAD-box protein *Drosophila* Vasa. *Cell* **125**: 287–300
- Sherlin LD, Bullock TL, Nissan TA, Perona JJ, Lariviere FJ, Uhlenbeck OC, Scaringe SA (2001) Chemical and enzymatic synthesis of tRNAs for high-throughput crystallization. *RNA* **7**: 1671–1678
- Shi H, Cordin O, Minder CM, Linder P, Xu RM (2004) Crystal structure of the human ATP-dependent splicing and export factor UAP56. *Proc Natl Acad Sci USA* **101**: 17628–17633
- Soma A, Ikeuchi Y, Kanemasa S, Kobayashi K, Ogasawara N, Ote T, Kato J, Watanabe K, Sekine Y, Suzuki T (2003) An RNA-modifying enzyme that governs both the codon and amino acid specificities of isoleucine tRNA. *Mol Cell* **12**: 689–698
- Stern L, Schulman LH (1978) The role of the minor base N4-acetylcytidine in the function of the *Escherichia coli* noninitiator methionine transfer RNA. *J Biol Chem* **253**: 6132–6139
- Story RM, Li H, Abelson JN (2001) Crystal structure of a DEAD box protein from the hyperthermophile *Methanococcus jannaschii*. *Proc Natl Acad Sci USA* **98**: 1465–1470
- Suzuki T (2005) Biosynthesis and function of tRNA wobble modifications. In *Fine-Tuning of RNA Functions by Modification and Editing*, Grosjean H (ed), Vol. 12, pp 24–69. New York: Springer-Verlag
- Sylvers LA, Rogers KC, Shimizu M, Ohtsuka E, Soll D (1993) A 2-thiouridine derivative in tRNA^{Glu} is a positive determinant for aminoacylation by *Escherichia coli* glutamyl-tRNA synthetase. *Biochemistry* **32**: 3836–3841
- Tanaka N, Schwer B (2006) Mutations in PRP43 that uncouple RNA-dependent NTPase activity and pre-mRNA splicing function. *Biochemistry* **45**: 6510–6521
- Tanner KG, Langer MR, Kim Y, Denu JM (2000) Kinetic mechanism of the histone acetyltransferase GCN5 from yeast. *J Biol Chem* **275**: 22048–22055
- Tanner NK, Linder P (2001) DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Mol Cell* **8**: 251–262
- Vetting MW, de Carvalho LPS, Yu M, Hegde SS, Magnet S, Roderick SL, Blanchard JS (2005) Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys* **433**: 212–226
- Yao M, Zhou Y, Tanaka I (2006) LAFIRE: software for automating the refinement process of protein-structure analysis. *Acta Crystallogr D Biol Crystallogr* **62** (Part 2): 189–196