The universal 3′-terminal CCA sequence of tRNA is built and/or synthesized by the CCA-adding enzyme, CTP:(ATP) tRNA nucleotidyltransferase. This RNA polymerase has no nucleic acid template, but faithfully synthesizes the defined CCA sequence on the 3′-terminus of tRNA at one time, using CTP and ATP as substrates. The mystery of CCA-addition without a nucleic acid template by unique RNA polymerases has long fascinated researchers in the field of RNA enzymology. In this review, the mechanisms of RNA polymerization by the remarkable CCA-adding enzyme and its related enzymes are presented, based on their structural features.

Keywords: tRNA, CCA, template-independent, nucleotidyltransferase, class-I and II
for both CTP and ATP incorporations, and the tRNA is fixed on the surface of the enzyme, and neither translocates nor rotates relative to the enzyme during CCA-addition (Shi et al., 1998a; Yue et al., 1998).

Over the past fifteen years, the crystal structures of class-I and class-II enzymes and their complexes with nucleotides of various tRNAs or mini-helices have been reported (Li et al., 2002; Augustin et al., 2003; Okabe et al., 2003; Xiong et al., 2003; Tomita et al., 2004, 2006; Xiong and Steitz, 2004; Toh et al., 2008, 2009, 2011; Pan et al., 2010; Yamashita et al., 2014). The structural information has solved most of the long-standing mysteries, but not all yet, about the mechanism of the remarkable, template-independent CCA-adding enzyme and its relatives (Schimmel and Yang, 2004; Weiner, 2004; Xiong and Steitz, 2006). Here, we review the current understanding of the mechanism of CCA-addition by the CCA-adding enzyme, and its related enzymes, based on their crystal structures.

Class-I ARCHAEAAL CCA-ADDING ENZYME

In this section, we review the mechanism of CCA-addition by the class-I archaeal CCA-adding enzyme, based on the crystal structures of the enzyme and its complexes with RNAs and nucleotides or nucleotide analogs (Okabe et al., 2003; Xiong et al., 2003; Xiong and Steitz, 2004; Tomita et al., 2006; Toh et al., 2008; Pan et al., 2010).

THE TEMPLATE DOES NOT RESIDE WITHIN THE class-I CCA-ADDING ENZYME

The crystal structures of class-I CCA-adding enzyme from *Archaeoglobus fulgidus* (AFCCA) and its complexes with CTP or ATP were reported (Okabe et al., 2003; Xiong et al., 2003). The structures of AFCCA consist of four domains: N-terminal, central, C-terminal, and tail domains, and the overall structure is U-shaped (Figure 2A). This architecture of AFCCA is different from that of the class-II CCA-adding enzyme as described below, and is rather similar to that of the eukaryotic PAP (Bard et al., 2000; Martin et al., 2000). This was anticipated from the comparisons of their primary amino acid sequences (Yue et al., 1996; Figure 1A).

The N-terminal domain of AFCCA consists of five β-strands and two α-helices, and three catalytic carboxylates (Glu59, Asp61, and Asp110) reside on the β-sheets. The three catalytic carboxylates are located in close proximity to each other and coordinate the catalytic Mg²⁺ ion. The N-terminal catalytic domain structure of AFCCA is homologous to those of other NT family members, including class-II CCA-adding enzyme, pol β and other polynucleotide polymerases (Sakon et al., 1993; Pelletier et al., 1994; Bard et al., 2000; Martin et al., 2000). This suggested that the catalytic cores of the class-I and class-II CCA-adding enzymes share a common ancestor, together with those of other NT family members, and that the class-I and class-II enzymes both catalyze nucleotidyl-transfer by the same metal–ion catalytic mechanism (Brautigam and Steitz, 1998). The central domain consists of four stranded β-sheets, and is topologically homologous to the RNA-recognition motif (RRM) of several RNA-binding proteins, such as ribosomal protein S6 (Okabe et al., 2003; Xiong et al., 2003).

The structures of AFCCA complexed with various nucleotides (Xiong et al., 2003) revealed that the nucleotide sits in the inter-domain region, between the N-terminal and central domains of AFCCA (Figure 2B). In the complex structures, all nucleotides bound to the catalytic pocket of the enzyme non-specifically, and the base moieties of the nucleotides were disordered in the complex structures. This observation implied that the nucleotide specificity is not dictated by the enzyme alone in the class-I CCA-adding enzyme. Instead, it suggested that the specificity is dictated by the RNA–enzyme complex, as conceptually suggested in the “collaborative templating” model (Shi et al., 1998a; Figure 1C). The detailed mechanism of nucleotide selection by the class-I CCA-adding enzyme was later clarified by the determination of several crystal structures of AFCCA complexed with an RNA primer in the presence of an incoming nucleotide, as described below in detail.

![Figure 1 Two classes of CCA-adding enzymes and the proposed models](image-url)
Mechanism of CCA-addition

RNA-PROTEIN TEMPLATE FOR CTP AND ATP SELECTION BY class-I CCA-ADDING ENZYME

The complex structures of AFCCA with a tRNA bearing a CCA-3′ terminus, and with various RNA primers mimicking the top-half of a tRNA molecule (tRNA mini-helix or double-stranded RNA) in the presence or absence of nucleotide, were reported (Xiong and Steitz, 2004; Tomita et al., 2006; Toh et al., 2008; Pan et al., 2010). These complex structures of AFCCA with various RNA primers, representing the sequential CCA adding reactions, revealed the detailed mechanism of nucleotide specificity and the dynamic CCA-adding reaction by the class-I CCA-adding enzyme.

AFCCA recognizes the acceptor-TΨC helix, the top-half of the tRNA, and does not interact with the tRNA anticodon region at all (Xiong and Steitz, 2004; Figure 2C). This is consistent with a previous biochemical study showing that a mini-helix RNA (and even the double-stranded RNA) corresponding to the top-half of the tRNA can be a primer for CCA-addition by the class-I CCA-adding enzyme (Shi et al., 1998b). The tail domain of AFCCA interacts with the elbow region in the TΨC loop of the tRNA. The tail domain functions as an anchor for the tRNA, and prevents the tRNA from dislodging from the enzyme surface during CCA-addition. The 3′-terminus of the tRNA enters the active pocket between the N-terminal and central domains of AFCCA (Figure 2C).

The ternary structures of AFCCA with a tRNA mini-helix (or double helix RNA) and an incoming nucleotide CTP (or ATP), representing the C75-adding (or A76-adding) reaction, were reported (Xiong and Steitz, 2004; Tomita et al., 2006). In the ternary complex structures, the geometries of the incoming CTP (or ATP) and the 3′-OH group of the ribose in the 3′-terminal nucleoside of the RNA, relative to the catalytic carboxylates (Glu59, Asp61, and Asp110) and a Mg^{2+} metal, suggested that the structures represent the nucleotide insertion stages of RNA polymerization.

In the ternary complex structure of AFCCA with a tRNA mini-helix ending with C74 (or double helix RNA) in the presence of CTP, representing C75-addition, the cytosine base of the CTP stacks with the cytosine base of C74 at the 3′-terminus of the RNA. The 4-NH2 group and the N3 atom of the CTP hydrogen-bond with the phosphate groups of C74 and A73 (discriminator nucleoside) of the RNA primer, and with Arg224 in the central domain, respectively (Figure 2D). In the ternary structure of AFCCA with the tRNA mini-helix (or double helix RNA) ending with C74C75 in the presence of ATP, representing the A76-adding reaction, the adenine base of the ATP stacks with the cytosine base of C75 at the
3′-terminus of the RNA. The 6-NH₂ group and the N₁ atom of the incoming ATP form hydrogen-bonds with the phosphate groups of C₇₄ and A₂₃ in the RNA primer and Arg₂₂₄ in the central domain, respectively (Figure 2D). Thus, the templates for CTP and ATP selection by AFCCA were found to be the phosphate backbone of the RNA primer and the protein, rather than solely the protein itself, as implicated (Shi et al., 1998a).

After the C₇₅-addition, tRNA mini-helix acceptor stem is fixed on the enzyme surface, and the tRNA mini-helix neither translocates nor rotates relative to the enzyme surface. Then, the 3′-terminus of tRNA mini-helix reorients and retracts into the enclosed active pocket (Xiong and Steitz, 2004; Tomita et al., 2006; Toh et al., 2008; Pan et al., 2010). The reorienting of the 3′-terminus of the tRNA in the pocket places the ribose 3′-OH group of C₇₅ in the RNA proximal to the active site of the enzyme. The structural changes in the active pocket of the enzyme, as well as the reorienting of the 3′-end of RNA, after C₇₅-addition, ensure that the active pocket is free of any nucleotide, for successive ATP accommodation in the pocket. Thus, a single active pocket can be utilized for both C-addition and A-addition by the class-I CCA adding enzyme.

These sequential structural analyses of AFCCA also revealed that the size and the shape of the nucleotide pocket, composed of the growing 3′-terminus of the RNA and the enzyme, successively change during the CCA-adding reaction (Figures 2E,F). At the C₇₅-adding stage, the size and the shape of the nucleotide binding pocket are suitable for CTP accommodation, and the larger ATP cannot snugly fit in the pocket. After C₇₅-addition, the 3′-terminus of the RNA reorients in the enclosed active site, and the size and the shape of the nucleotide binding pocket become suitable for ATP. Although the smaller CTP could bind in the pocket, it does not snugly fit (Tomita et al., 2006; Pan et al., 2010). Thus, the nucleotide specificity of the class-I CCA adding enzyme shifts from CTP-specific to ATP-specific during the successive CCA-adding reactions.

Together, these extensive crystallographic analyses suggest that the template of the class-I CCA-adding enzyme is neither the protein nor the RNA, but the RNA–protein complex. The RNA primers neither translocate nor rotate relative to the enzyme surface, and a single active pocket is utilized for both C- and A-addition, by successive reorients of the 3′-terminal nucleoside in the enclosed active pocket. The successive reorients of the 3′-terminus of the tRNA during polymerization changes the nucleotide specificity of the class-I CCA-adding enzyme from CTP to ATP. These structural features explained the previous biochemical studies well (Shi et al., 1998a; Yue et al., 1998).

**DYNAMICS OF CCA-ADDITION BY THE class-I CCA-ADDING ENZYME**

The binary complex structures of AFCCA with a tRNA mini-helix in the absence of incoming nucleotides were also reported (Tomita et al., 2004; Toh et al., 2008). The comparison of the binary and ternary structures highlights the dynamic change in the orientation of the N-terminal domain of the enzyme and the 3′-terminus of the RNA during CCA-adding (Figures 3A,B).

In the binary complex of AFCCA with a tRNA mini-helix ending in either D₇₃ (D is the discriminator nucleoside) or C₇₄, the nucleobase of the 3′-terminal nucleoside stacks onto the preceding nucleobase, and the ribose 3′-OH group of the 3′-terminal nucleoside is far from the active site (three catalytic carboxylates). This structure represents an inactive form. At the C₇₅-adding reaction stage, upon binding the incoming CTP in the active site, the N-terminal domain of AFCCA relocates toward the central domain, leading the enzyme to transit from an open conformation to a closed conformation (Figure 3A). This allows the 3′-nucleoside C₇₄ of the RNA to flip, and positions the ribose 3′-OH group of the 3′-terminal nucleoside proximal to the catalytic residues and the triphosphate group of the incoming CTP. This structure represents a catalytically active form (Figure 3C). Although the structure representing C₇₄-addition was not determined, the binary complex of AFCCA with tRNA mini-helix ending in D₇₃ showed that the 3′-terminal D₇₃ of RNA and three catalytic carboxylates well superimposed onto the 3′-terminal C₇₄ of RNA and the catalytic carboxylates, respectively, in the binary complex of AFCCA with tRNA mini-helix ending in C₇₄, as described below (Figure 3D). Thus, C₇₄-addition reaction would proceed in the same mechanism as C₇₅-addition reaction.

At the C₇₄-addition reaction stage, the mini-helix RNA ending with D₇₃ adopts an extended form (Tomita et al., 2006; Figure 3D). After the C₇₄-addition reaction is completed, the N-terminal domain of AFCCA relocates outward, and the enzyme transits to an active form. Then, the 3′-terminal nucleoside (C₇₄) of the tRNA mini-helix flips back into the active site, and the acceptor helix of the tRNA mini-helix shrinks back from the extended form. The change in the tRNA mini-helix from the extended form to the shrunken form allows the active pocket to become nucleotide free, for successive CTP accommodation and C₇₅-addition. CTP binding in the active pocket for C₇₅-addition induces the relocation of the N-terminal domain of the enzyme toward the central domain again (the enzyme transits to an active closed form again; Figure 3A). The 3′-nucleoside of the tRNA flips again, and the C₇₅-addition reaction proceeds (Figure 3C). Thus, in both the C₇₄-addition and C₇₅-addition reactions, CTP binding in the active site dynamically induces the conformational change of the enzyme from an inactive open form to an active closed form, and only the correct nucleotide, CTP, can allow the transition of the enzyme.

After the C₇₅-addition reaction, the enzyme is fixed in an active closed form (Figure 3B). The 3′-terminus of the tRNA mini-helix reorients in the enclosed active site, and a newly shaped nucleotide binding pocket is created by the enzyme and the 3′-terminus of the RNA (Figure 3E). ATP can bind in the nucleotide pocket, and A₇₆-addition proceeds without the open to closed conformational change of the enzyme. The fixation of the enzyme in a closed conformation after C₇₅-addition is facilitated by the interaction between the β-turn in the N-terminal domain and the 3′-terminus of the tRNA mini-helix. The mutation of a key amino acid residue, Arg₂₂₄, reduced C₇₄C₇₅-addition, but not A₇₆-addition, in vitro, indicating that Arg₂₂₄ in the pocket does not discriminate ATP from CTP in the A₇₆-addition reaction (Tomita et al., 2006; Pan et al., 2010). Thus, the A₇₆-addition reaction is static, and is distinct from the C₇₄C₇₅-addition reactions, which are accompanied by the dynamic open to closed conformational transition of the enzyme.
and require the proper conformation of Arg244 in the pocket. Consecutive conformational changes of the β-turn in the N-terminal domain accompany the refolding of the tRNA 3′-terminus during the reaction (Figures 3C,E). The β-turn in the N-terminal domain monitors the 3′-terminal sequence of the tRNA for correct CCA-addition (Toh et al., 2008).

After A76-addition, the N-terminal domain relocates outward, triggered by pyrophosphate release, and the enzyme adopts the open conformation. At this stage, there is no room to accommodate another nucleotide in the active pocket. Finally, a tRNA with a CCA-3′-terminus dissociates from the enzyme, and the CCA-adding reaction is completed (Xiong and Steitz, 2004; Tomita et al., 2006).

The dynamic sequence of the CCA-adding reaction by the class-I CCA-adding enzyme, revealed by the crystallographic analyses of complexes of class-I CCA-adding enzyme with various RNA primers with or without nucleotides, is presented in Figure 4.

**THE class-II CCA-ADDING ENZYME AND ITS RELATED ENZYMES**

In this section, we review the mechanism of CCA-addition by the class-II eubacterial/eukaryotic CCA-adding enzyme, and its related class-II eubacterial CC-adding and A-adding enzymes, based on the crystal structures of the enzymes and their complexes with RNAs (Li et al., 2002; Augustin et al., 2003; Tomita et al., 2004; Toh et al., 2009; Yamashita et al., 2014).

**PROTEIN-BASED TEMPLATE FOR CTP AND ATP SELECTIONS BY class-II CCA-ADDING ENZYME**

The crystal structures of the class-II CCA-adding enzymes from *Bacillus stearothermophilus* (BstCCA), *Thermotoga maritima* (TmCCA) and human mitochondria (HmtCCA) were reported (Li et al., 2002; Augustin et al., 2003; Toh et al., 2009).

The class-II CCA-adding enzymes adopt a sea-horse-shaped structure, and consist of four domains – the head, neck, body and tail domains (Figure 5A). The overall architecture of the class-II CCA adding enzyme is different from that of the class-I CCA-adding enzyme (Okabe et al., 2003; Xiong et al., 2003; Figure 2A), and it is rather similar to that of the eubacterial PAP from *Escherichia coli* (EcPAP; Toh et al., 2011; Figure 5A). This was anticipated by the comparisons of their amino acid sequences (Yue et al., 1996; Figure 1), although EcPAP adopts a sea-otter-shaped structure.

The head domain of the class-II CCA-adding enzyme comprises five-stranded β sheets connected by two α helices, with three conserved catalytic carboxylate residues (Asp55, Asp57, Asp99 of...
FIGURE 4 | Mechanism of CCA-addition by class-I CCA-adding enzymes. Schematic representation of the CCA-addition reaction by class-I CCA-adding enzymes. The N-terminal, central, C-terminal, and tail domains are colored magenta, green, cyan, and orange, respectively. tRNAs are colored gray. Catalytic sites are colored yellow in the N-terminal domains.

The mechanism of nucleobase recognition by the class-II CCA-adding enzyme is distinct from that observed in the class-I CCA-adding enzyme (Figures 2D,E). The template for the CCA-addition by the class-II CCA-adding enzyme is composed of the protein itself, rather than the RNA–protein complex as in the class-I CCA-adding enzyme. The protein-template of the class-II CCA-adding enzymes was confirmed by the mutations of Asp and Arg in the neck domain. The rational mutagenesis of the two key residues in the neck domain allowed the enzyme to add other nucleotides in vitro (Cho et al., 2007).

PROTEIN-BASED TEMPLATE FOR ATP-SELECTION BY THE CLASS-II A-ADDITION ENZYME

Although the structures of the class-II CCA-adding enzyme and its complexes with CTP and ATP are available (Li et al., 2002; Toh et al., 2009), the structures of the class-II CCA-adding enzyme complexed with tRNA (or RNA) have not been reported yet. Thus, the detailed RNA polymerization mechanism of CCA-addition by the class-II CCA-adding enzyme remained enigmatic.

Compounding the unsettled questions on the polymerization mechanism by the class-II CCA-adding enzyme, in some eubacteria such as *Aquifex aeolicus*, the CCA-adding activity is split between two distinct, but closely related, enzymes – one adds C74C75 and the other adds A76 (Tomita and Weiner, 2001, 2002; Bralley et al., 2005; Neuenfeldt et al., 2008; Figure 1). In *A. aeolicus*, which is placed at the deepest root of the 16S rRNA-based phylogenetic tree (Pace, 1997), the CC-adding and A-adding enzymes collaboratively synthesize the CCA-3′ (Tomita and Weiner, 2001).

The complex structure of AaL with tRNA lacking the terminal A76 and an ATP analog was reported (Tomita et al., 2004). AaL also adopts a sea-horse-shaped structure, as found with the other class-II CCA-adding enzymes (Figure 5C). As anticipated from the sequence similarity and closely related phylogeny (Tomita and Weiner, 2002), the overall structure of AaL superimposed well onto that of TmCCA (Toh et al., 2009).

In the complex structure of AaL with tRNA lacking the terminal A76 and an ATP analog (Figure 5C), the acceptor and TΨC helices of the tRNA are recognized by the enzyme, and the anticodon region does not interact with the enzyme. As in the class-I CCA-adding enzyme (Figure 2C), the tail domain of AaL interacts with the elbow region of the tRNA, and functions as an anchor.

The 3′-terminus of the tRNA enters the active pocket, which resides between the head and neck domains of AaL. The geometry of the incoming ATP analog, the 3′-OH group of C75 of tRNA, relative to the catalytic carboxylates (Asp31, Asp33, and...
FIGURE 5 | Structures of the class-II CCA-adding enzyme and its related A-adding enzyme. (A) Overall structure of Thermotoga maritima CCA-adding enzyme (TmCCA). The head, neck, body and tail domains are colored magenta, green, cyan, and orange, respectively. For comparison, the structure of Escherichia coli polynucleotide (EcPAP) is depicted (inset). (B) Structures of the catalytic pocket of TmCCA with CTP (left) and ATP (right). Nucleotides are depicted by stick models. (C) Overall structure of Aquifex aeolicus A-adding enzyme (AaL) complexed with tRNA lacking the terminal A76 and an ATP analog. The anticodon region in the structure was disordered. (D) Structure of the catalytic core of AaL, in the presence of a tRNA primer lacking the terminal A76 and an ATP analog. The ATP analog and the tRNA are depicted by stick models. (E) The flexible loop (colored yellow, amino acid residues 107–115) in the head domain would interact with the 3′-terminal region of the tRNA. The 3′-terminal region of tRNA and the ATP analog in the AaL complex in (C) were modeled onto the structure of TmCCA in (A).

Glu74), suggested that the structure represents the insertion stage of A76-addition (Figure 5D). The adenine base of the ATP analog is sandwiched by the cytosine base of C75 of tRNA and by hydrogen-bonds between Asp149 and Arg152. Thus, the binding pocket of ATP is composed of the 3′ end of the tRNA and the protein. In the ternary complex structure, as observed in the complex structure of TmCCA (or BstCCA) with ATP (Figure 5B), the adenine base is recognized by the side chains of the conserved amino acids, Asp149 and Arg152, through Watson-Crick-like hydrogen-bonds (Figure 5D). The 6-NH2 and the N1 atom of ATP form hydrogen-bonds with Asp149 and Arg152, respectively. Thus, the specificity for ATP by AaL is determined by the side chain of the protein itself, as in the class-II CCA-adding enzymes.

Biochemical and genetic studies revealed that a flexible loop in the head domains of the CCA-adding and A-adding enzymes is involved in the A76-addition reaction, but not the C74C75-addition reaction (Tomita et al., 2004; Neuenfeldt et al., 2008; Toh et al., 2009). In most of the reported crystal structures of the class-II CCA-adding and A-adding enzymes, the loop structure is disordered (Li et al., 2002; Augustin et al., 2003; Tomita et al., 2004). The loop region was only clearly visible in the crystal structure of apo TmCCA (Toh et al., 2009; Figure 5E). The loop extends from the head domain to the neck domain, bridging the two domains.

The superimposition of the structures of apo TmCCA and AaL complexed with tRNA revealed that the loop would interact with the 3′-part of the tRNA (Toh et al., 2009; Figure 5E). It is likely that the loop recognizes the growing 3′-CC sequence of the tRNA, and fixes the conformations of two key residues, Asp174 and Arg177, for the specific recognition of ATP. The corresponding loops in the CC-adding enzymes are shorter than those in the CCA-adding and A-adding enzymes, and the shorter loop was suggested to be one of the hallmarks of the CC-adding enzyme (Neuenfeldt et al., 2008).

TRANSLOCATION AND ROTATION OF tRNA DURING CC-ADDITION BY THE CC-ADDING ENZYME

More recently, the structures of Aquifex aeolicus CC-adding enzyme, AaS (Tomita and Weiner, 2001), in its apo form and in complexes with various tRNAs were reported (Yamashita et al., 2014). AaS also adopts a sea-horse-shaped structure similar to the other class-II CCA-adding and A-adding enzymes (Figure 6A). Although the structures of the head and neck domains of AaS are homologous to those of the class-II CCA-adding and A-adding enzymes, the structure of the body domain of AaS slightly differs from those of the CCA-adding and A-adding enzymes (Figures 5A and 6A). The overall structure of AaS adopts a relatively closed form, by the insertion of an additional α-helix between the head and neck.
FIGURE 6 | Structure of the class-II CC-adding enzyme. (A) Overall structure of *Aquifex aeolicus* CC-adding enzyme (AaS). The head, neck, body, and tail domains are colored magenta, green, cyan, and orange, respectively. Unique insertion helices are colored red and yellow. (B) Complex structures of AaS with tRNA lacking CCA. The tRNA is depicted by a stick model. Detailed view of the interaction between the acceptor helix of tRNA and AaS (inset). (C) Structures of the catalytic pocket of AaS at the C74-adding (left) and C75-adding (middle) stages. CTPs are depicted by stick models. The superimposition of the structures at C74-adding (magenta) and C75-adding (cyan) stages (right). (D) Comparison of the overall complex structures of AaS at the C74-adding (cyan) and C75-adding (pink) stages. (E) Detailed view of the superimposed structures of the tRNA acceptor helices at the C74-adding (cyan) and C75-adding (pink) stages in (D).

domains, and the body domain of AaS contains an additional α-helix and forms a bulging structure.

The complex structures of AaS with tRNA and an incoming CTP, representing the C74-adding and C75-adding stages, were also reported (Yamashita et al., 2014; Figures 6B,C). As observed in the complex structure of AaL with tRNA (Tomita et al., 2004; Figure 5C), AaS also recognizes the top-half region of the tRNA, and does not interact with the anticodon region (Figure 6B). The TΨC loop and D-loop of the tRNA interact with the tail domain of AaS.

In the C74-adding structure, the base-pair at the top of the tRNA acceptor helix stacks with Phe83 and Phe95 on the β-sheet in the head domain, and the 3′-terminal discriminator nucleoside A73 enters the active pocket. The cytosine base of the incoming CTP and the adenine base of A74 are stacked, and the triphosphate of the CTP and the ribose 3′-OH group of A73 are proximal to the catalytic carboxylates (Asp58, Asp60, and Asp112) and Mg2+ ion. In the ternary structure, the 4-NH2 and the N3 atom of CTP form hydrogen-bonds with Asp182 and Arg185, respectively.

The O2 atom of CTP also form a hydrogen-bond with Arg185 (Figure 6C). The mechanism of CTP recognition by AaS is the same as those observed in the complex structures of class-II CCA-adding enzyme with CTP in the absence of tRNA (Li et al., 2002; Toh et al., 2009). In the complex structure of AaS, representing the C75-adding stage, the mechanism of CTP recognition is the same as that of CTP recognition at the C74-adding stage (Figure 6C). Thus, the C74 and C75-adding reactions both proceed by the same mechanism, using the same active pocket. The size and the shape of the nucleotide binding pocket at the C74-adding and C75-adding stages are suitable to accommodate CTP, but not the other three nucleotides. CTP is selectively accommodated as a consequence of competition between nucleotides, using the conserved Asp182 and Arg185 in the pocket.

A comparison of the complex structures of the C74-adding and C75-adding stages revealed the translocation and rotation of the tRNA relative to the enzyme (Figure 6D). As a consequence of its backward translocation, the tRNA rotates and the relative orientation of its anticodon region on the enzyme changes by

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approximately twenty five degrees, as compared to that of the tRNA in the C74-addition stage. In the complex structure, the positions that were formerly occupied by the terminal base-pair (G1–C72) of the tRNA acceptor stem at the C74-addition stage are now empty. A73 translocates out of the catalytic pocket, and the position that was occupied by A73 at the C74-adding stage accommodates the newly incorporated C74 (Figure 6E). The release of pyrophosphate, a byproduct of RNA polymerization, from the active site of AaS triggers the backward translocation of the tRNA, as observed in T7 RNA polymerase (Yin and Steitz, 2004).

Upon pyrophosphate release from the active site after C75-addition, the tRNA translocates further toward the tail domain and rotates relative to the enzyme. A tRNA ending with C74C75 could no longer be retained on the enzyme, due to the further translocation and rotation of the tRNA relative to the enzyme, and would dissociate after C74C75 synthesis. Thus, the enzyme would terminate RNA synthesis. The tRNA ending with C74C75 then binds to AaL, the terminal A76 is added, and the CCA-3′ synthesis of tRNA is completed.

Previous biochemical studies using class-II CCA-adding enzymes and a tRNA mini-helix, corresponding to the top-half of tRNA, suggested that C74 addition, like C75 and A76 addition, involves neither tRNA translocation nor rotation (Cho et al., 2006). The mechanisms of CC-addition by the class-II CC-adding and CCA-adding enzymes might be different. Alternatively, the previous biochemical study using a tRNA mini-helix might not represent the actual nature of C74-addition onto tRNA, since the tRNA mini-helix lacks the interactions between the TΨC loop and the D-loop in tRNA.

The dynamic sequence of the CC-addition reaction by the class-II CC-adding enzyme, revealed by the crystallographic analyses of the complexes of the CC-adding enzyme with various tRNAs with or without and CTP, is presented in Figure 7.

**CONCLUSIONS AND PERSPECTIVES**

The detailed and extensive crystallographic analyses of the class-I CCA-adding enzyme complexed with various RNA primers explained the previous biochemical results well (Shi et al., 1998a; Yue et al., 1998; Okabe et al., 2003; Xiong et al., 2003; Xiong and Steitz, 2004; Cho et al., 2006; Tomita et al., 2006; Toh et al., 2008; Pan et al., 2010). As previously suggested by biochemical studies using the class-I CCA-adding enzyme (Shi et al., 1998b; Yue et al., 1998; Cho et al., 2006), structural studies of the class-I CCA-adding enzyme revealed that the tRNA neither translocates nor rotates relative to the enzyme during the CCA-addition reaction. The reaction proceeds in a single active pocket, and the template for CTP and ATP is the RNA-protein complex, rather than the protein itself. The crystallographic analyses also showed that the size and the shape of the nucleotide binding pocket, formed by the growing 3′-terminus of the RNA and the enzyme, successively change during the CCA-addition reaction, thus switching the nucleotide specificity, and that the CCA-addition reaction proceeds via two modes – dynamic CC-addition and static A-addition.

On the other hand, regarding the class-II CCA-adding enzyme, only the apo structures of the enzyme and its complexes with CTP and ATP are available (Li et al., 2002; Toh et al., 2009). Until now, the structure of a class-II CCA-adding enzyme complexed with tRNA (or RNA) has not been reported, and the detailed molecular mechanism of nucleotide specificity switching during CCA-addition has remained elusive. The complex structures of the class-II CCA-adding enzyme with CTP and ATP revealed that both CTP and ATP are recognized in the same active pocket, through Watson-Crick-like base pairings between the nucleobases and the conserved Asp and Arg residues in the active pocket (Li et al., 2002; Toh et al., 2009). Thus, the template for CTP and ATP is the protein itself, rather than the RNA-protein complex. This is distinct from the mechanism of nucleotide selection by the class-I CCA-adding enzymes.

The complex structures of the A. aeolicus class-II CC-adding and A-adding enzymes with tRNA and a nucleotide were reported (Tomita et al., 2004; Yamashita et al., 2014). The recognition mechanisms of CTP and ATP by the A. aeolicus CC-adding and A-adding enzymes at the insertion stage of...
RNA polymerization, respectively, are the same as those of the class-II CCA-adding enzyme, as revealed by the complex structures of these enzymes with nucleotides (Li et al., 2002; Toh et al., 2009).

The detailed molecular basis for the different activities between the A-adding enzyme and the CC-adding enzymes is not fully understood. The mechanism by which the CC-adding enzyme adds only C74C75, and then terminates RNA polymerization without adding A76, was explained well by the structural analyses (Yamashita et al., 2014). However, the mechanism by which the A-adding enzyme adds only A76, but not C74C75, has not been clarified yet, even though the complex structure of the *A. aeolicus* A-adding enzyme with tRNA and a nucleotide analog is available (Tomita et al., 2004).

The short loop in the head domain is suggested to be a hallmark of the CC-adding enzymes (Neuenfeldt et al., 2008). However, transplantation of the corresponding flexible longer loop from the A-adding enzyme (or CCA-adding enzyme) into the corresponding position of the CC-adding enzyme did not always transform the CC-adding enzyme into a CCA-adding enzyme, *in vitro* as well as *in vivo* (Toh et al., 2009). Thus, the longer loop in the head domain of the A-adding enzyme itself is not the main determinant for the A-adding enzyme to add only terminal A76. The C-terminal body and tail domains of the A-adding enzymes reportedly inhibit C74C75 addition *in vitro* (Tretbar et al., 2011). Since the overall structures of *A. aeolicus* A-adding enzyme and *T. maritima* CCA-adding enzyme superimposed well, the inhibitory effects of the C-terminal region of all A-adding enzymes on CC-addition are not fully understood.

**FIGURE 8 | Hypothetical mechanisms of nucleotide addition by class-II enzymes.** (A) CC-addition by CC-adding enzyme possessing two tRNA binding sites (sites A and B). Sites A and B are used for C74- and C75-addition, respectively. When a tRNA ending with the discriminator nucleoside (D73) is on site B, the 3′-terminal D73 cannot reach the active site for catalysis (inactive state). A tRNA ending with C74C75 cannot bind either site A or B. (B) A-addition by A-adding enzyme, possessing a single tRNA binding site. The neck domain is not flexible. The tRNA binds the enzyme, using the single site. On this site, the 3′-terminal nucleoside of a tRNA ending in either a discriminator nucleoside (tRNA-D73) or C74 (tRNA-C74) cannot reach the active site (inactive state). Only the 3′-terminal nucleoside of tRNA ending in C74C75 can reach the active site for the catalysis (active state). (C) CCA-addition by CCA-adding enzyme, possessing a single tRNA binding site. The head domain of the CCA-adding enzyme is flexible, the head domain of the CCA-adding enzyme could relocate toward the neck domain to catalyze C74 and C75 addition. The head, neck, body, and tail domains of the enzymes are colored magenta, green, cyan, and orange, respectively. tRNAs are colored gray. Catalytic sites are colored yellow in the head domains.
probably do not dictate the specificity for the enzymes to add only terminal A76, in general.

The C74C75-addition by the CC-adding enzyme involves the translocation and rotation of the tRNA relative to the enzyme (Yamashita et al., 2014). Apparently, there are two tRNA binding sites on the surface of A. aeolicus CC-adding enzyme. One is for C74-addition and the other is for C75-addition (Figure 8A). The body domain of A. aeolicus CC-adding enzyme adopts a bulging structure and the overall structure has a more closed conformation, as compared with the A. aeolicus A-adding enzyme and other CCA-adding enzymes (Li et al., 2002; Tomita et al., 2004; Toh et al., 2009; Yamashita et al., 2014). Thus, the distinct structures of the body domain of the CC-adding enzyme, with two tRNA binding sites, allow the tRNA to translocate and rotate during the CC-adding reactions and to terminate the RNA polymerization after CC-addition.

A chimeric enzyme of A. aeolicus A-adding enzyme and the closely related T. maritima CCA-adding enzyme (Tomita and Weiner, 2001, 2002), designed based on their crystal structures, was constructed, and the A-adding enzyme was transformed into an enzyme that could perform CCA-addition in vitro as well as in vivo (Toh et al., 2009). These biochemical and genetic analyses suggested the importance of the flexibility of the neck domain, in defining the number of nucleotides added onto the 3′-end of the tRNA and the nucleotide specificity by the class-II CCA-adding enzyme.

It could be hypothesized that only a single tRNA binding site exists in both the class-II A-adding and CCA-adding enzymes (Figures 8B,C). On the single tRNA binding site, the 3′-terminus of tRNA lacking CCA or CA could not reach the active site without structural change of the enzyme, while the 3′-terminus of tRNA lacking A76 could reach it. The absence of flexibility in the neck domain of the A-adding enzyme would not allow the head domain of the enzyme to relocate toward the neck domain for CC-addition, when tRNA binds the single tRNA binding site. Hence, the A-adding enzyme could not add C74C75, but could only add A76 (Figure 8B). On the other hand, the CCA-adding enzyme, with a flexible neck domain, could add CC as well as A, by relocating the catalytic active head domain toward the neck domain for catalysis (Figure 8C), even though there is only one tRNA binding site, as in the A-adding enzyme. In these models, neither translocation nor rotation of tRNA is involved in the CCA-addition by the class-II CCA-adding enzyme, as previously suggested (Cho et al., 2006).

In the future, the complex crystal structures of a class-II CCA-adding enzyme with various tRNAs, representing sequential CCA-addition, and the comparison of the structures with those of the A-adding enzyme and the CC-adding enzyme complexed with tRNAs, will provide clear and definitive answers to all of the above-mentioned unsettled questions about the mechanisms of class-II CCA-adding enzyme and its relatives.

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