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How the CCA-Adding Enzyme Selects Adenine over Cytosine at Position 76 of tRNA

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Abstract

CCA-adding enzymes [ATP(CTP):tRNA nucleotidyltransferases] add CCA onto the 3' end of transfer RNA (tRNA) precursors without using a nucleic acid template. Although the mechanism by which cytosine (C) is selected at position 75 of tRNA has been established, the mechanism by which adenine (A) is selected at position 76 remains elusive. Here, we report five cocrystal structures of the enzyme complexed with both a tRNA mimic and nucleoside triphosphates under catalytically active conditions. These structures suggest that adenosine 5'-monophosphate is incorporated onto the A76 position of the tRNA via a carboxylate-assisted, one-metal-ion mechanism with aspartate 110 functioning as a general base. The discrimination against incorporation of cytidine 5'-triphosphate (CTP) at position 76 arises from improper placement of the α phosphate of the incoming CTP, which results from the interaction of C with arginine 224 and prevents the nucleophilic attack by the 3' hydroxyl group of cytidine75.

CCA-adding enzymes are nucleotidyltransferases that catalyze the posttranscriptional addition of CCA onto the 3' terminus of immature tRNAs without using a nucleic acid template (1). They are essential in all three kingdoms of life and are divided into two classes based on their sequences. The enzymes from Archaea belong to class I, whereas those from eubacteria and eukaryotes belong to class II (2). These two classes of enzymes may have quite different catalytic mechanisms.

During the past several years, crystal structures of CCA-adding enzymes and their tRNA complexes from Archaea (3–7), bacteria (8, 9), and humans (10) have been determined. These structures show that both families have only one site for binding either cytidine 5'-triphosphate (CTP) or adenosine 5'-triphosphate (ATP). Though the crystal structures shed light on how class I CCA-adding enzymes achieve their specificity for addition of the second C at position 75 without the use of a nucleic acid template (11), the question of how A, rather than C, is added at position 76 remains controversial. Based on the crystal structures of ternary complexes prepared by diffusing nucleotides into preexisting crystals of an RNA-protein binary complex, two different models have been suggested. One proposal

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Materials and Methods

Fig. S1

Table S1

References

from studies of class I enzymes is that the specificity is conferred by the size and shape of the nucleotide-binding pocket that is determined by the movement of the flexible head domain (5). In contrast, another set of crystal structures of class I enzymes led to the conclusion that the discrimination is dictated by a single, flexible amino acid side chain (Arg²²⁴) of the enzyme for addition of A76 (6).

To understand the structural basis of a biological process, structures of the entire assembly captured at each step in the process are necessary (12). The greatest challenge is to control the catalytic reaction of the nucleotide incorporation so that crystals of the whole assembly can be obtained at each step. The initial assembly is the ternary complex of the polymerase, the RNA primer, and the nucleotide triphosphate (NTP) substrate. Capturing this complex requires stopping its catalytic activity with strategies such as mutation of key amino acid residues (13), nonhydrolyzable NTP analogs (14–16), or a polynucleotide primer that is incapable of nucleotide incorporation (17, 18). Though these approaches have successfully yielded structures, they also have major limitations. For instance, the structural information may be altered by the modifications that are used to inactivate the complex, and the complex may not be trapped in the correct conformation. Further, structures of the initial ground state of the catalytic process may not allow correct extrapolation to the subsequent intermediate states. Therefore, illuminating the correct mechanism of polymerization could be facilitated by accurate and detailed structural information from catalytically active complexes.

We have determined the cocrystal structures of five ternary complexes of the archaeal CCA-adding enzyme from *Archaeoglobus fulgidus* (abbreviated AfCCA) that illuminate the mechanism and specificity by which the enzyme incorporates A, and not C, at position 76 of tRNA. The divalent metal ions in the buffer were either Mg²⁺, Ca²⁺, or Mn²⁺. The reaction equilibrium of nucleotide addition can be altered by the use of different divalent metal ions, and we took the advantage of this to trap the different steps in the reaction (19). The RNA primers were nucleotide hairpin mimics of *Mycoplasma pneumoniae* tRNA^{Ile} composed of the TΨC stem loop and the acceptor stem whose 3' ends were at the positions of 73 (called A73) or 75 (called ACC75) (Fig. 1), which have been demonstrated to be good substrates for the addition of CCA (20). The specific combinations of reactants and divalent metal ions cocrystallized were: [1] AfCCA(Mg²⁺)+A73+ [CTP+AMPcPP] (preinsertion complex), in which AMPcPP (α,β-methylene ATP) is the nonreactive ATP analog (and AMP is adenosine 5' monophosphate); [2] AfCCA(Mn²⁺)+ACC75+ATP (initial complex); [3] AfCCA(Mg²⁺)+A73+[CTP+ATP] (intermediate complex); [4] AfCCA(Ca²⁺)+ACC75+ATP (product complex); and [5] AfCCA(Ca²⁺)+ A73+CTP (CTP complex).

The crystal structures of complexes [1], [3], and [5] show that the RNA primer A73 has been extended to ACC75 in all three complexes during crystallization (Fig. 2). In the intermediate complex AfCCA(Mg²⁺)+A73+[CTP+ATP], ~50% of ATP remains in a preinsertion site without being incorporated, and the other 50% is incorporated into the primer ACC75 to form the product ACCA76 and pyrophosphate (fig. S1). In the structures of complexes [1] and [5], the reaction stalls after the incorporation of C75 with all of the nonreactive AMPcPP or noncognate substrate CTP in the pre-insertion site (Fig. 2, B and E). The activity of complex [5] is different from the poly(C) polymerase activity observed in the CCA-adding enzymes from *Escherichia coli*, *Sulfolobus shibatae*, and *Methanococcus jannaschii* (21), which may result from the different conditions that we used in the crystallographic and biochemical studies. Similarly, the primer ACC75 has been extended to ACCA76 in the product complex AfCCA(Ca²⁺)+ ACC75+[ATP] (Fig. 2D). However, somewhat surprisingly, the ATP stays in the preinsertion site in the initial complex AfCCA(Mn²⁺)+ACC75+[ATP] without incorporation (Fig. 2C).

The preinsertion complex AfCCA(Mg²⁺)+ A73+[CTP+AMPcPP] cannot incorporate A76 because AMPcPP is unreactive. This structure reveals the essential network of interactions in the active site before catalysis begins. AMPcPP forms hydrogen-bonding interactions in the nucleotide-binding pocket with Arg²²⁴, His¹³³, and Arg⁵⁰ of the enzyme and with the phosphates of A73 and C74 of the RNA primer (Fig. 3A). In addition, Nη2 of Arg²²⁴ forms hydrogen bonds to the phosphate groups of C72 and A73, and its Nε hydrogen bonds to the O1P of C72. Similar interactions between ATP and the enzyme have been observed in the initial, intermediate, and product complexes, as well as in the previous structural studies of the A-adding step (5, 6).

A metal ion that is coordinated to the triphosphate moiety of ATP (metal ion B) is observed in the active site of all of the structures reported here, except for that of the product complex. In the preinsertion complex, the metal ion (Mg²⁺) is coordinated by the triphosphate moiety of AMPcPP and the two carboxylates of Glu⁵⁹ and Asp⁶¹ (Fig. 3B). A third carboxylate, Asp¹¹⁰, forms a strong hydrogen bond with the 3' OH of the primer terminal C75. In the product complex in which AMP has been incorporated into the RNA primer, the pyrophosphate rotates ~180° away from the active site relative to the phosphate moiety of AMPcPP in the preinsertion complex and loses its interaction with metal ion B. Nonetheless, the pyrophosphate maintains essentially the same hydrogen-bonding interactions with the head and neck domains of the enzyme (Fig. 3E).

The superposition of the structures of the pre-insertion, initial, and intermediate complexes shows the process of ATP incorporation (Fig. 3C). The distance between the primer terminal O3' of C75 and the Pα of ATP decreases, and the angle formed by the O3', the Pα, and the bridging oxygen atom between Pα and Pβ (O3'-Pα-O3A) increases from 155° toward 180° from the preinsertion state to the reaction intermediate state, illustrating the process of a nucleophilic attack of the O3' on the Pα. In the intermediate complex, where both the reactants and the products coexist, the primer terminal O3' atom remains in the same position, whereas the AMP moves toward the O3' atom to form the O3'-Pα bond (Fig. 3D). A similar movement of the nucleotide substrate has been observed in the structures of ternary substrate [Protein Data Bank identification number (PDB ID): 1S76] and product (PDB ID: 1S77) complexes of T7 RNA polymerase (14).

Only one metal ion is observed in the active sites of the three A-adding complexes, which is consistent with previous structures (5, 6). This metal ion corresponds to metal ion B of the classic two-metal-ion mechanism, which functions to coordinate and stabilize the leaving pyrophosphate group (22). The position of the 3' terminal nucleotide C75 in the nucleotide-binding pocket sterically excludes the metal ion A from binding to the active site and facilitates the addition of A76. After the incorporation of C75 by a two-metal-ion mechanism (5), the acceptor stem of the tRNA does not translocate as first observed biochemically (23), but rather the previous primer terminal nucleotide, C74, is repositioned from its helical stacking position to a bulged position to allow the new primer terminal C75 nucleotide to now occupy the same priming-site position. The bulged out C74 lies in a nucleotide-binding pocket of limited size, which positions the 3' OH of C75 more than 3 Å closer to the catalytic carboxylates than the 3' OH of C74 in the previous addition step. Consequently, the O3' of C75 occupies the metal ion position (Fig. 3F). The carboxylate side chain of Asp¹¹⁰ forms a new hydrogen bond with the O3' of C75, whereas the other two catalytic carboxylates, Glu⁵⁹ and Asp⁶¹, coordinate metal ion B (Fig. 3B).

This model suggests that class I CCA-adding enzymes catalyze the addition of A76 by a carboxylate-assisted, one-metal-ion mechanism rather than a two-metal-ion mechanism. In the classic two-metal-ion mechanism, metal ion A facilitates the nucleophilic attack of the primer terminal 3' OH on the α-phosphate of the incoming nucleotide by lowering its pK_a

(where K_a is the acid dissociation constant), whereas metal ion B assists the leaving of the pyrophosphate group (22). The absence of metal ion A raises the question of what activates the nucleophilic attack of the 3' OH to form the O3'-P α bond. On the basis of the strong H-bond between Asp¹¹⁰ and the 3'-hydroxyl group of C75, we suggest that the third carboxylate residue in the active site, Asp¹¹⁰, could function as a general base. Consistent with this proposal, biochemical studies have shown that mutation of Asp¹¹⁰ impairs addition of A76 but does not affect the addition of either C74 or C75 (24, 25). Thus, the one-metal-ion mechanism requires all three carboxylates: two that function in binding metal ion A and a third required for A76 addition only.

The structure of the ternary CTP complex, AfCCA(Ca²⁺)+A73+[CTP], showed that, in position 76, the CTP base makes the same hydrogen-bonding interaction with Arg²²⁴ as the base of ATP does in the preinsertion complex (Fig. 4A), which requires the movement of CTP toward Arg²²⁴ (Fig. 4D). Superposition onto the previously reported binary enzyme + tRNA complex AfCCA+ACC75 (6) shows that, in the CTP complex, the key amino acids in the binding pocket, Arg²²⁴, His¹³³ and Arg⁵⁰, move toward the incoming CTP, which fits snugly in the pocket (Fig. 4, B and C). In addition, oxygen atoms of the phosphate groups of A73 and C74 move toward CTP to engage in stronger hydrogen-bonding interactions with N4 of CTP. Such "induced fit" behavior of the binding pocket shows the effects of the incoming nucleosides on catalytic activity of nucleotidyltransferases.

The ability of the CCA-adding enzyme to insert ATP and not CTP at position 76 is a result of its properly positioning the α phosphate of ATP, but not that of CTP, in the active site for a nucleophilic attack by the 3' OH of C75. CTP at position 76 has the same hydrogen-bonding interaction network as does ATP in the preinsertion complex. Because CTP is smaller, these interactions cause it to be located closer to Arg²²⁴ and cause Arg²²⁴ to move closer to CTP (Fig. 4D). As a result, the distance between the α -phosphate of the CTP and the 3' oxygen atom O of C75 is 4.5 Å, which is too far for a reaction to occur. Furthermore, the 3' terminal primer nucleotide C75 moves upward relative to the base of CTP, presumably to produce a better base-stacking interaction. This change moves the primer terminal 3' OH out of interaction range with the catalytic carboxylate of Asp¹¹⁰ (Fig. 4D). Thus, the nucleophilic attack of O3' of C75 on the α -phosphate of CTP is prevented.

Our results differ from those of a previous study in which CTP or ATP was soaked into the crystals of the binary complex, drawing the conclusion that Arg²²⁴ remains in the same configuration for the addition of nucleotide 76 onto ACC75, regardless of whether ATP or CTP is soaked into the crystal, and the base of CTP is positioned too far from Arg²²⁴ to interact with the guanidinium group (6). It could be that crystal-lattice constraints prevented the structural changes that we observe. Consistent with this, the soaking experiments did not result in extension of AC74 or ACC75 (6).

The present study resolves the disagreement between the two mechanisms that have been proposed for how the class I CCA-adding enzymes discriminate between CTP and ATP at the final addition step (5, 6). The discrimination was first attributed to the size and shape changes of the nucleotide-binding pocket, caused by the movement of the head domain of the enzyme (5). However, in a subsequent study, the Arg²²⁴ residue was proposed to be the principal determinant of the discrimination between A and C incorporation at position 76 (6). In this model, the enzyme conformation did not change so that the smaller CTP could not react with Arg²²⁴. Based on the five cocrystal structures, we show that the flexible side chain of Arg²²⁴ repositions in response to an incoming nucleotide base to hydrogen bond with the Watson-Crick edge of either C or A. The discrimination against incorporation of C arises, not from its lack of interaction with A224, but because the protein's flexibility to accommodate these interactions results in the improper geometry of CTP in the active site.

Supplementary Material

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Acknowledgments

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26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Fig. 2.

(**A**) Global view of the molecular structures determined in the present study. (**B** to **E**) The 3'-termini of tRNA mimics used in different complexes with the incoming NTP in the preinsertion site and the incorporated nucleotides superimposed on difference electron-density maps (Fo-Fc) contoured at 3.5σ. (**B**) AMPcPP, C74, and C75 and in the preinsertion complex. (**C**) ATP in the initial complex (Mn⁺² ion). (**D**) A76 and pyrophosphate in the product complex (Ca⁺² ion). (**E**) CTP, C74, and C75 in the CTP complex. Pa, α-phosphate; PPi, pyrophosphate.

Fig. 3. (A) Hydrogen-bonding network between ATP and the nucleotide-binding pocket in the present structures (26). (B) Hydrogen-bonding network for the addition of A76 in the active site. (C) Comparison of conformational differences among the preinsertion complex (green), the initial complex (yellow), and the intermediate complex (magenta) in the active site. (D) Comparison of the conformations of substrates (magenta) with those of the products (orange). (E) Interactions between the pyrophosphate product and the amino acids in the head and neck domains. (F) Superposition of an AfCCA complex with an RNA substrate ending in C74 and CTP (green) (PDB ID 1tfy) on the preinsertion complex in the present study (magenta).

Fig. 4.

(A) Hydrogen-bonding network in the nucleotide-binding pocket in the CTP complex with CTP and Arg²²⁴ superimposed on a difference electron-density map (Fo-Fc) contoured at 3.5σ. (B) Superposition of the CTP complex (orange) onto a binary complex of AfCCA and an RNA substrate ending in C75 (PDB ID 2dr9) (green). (C) The size of the binding pocket in the CTP complex with the enzyme shown in the surface representation. (D) Conformational differences between the CTP complex (magenta) and the preinsertion complex (green) in the nucleotide-binding pocket.